Process design tools for reductive biocatalysis

A thesis submitted to the University of London for the degree of Doctor of Philosophy

by
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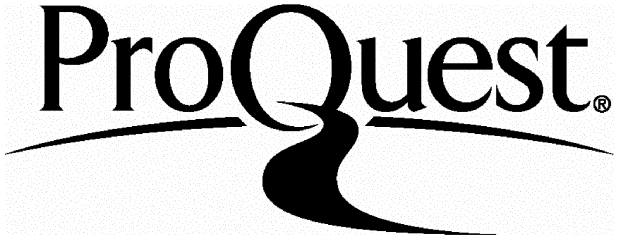

Acknowledgements

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Abstract

Reductive biocatalysis is potentially a very useful tool as it creates a chiral centre. Enzyme catalysed reduction reactions generally require the addition of expensive nicotinamide cofactors in stoichiometric amounts. To reduce the cost of these cofactors they can be recycle by a second substrate or enzyme or by chemical or electrochemical methods.

A decisional tool is proposed to assist the choice of the optimal enzymatic cofactor regeneration system for reductive biocatalysis. The tool comprises a series of questions addressing a potential process involving an enzymatic cofactor regeneration system and offers possible solutions to guide an engineer to an economically feasible process.

A model system was chosen with which to test the proposed decision tool which was the reduction of 6-bromo-β-tetralone to (S)-6-bromo-β-tetralol by the yeast *Trichosporon capitatum.*

An NADH-specific reductase enzyme from the yeast was isolated to apparent homogeneity with an enantiomeric excess of 99.54%. Purification involved protamine sulphate precipitation, anion exchange chromatography on Q Sepharose FF, affinity chromatography on Amicon Affinity Dyematrex Red A and gel filtration on Superdex 200 HR. The final purification stage resulted in a 54-fold increase in specific activity, to 430U/mg with a yield of 1.34%.

The optimum conditions for the operation of a bioconversion using the isolated tetralone reductase enzyme were investigated. The enzyme was immobilised onto Eupergit C in the presence of 0.5M phosphate buffer at pH7 with an optimum enzyme challenge of 0.5U/g beads; both the free and immobilised forms of the enzyme were investigated under process conditions. The optimum pH for activity of the free enzyme was in the range 6-7 and 6-8.5 for the immobilised enzyme. Both forms of the enzyme were at their most stable over this range. The temperature optima, a trade off between optimum activity and stability were found to be 21°C and 31°C for the free and immobilised forms respectively. Optimum solvent regime was 50% hexane for the free enzyme and 50% octanol for the immobilised enzyme.

A second enzyme for cofactor regeneration was selected and a combined, two enzyme system was optimised using a semi-automated, high throughput screening method. The regenerative enzyme, formate dehydrogenase, converts formate to CO_2 and recycles NADH. The optimum condition of pH (6.8), temperature (26°C) and solvent (30% octanol) were found by factorial experiments using a robotic liquid handling system to produce a semi-automated process.

The difficulties associated with operating the robotic system for enzyme reaction characterisation have been investigated with respect to bead handling and accuracy of the system. The main problems identified with bead handling for immobilised enzymes were pipette tips becoming clogged with beads and the bead slurry settling out and therefore not being homogeneous for pipetting. The accuracy of the system is important to investigate for the quantitative use of robotic systems otherwise insufficient data or too much data could be collected which would lead to wasteful redundancy of the data.

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Abbreviations

Abs	absorbance
ATP	2-deoxyadenosine 5-triphosphate
BSA	bovine serum albumen
CoA	coenzyme A
CV	coefficient of variation
DCW	dry cell weight
DSP	downstream processing
FAD	flavin adenine dinucleotide
FDH	formate dehydrogenase
FMN	flavin mononucleotide
g	grams
mg	milligrams
hrs	hours
L	litres
M	molar concentration
mM	milli molar concentration
mins	minutes
mL	millilitres
NAD^+	β-nicotinamide adenine dinucleotide
NADH	reduced β- nicotinamide adenine dinucleotide
NADP	β-nicotinamide adenine dinucleotide phosphate
NADPH	reduced β- nicotinamide adenine dinucleotide phosphate
OD	optical density
PM	production medium
SD	standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tetralol	(S)-6-bromo-β-tetralol
Tetralone	6-bromo-β-tetralone

Nomenclature

A	absorbance	min^{-1}
c	concentration	M
ε	molar absorption coefficient	L mol^{-1} cm^{-1}
L	length of confidence interval	%
λ	light path length	cm
N	number of repeats	-
σ	standard deviation	-
t	student's t-test value	-

1. Introduction

1.1 Bioconversions

1.1.1 General

A bioconversion is the transformation of an organic compound into a recoverable product by simple, defined, enzyme catalysed reactions. In contrast to many chemical processes the substrates and products of the reaction are structurally similar to each other. Bioconversions can be brought about by the enzymes in whole cells, spores or isolated enzymes. In all of these cases it is the enzymes that actually perform the catalysis.

One of the characteristic features of enzymes is their specificity and selectivity in the following respects:

- Chemospecificity – the ability of an enzyme to use only one specific substrate or a range of related substrates and to catalyse only one step with that substrate
- Regiospecificity – regiospecific resolution, is when a compound contains two identical groups but only one is altered by the enzyme, regiospecific synthesis is when a group is introduced in only one specific position
- Enantiospecificity – the ability of an enzyme to resolve (separate) the components of a racemate
- Enantioselectivity – the ability of an enzyme to introduce a new chiral centre

Specificity and selectivity are not the only advantage of enzymes. It may be possible to synthesise novel molecules, especially ones of a complex nature which have not been possible to produce chemically. Enzymes also typically catalyse reactions under mild conditions of temperature, pH and pressure. The advantage over chemical reactions which require more extreme conditions is that there is less chemical change due to isomerisation, epimerisation and racemisation, so that the yield of the required product can be higher. The moderate reaction conditions also help to make processes based on bioconversions provide an environmentally improved route to existing chemicals. However, these conditions also have their

disadvantages as enzymes are not tolerant to changes in temperature and pH (Ballesteros *et al*, 1994).

Bioconversions are not likely to replace existing methods of production as their development is costly and still in its infancy. However, bioconversions can be a useful tool to use alongside chemical processes particularly for the production of single enantiomer drugs (Cannarsa, 1996 and Davies & Reider, 1996). For this reason, bioconversion processes must be designed with this in mind and must be suitable to fit within a sequence of chemical processes (Myles *et al*, 1991).

1.1.2 Process Development

Process development and reactor design are generally slow and expensive processes because there are so many interrelated problems to be considered. Unlike the chemical industry, which has been established for longer than the biochemical industry, there have been few structured approaches to process design. This contrasts with the need within the pharmaceutical industry, which biochemical engineering largely serves, for a swift time to market of their products. The selection of a suitable product and process selection tend to be separated in biochemical engineering. This strategy contributes to the slowness of process development because many unnecessary experiments are done. A more strategic approach has been developed where both areas of research are combined, which should reduce the number of experiments necessary to find the most suitable process for a particular reaction and provide an informed choice for reactor design and configuration. The first stage is to characterise the reaction to identify certain constraints on the use of a particular process or reactor configuration. Then a change can be made to some aspect of the process which will provide a new set of constraints, but hopefully at a higher level of productivity. For example, if product inhibition is a constraint, *in situ* product removal strategies can be adopted (Freeman *et al*, 1993). Other changes which can be made include changes to the enzyme such as immobilisation, the use of genetic engineering or protein evolution techniques or changes to the process conditions, such as substrate feeding strategies. The process of step changes and re-characterisation

continues until the process has been fully developed (Figure 1.1) (Woodley & Lilly, 1994).

1.1.3 Process development tools

Alongside a more structured approach, tools to aid process development have also been developed including windows of operation (Woodley & Titchner-Hooker, 1996). Relevant parameters for the process are chosen and form the axes of a graph, on which are imposed conditions such as enzyme stability and activity and constraints imposed on the process. Thus many different objective functions may be expressed on the same diagram. This allows complex information about the process to be assessed and used to aid design.

Modelling of the process can also be used in development to help predict information about the reaction and look at the best conditions without doing a large number of experiments.

Other areas of pharmaceutical drug development employ techniques such as high throughput screening (HTS) to help increase time to market. Combinatorial chemistry has driven the development of such screening techniques which is both cheaper and quicker than traditional methods of drug discovery (Persidis, 1998). There are currently two different avenues of development within HTS: microtitre plates and 'lab on a chip' systems. 'Chips' can perform functions such as parallel solute quantification and analysis. The 'chip' innovation utilises nanotechnology to reduce the scale of traditional lab equipment so that it fits onto a support a few centimetres square. The unit operations which have been miniaturised successfully in this way include chromatography, electrophoresis, polymerase chain reaction vessels, pumps and valves (Cowen, 1998). Chip technology could have many useful applications in on-line analysis for process monitoring. However, the microtitre plates will be useful for micro-scale down applications as they are frequently used to house large numbers of reactions on a small scale.

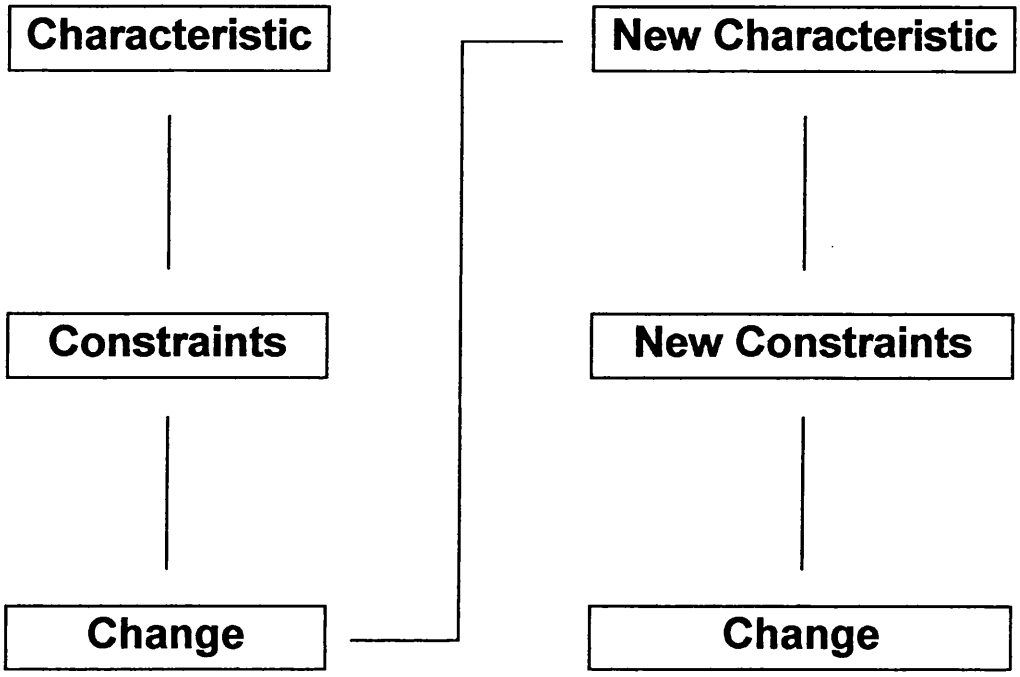


Figure 1.1 A structured approach to process development

1.1.4 Catalyst Choice

The first choice of catalyst for bioconversions may be whole cells, as they are easiest to produce and require little processing from fermentation to use in bioconversion. The substrate for the bioconversion may often be added straight to the fermentation broth in the fermenter, once the cells have grown sufficiently. Whole cells are also most suitable when there are more than two enzymatic steps especially if the enzymes are membrane bound or if the isolated enzyme is not stable. Whole cells do however, have certain disadvantages. Low molecular weight compounds are limited in the rate at which they can travel across the cell membrane, leading to low productivity (Angelova & Schmauder, 1999). Many enzymes are present in a cell and so side reactions can occur, reducing the yield of the desired product. Therefore, with increasing need for extremely pure chiral drugs, isolated enzymes are becoming the favoured catalyst option for bioconversions (Cannarsa, 1996).

To date, most enzymes used in industrial applications have been those that catalyse hydrolytic reactions or isomerisations (Faber, 1997). Potentially more useful enzyme reactions such as carbon-carbon bond formation and redox reactions have been slower to be exploited. Redox reactions, catalysed by cofactor dependent enzymes, have not been developed on a large scale, partly due to the prohibitive cost of the cofactors which means that they cannot be used in stoichiometric amounts. Where cofactor dependent reactions have been used, the catalyst is usually a whole cell.

There is also a choice to be made between free or immobilised catalyst, for both whole cells and isolated enzymes. Immobilisation increases the options for operation and choice of reactor. It allows for easier recovery and reuse of the catalyst and retention of the catalyst within the reactor, so that continuous stirred tank and plug flow set ups can be used (Carleysmith *et al*, 1980). The immobilisation matrix can be very expensive however, and the immobilisation process can lead to a loss in activity of the enzyme.

Genetic engineering has been used to improve biocatalysts. For example, to make overproducing recombinant organisms which provide much higher levels of the

required protein than the wild type organism (Schreiber & Verdine, 1991). The gene for the enzyme transketolase has been transformed back into *E. coli* under the control of a strong promoter so that much higher levels of the enzyme are produced (French & Ward, 1995 and Lilly *et al*, 1996).

Protein engineering can be used to produce enzymes with increased stability or with wider or different substrate or cofactor specificity (Ulmer, 1983). For example, widening the substrate specificity of cytochrome P-450 (Di Primo *et al*, 1990, Fowler *et al*, 1994 and Petersen & Martel, 1994) or changing the cofactor requirement of glutathione reductase from NADPH to NADH which is cheaper to use (Scrutton *et al*, 1990).

1.1.5 Process choice

The catalyst is not the only area for improvement. Improvements can be made to the whole process by altering the reactor configuration. The substrate may be inhibitory at the high concentrations necessary for an optimum process or may be toxic at relatively low concentrations and so in both cases the rate of the reaction will be reduced (Ballesteros *et al*, 1994). These problems can be overcome by using a fed-batch (Gbewonyo *et al*, 1991) or continuous flow operating system. These both keep the substrate concentration which the enzyme is exposed to at any moment in time fairly low, but in total, a large amount of the substrate is still converted to product (Cabral & Tramper, 1994). In some cases the product may be inhibitory and so a high product concentration, which allows for a low volume reactor to be used, cannot be achieved. This can be avoided by using *in situ* product removal to keep the concentration of product which the enzyme is exposed to low and thus retaining a high reaction rate (Freeman *et al*, 1993).

1.2 Redox reactions

1.2.1 General

Enzymes employed in redox reactions are classified into three categories: dehydrogenases, oxygenases and oxidases. Dehydrogenases have been widely used

for the reduction of carbonyl groups of aldehydes or ketones and of carbon-carbon double bonds, as well as in the recycle of nicotine adenine cofactors. Both of these reactions offer potential asymmetric synthesis leading to a chiral product, a reaction which is potentially very useful for the production of optically pure pharmaceuticals and other fine chemicals. The reverse reaction, i.e. oxidation, results in the destruction of a chiral centre but resolution of a racemic mixture, which is a less useful reaction. Dehydrogenase enzymes require nicotine adenine cofactors to carry the hydride group to be transferred to or from the substrate (Faber, 1992).

1.2.2 Bioreduction

Figure 1.2 demonstrates that reduction can produce products (2) or (3) from substrate (1). An enzyme catalysed reduction may favour either product (2) or (3), thus increasing the enantiomeric excess of the product. Oxidation can resolve the racemate mixture of (2) and (3), but will also destroy the chiral centre. As the ability to distinguish between chiral molecules is almost exclusive to enzyme (as opposed to chemically) catalysed reactions, resolution is potentially a more powerful tool for a biochemical engineer to have at their disposal.

Many companies have investigated the use of biologically catalysed reduction to produce chiral products, but these all involve whole cells as the catalysts (Table 1.1). To date, none have been investigated for use as an isolated enzyme, due to the difficulties associated with enzyme isolation and the use of cofactors.

1.3 Cofactors

1.3.1 Definition

The terms coenzyme and cofactor refer to low molecular weight molecules associated with certain enzymes which are essential for their activity. However, the term cofactor is more appropriate as these molecules are not proteins and also do not function as a catalyst as they are chemically altered during the reaction. Each cofactor is a carrier of a small chemical group, linked by reactive bonds to the rest of the cofactor so that they can be transferred easily to other molecules (Table 1.2). The

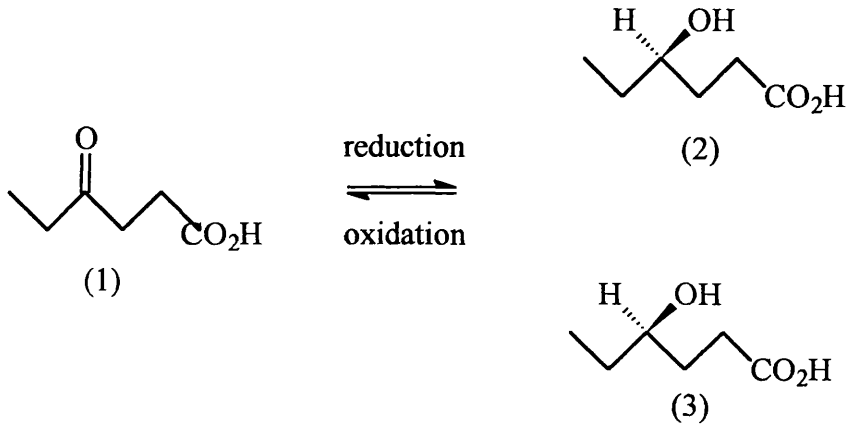


Figure 1.2: Reduction and oxidation of chiral molecules.
(1) 4-oxo-hexanoic acid, (2) (R)-4-hydroxy-hexanoic acid, (3) (S)-4-hydroxy-hexanoic acid

Table 1.1: Recent industrial research into biologically catalysed redox reactions

Company	Organism	Reaction	Reference
Eli Lilly & Co.	*Zygosaccharomyces rouxii*	enantioselective reduction	Anderson *et al*, 1995
Konan Chemical Industry Co. Ltd.	Immobilisaed *Daucus carota*	enantioselective reduction of prochiral ketones	Akakabe *et al*, 1995
Luso Farmaco s.p.a.	immobilised *Saccharomyes cerevisiae*	enantioselective reduction to form β-lactam antibiotics	Banfi *et al*, 1994
Merck	*Mortierella alpina*	asymmetric bioreduction of β-ketoesters	Chartrain *et al*, 1995
Merck	*Rhodotorula rubra*	asymmetric bioreduction of ketosulphone	Katz *et al*, 1996
Merck	*Microbacterium sp.*	Asymmetric bioreduction of ketoester	Roberge *et al*, 1996
Merck	*Trichosporon capitatum*	asymmetric bioreduction of ketoester	Reddy *et al*, 1996
Smithkline Beecham Pharmaceuticals	*Rhodotorula rubra*	enantioselective reduction of C=C bonds	Cantello *et al*, 1994
Tenabe Seiyaku Co.	*Pseudomonas polycolor*	asymmetric oxidation	Takahashi *et al*, 1995
Tenabe Seiyaku Co.	*Micrococcus freudenrichii*	asymmetric reduction	Takahashi *et al*, 1995
Zeneca	*Zygosaccharomyces rouxii*	enantioselective reduction of β-ketoesters	Hallinan *et al*, 1995

Table 1.2: Cofactors involved in group-transfer reactions

Cofactor		Group transfered
nicotinamide adenosine diphosphate	NAD(P)(H)	hydride ion
flavin adenine nucleotide	FAD(H)	hydride ion
adenosine tri-phosphate	ATP	phosphate
coenzyme A	CoA	acetyl
S-adenosyl-L-methionine	SAM	methyl
3'-phosphoadenosine 5'-phosphosulphate	PAPS	sulphate
biotin		carboxyl

same carrier molecules may participate in many different reactions within a cell, and so there may be many different enzymes which require the same cofactor. Some cofactors are covalently linked to the enzyme, these are called prosthetic groups. A few cofactors are self-regenerating, such as pyridoxal phosphate and thiamine pyrophosphate (Baricos *et al*, 1976), but most require a complementary reaction to regenerate the active form. Certain metal ions which interact with enzymes and are essential for their activity may also be regarded as cofactors.

Biologically the most important group of coenzymes are the adenine coenzymes: NAD, NADP, ATP, FAD and CoA. These are required by one third of the 2000 enzymes registered by the International Union of Biochemistry. This project concentrates on the nicotinamide adenine cofactors NAD(P)(H)[1].

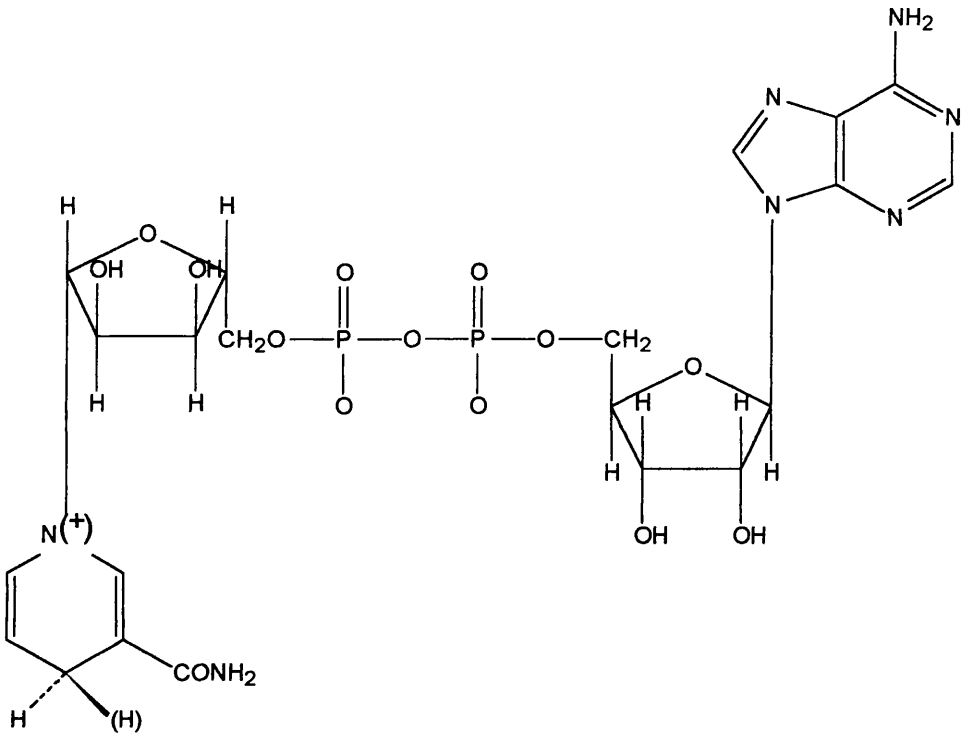
1.3.2 Chemistry of NAD(P)(H)

The nicotinamide adenine family of cofactors are nucleosides. In both cofactors the electron transfer involves only the nicotinamide moiety. Figure 1.3 shows the structure of all four of the native forms of the nicotine adenine cofactors. Enzymatic reduction of NAD(P) directs the hydride attack at C-4 of the pyridinium ring and forms exclusively the 1,4-dihydropyridine product, which is the only enzymatically active form of NAD(P)H.

The nicotinamide cofactors are unstable in aqueous solution, especially at extremes of pH (Chenault & Whitesides, 1987). Acidic conditions catalyse the hydration and anomerization of the reduced cofactors (Wong & Whitesides, 1981). Basic condition catalysed the hydrolysis of the nicotinamide-ribose bond of the oxidised cofactors (Guilbert & Johnson, 1977) (figure 1.4).

[1] Parentheses indicate the generic species within a class of the nicotinamide cofactors. For example, NAD(P) refers to both oxidised forms of the cofactors NAD and NADP; NAD(H) refers to the oxidised and reduced form of NAD and NADH; NAD(P)(H) denotes both redox forms of both cofactors.

NAD(H)



NADP(H)

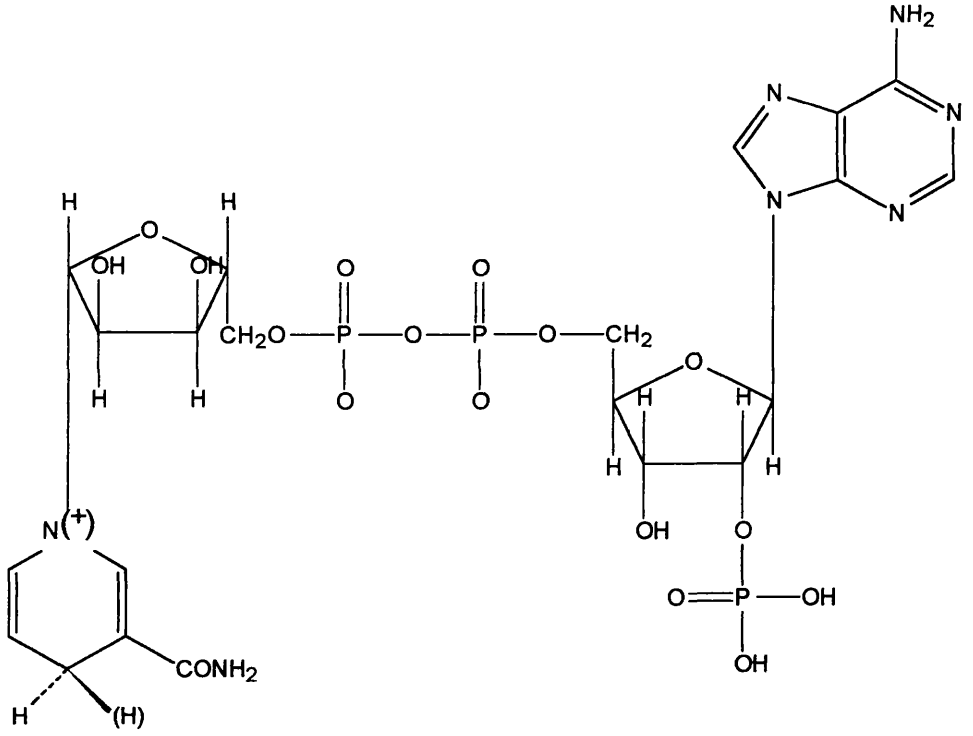


Figure 1.3: Structure of NAD(H) and NADP(H)

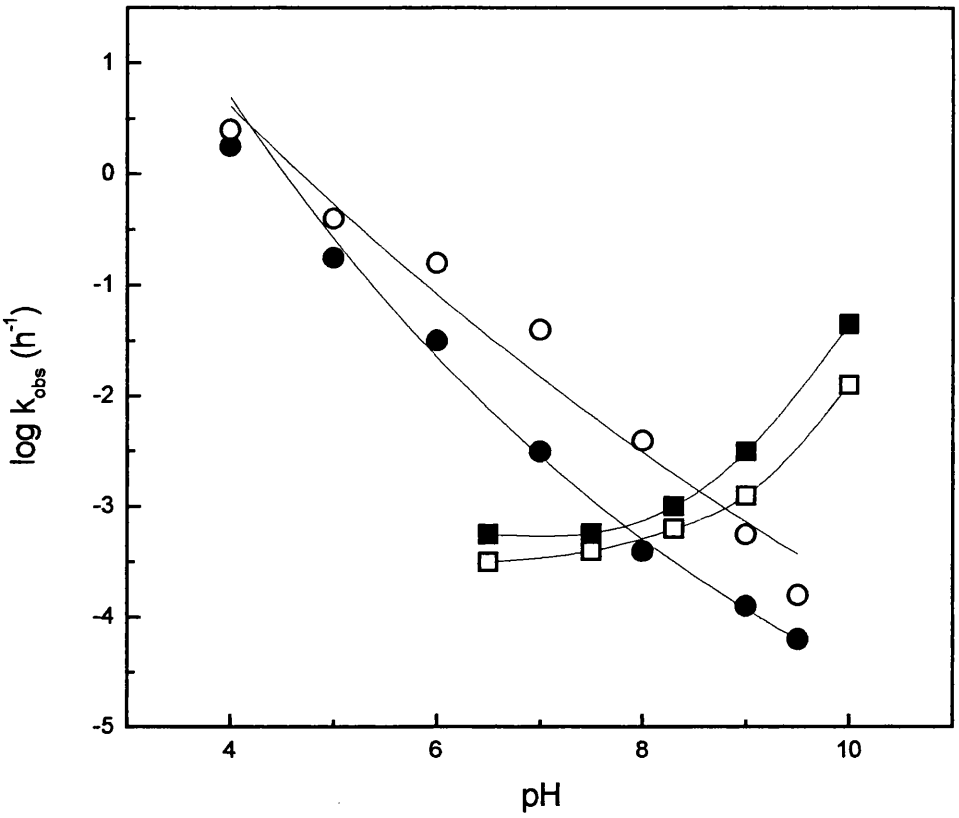


Figure 1.4 Stability of NAD^+ (■), NADP (□), NADH (●) and NADPH (○) as a function of pH. Data taken from Wong & Whitesides, (1981).

The reduced nicotinamide cofactors, and especially NADPH, are particularly unstable in phosphate buffers (Wong & Whitesides, 1981).

In cells, ATP and NAD(P)(H) play an intermediary role in the transfer of energy. Chemical energy derived from the oxidation of glucose is transformed into the energy rich bonds of ATP and NAD(P)H. The energy derived in this way can then be used in other biochemical pathways. NAD(P)(H) mediates two electron redox reactions. Unlike flavin cofactors, nicotinamide cofactors are readily dissociable from their enzymes. The hydride ion to be transferred from the cofactor is part of the nicotinamide ring and is easily lost because the ring can achieve a more stable aromatic state without it. Most redox reactions *in vivo* involve the association of NAD(P)(H) cofactors.

NAD(H) and NADP(H) differ only slightly chemically. NADP(H) has an extra phosphate group which is far away from the nicotinamide moiety where the hydride ion is lost and does not affect the transfer of this ion. It does however play a part in the recognition of the cofactor by the enzyme. Enzymes tend to be quite specific with regard to the species of nicotinamide cofactor they require and only show useful rates with the correct species. In general, enzymes involved in catabolic reactions, such as dehydrogenase-catalysed reactions of respiration, require NAD(H) and enzymes involved in anabolic reactions, by transferring electrons to intermediates of biosynthesis, require NADP(H). In this way the catabolic and anabolic pathways within a cell can be regulated independently by varying the levels of NAD(H) and NADP(H).

1.4 Cofactor recycling

1.4.1 Definition

Cofactor recycling can be separated into two distinct processes which must both occur to ensure that recycling takes place. The first process is the chemical reformation of the active form of the cofactor which is necessary for the proposed reaction: this will be called regeneration. The second process is retention of the cofactor. This process becomes important when employing a continuous or plug flow

reactor configuration as the cofactor could be removed from the system with the product. For regeneration, and therefore recycling to occur, the cofactor must remain in or be returned to the reaction environment.

1.4.2 Economics

No attempt will be made to offer an accurate cash figure for the cost of the nicotinamide adenine cofactors, although table 1.3 gives an idea of the possible order of magnitude and the relative costs of each to the other. This is because quoted bulk figures for the cost of cofactors are out of date (Coughlin *et al,* 1988 and Chenault & Whitesides, 1986) and chemical suppliers, such as Sigma Co. tend to sell these cofactors in analytical quantities only. Even bulk prices quoted may well be artificially high as there has only been a market for these chemicals in small quantities to date. Furthermore a company wishing to develop a large scale process could choose to produce the cofactors themselves and therefore the cost in a company's internal market is impossible to estimate.

Despite these problems, it can be reasonably assumed that the cost of cofactors is high, often the most dominant cost in the process and higher than the value of the product to be produced. Therefore it is necessary to use the cofactor more than once in a process and as the cofactor is chemically altered during the reaction, it must be regenerated to its active form.

The number of times a cofactor is recycled in a process is called the turnover number, this refers to the number of moles of product formed per mole of cofactor per unit time:

$$\text{Turnover Number, TN} = \frac{\text{cofactor cycles}}{\text{time}} = \frac{\text{moles of product}}{(\text{moles of cofactor})(\text{time})}$$

Turnover number refers to a rate and has units s-1. The total turnover number is defined by:

Table 1.3: The order of magnitude for the cost of nicotinamide adenine cofactors.

Cofactor	$/g mol
NAD	100
NADH	1 000
NADP	10 000
NADPH	100 000

$$\text{Total Turnover Number, TTN} = \frac{\text{moles of product formed}}{\text{moles of cofactor present in reaction}}$$

Total turnover number emphasises the total number of moles of product formed per mole of cofactor during the whole process, and therefore the total number of times a mole of the cofactor will be regenerated in a particular process.

The number of turnovers required depends on the relationship between the cost of the cofactor and the value of the product. Generally, as the cost of the cofactor increases, then the number of turnovers required for an economic process increases. There are three categories which cofactor and product costs can fall into:

- cofactor costs more than the product value
- cofactor costs about the same as the product value
- cofactor costs less than the product value

When examining the economics of a system, the cost of the cofactor must include the cost of recycling if it is to be recycled. Therefore, when comparing the cost of a cofactor added in stoichiometric amounts to one recycled, the initial cost may be more for the recycled cofactor as extra equipment and consumables will be necessary. However, the cost must be spread over the gross production of the product and not a single batch. Also, when comparing the cofactor cost and the product value, it is not sufficient for the total cost of the cofactor to equal the total value of the product. For the process to be economically viable, the cofactor must cost a fraction of the product value. This will also increase the number of times which the cofactor must be recycled. Therefore, when calculating the required number of turnovers of the cofactor, a term must also be added to take account of the proportion of the product cost which should be represented by the cofactor:

$$\text{Number of Cycles} = \frac{\left(\begin{array}{c}\text{value per}\\ \text{mole of product}\end{array}\right)\left(\begin{array}{c}\text{\%age contribution of}\\ \text{cofactor to product value}\end{array}\right)}{\text{cost per mole of cofactor}}$$

Therefore, the number of turnovers required will increase if the cofactor cost increases, if the product value decreases or if the cofactor is a small contribution to the product value. Even if the cofactor costs the same as or slightly less than the product value, it may still need to be recycled if it is to make only a small contribution to the total product value. In most processes it is necessary for the cofactor to be regenerated between 10^2 and 10^6 times to be economical (Simon *et al*, 1985).

With a high turnover rate, there is a need for a high selectivity for the regeneration process. If a large number of side reactions occur, then relatively high concentrations of inactive cofactors will build up, which may act as inhibitors of the enzyme since they may retain an ability to bind to the enzyme without being involved in the reaction. Therefore, at each regeneration cycle a high proportion of the cofactor present must be correctly regenerated. It is necessary for the method of regeneration to be more than 99% efficient at regenerating active cofactor. Figure 1.5 shows that even for only 10% of the original cofactor to remain active after as few as 10 cycles, the regeneration method must be about 80% effective. More realistic figures of 50% of the original cofactor to remain active after 10^4 cycles requires a regeneration efficiency of 99.993% and after 10^6 cycles of 99.99993%. Cofactor activity may also be lost for other reasons including instability due to heat and pH. For example, NAD is stable below pH 6, whereas NADH is stable above pH 8 (Wong & Whitesides, 1981 and Lowry & Passoneau, 1972) (see section 1.3.2).

The amount of cofactor needed for a process, and therefore the cost of the cofactor will be a compromise between space-time yield and total turnover number. The cofactor concentration should be high enough to avoid a significant drop in space-time yield after a slight drop in cofactor activity but low enough to provide high turnover numbers (Kragl *et al*, 1993).

In addition to the cost benefits of recycling there are also other economic benefits which could possibly be gained. The equilibrium position of a reaction can be affected by coupling a thermodynamically unfavourable target reaction to a

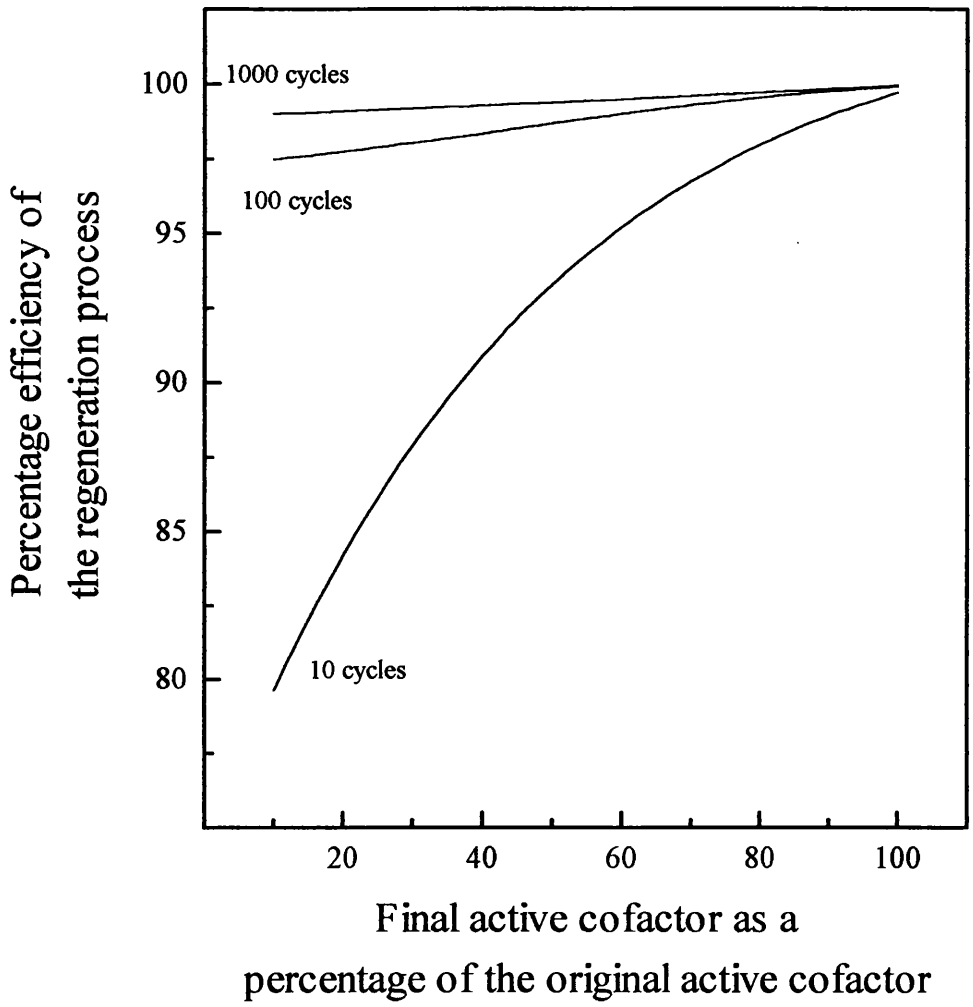


Figure 1.5: Percentage efficiency of regeneration vs. final active cofactor. As the number of cycles increases, it becomes increasingly important to have a high efficiency for the regeneration of active cofactor.

favourable regeneration reaction so that the target reaction is driven towards a product. Recycling will also prevent the accumulation of the by-products of used cofactors which may inhibit the forward reaction as they can still bind to the enzyme although they cannot transfer a group in the reaction. The reduction in the amount of cofactor added due to the elimination of the need for stoichiometric amount of cofactor will not only reduce the cost of the total cofactor but may also simplify the work up, although additives may be necessary with regeneration (Wong & Whitesides, 1994).

1.5 Cofactor Options

1.5.1 Pre-regeneration

Cofactor recycling may not be the first option that should be assessed when setting up a bioconversion using a cofactor requiring enzyme. If the cost of the cofactor does not make it economically unfavourable to do so, it may be possible to use the cofactor in stoichiometric amounts, as if it were a second substrate. Although this is unlikely to be a reality at the moment, it may be a realistic option in the future. Measures to reduce the cost of the cofactors could make this pragmatic, for instance, by increasing the stability of the cofactors and devising a cheaper method to produce them. The current prices may be artificially high as the demand is low and most cofactors are used in analytical quantities. If the demand was raised by their use in industrial processes the prices could be further reduced.

Another option could be to use an artificial cofactor that is cheaper than the natural cofactors. Some work has been done in this area, for example by using a modified triazine dye template, such as Blue N-3 (Burton *et al*, 1996 and Dilmaghanian *et al*, 1997). These artificial cofactors perform well in comparison to the natural cofactor, but they are still fairly expensive and so not suitable for immediate application.

In addition to changes to the cofactor, changes could also be made to the enzyme by genetic or protein engineering. The affinity of an enzyme can be switched from one form of nicotinamide cofactor to another with the same redox status (Scrutton *et al*, 1990). Although such techniques could be useful and very powerful, they are slow

and rather random at the moment. They also tend only to make a change from one expensive cofactor to a similarly expensive cofactor, although NAD^+ is about one hundredth the cost of $NADP^+$ and therefore, in some circumstances, this may be a significant change.

1.5.2 Methods of regeneration

There are numerous methods which have been described for the regeneration of cofactors and several good reviews have been written on the subject including Chenault *et al* (1988) and Lee & Whitesides (1985). Most of the regeneration methods are for NAD(P)(H), but there are also a few for ATP (Bednarski *et al*, 1988, Pollak *et al*, 1977 and Shih & Whitesides, 1977) and coenzyme A (Billhardt *et al*, 1988). These methods follow a few basic principles for regeneration which are based on the following:

- whole cell / organelle methods
- enzymatic methods
- chemical methods
- photochemical methods
- electrochemical methods

Not all these principles are suitable for the regeneration of all cofactors, for example electrochemical methods are only really applicable to NAD(P)(H). Almost all of these methods can be used in organic solvents, although the cofactors themselves are only soluble in aqueous solution and so if organic solvents are used, there must be some aqueous component (Adlercreutz, 1996).

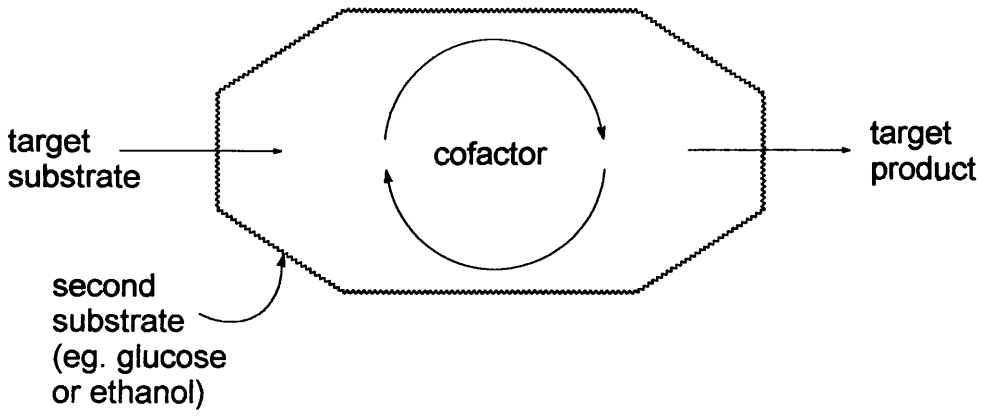
It is not easy to compare the relative efficiencies of different cofactor regeneration methods from the literature. This is because many terms are used to describe the efficiency of the cofactor regeneration methods. The most common of these is the total turnover number (TTN), as described in section 1.4.2. However some papers call this same term turnover number, cofactor recycle number and number of cycles of the cofactor. In addition, the data necessary to calculate the TTN is not always presented. In instances where the TTN is given it is still not a reliable measure of the

actual efficiency of the regeneration process because the process is not always optimised to make the best use of the cofactor. As the initial amount of cofactor added increases above the minimum amount required, the TTN decreases and therefore the regeneration process appears to be less efficient. However, recycling processes which employ a continuous reactor configuration appear to have higher TTN number and in addition a higher rate of recycle (TN).

1.5.2.1 Whole cell / organelle methods

The cheapest and most efficient method to recycle any cofactor is to allow the regeneration to occur by the normal biochemical processes within the cell. In some examples the target bioconversion and regeneration occur within the same cell e.g. yeast cells fed on ethanol for reduction reactions catalysed by baker's yeast (Kometani *et al*, 1994) (Figure 1.6). In other examples the target bioconversion is catalysed by an isolated enzyme while the regeneration alone is achieved by a preparation of whole cells, e.g. the use of anaerobically grown yeast cells for the regeneration of NADH using an alcohol dehydrogenase test system (Godbole *et al*, 1983) or the regeneration of NAD(P)H by hydrogenase enzyme within a preparation of whole cells with H2 gas added (Klibanov & Puglisi, 1980). In some cases dried preparations of whole cells can be used for regeneration, e.g. the use of immobilised dried yeast cells for. In this case ATP was regenerated within the whole cells, but NAD was not and had to be added to the system (Kimura *et al*, 1981). Immobilised *E. coli* and *Leuconostoc mesenteroides* have been used to regenerate NAD(P) (Burgstein *et al*, 1981 and Ergan *et al*, 1984). However, using whole cells as catalysts can have disadvantages, as discussed in section 1.1.3.

Methods involving the use of isolated whole organelles have also been described. Karube and co-workers (1980) used isolated spinach chloroplasts immobilised in agar to regenerated NADPH, although the isolated chloroplasts were very unstable. This method used the natural process of photosynthesis, which produces NADPH in green plants from the sun's energy. The advantages of these methods is that many enzymes and enzyme complexes which are involved in the regeneration of NAD(P)(H) *in vivo* are not stable once they are isolated, particularly as the activity of many of the



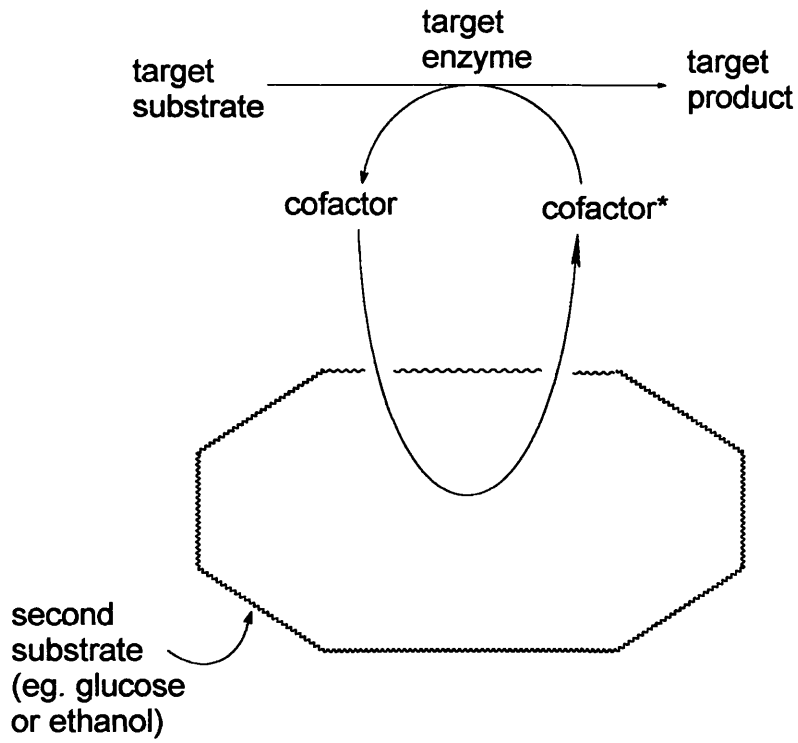


Figure 1.6: Principle of whole cell regeneration.
*' * ' indicates changed cofactor, whether reduced or oxidised*

enzymes is dependent on their position within a membrane bound cascade. None of the methods described for isolated organelles have been demonstrated on a preparative scale and all suffer from a lack of stability once the organelles have been isolated.

1.5.2.2 Enzymatic methods

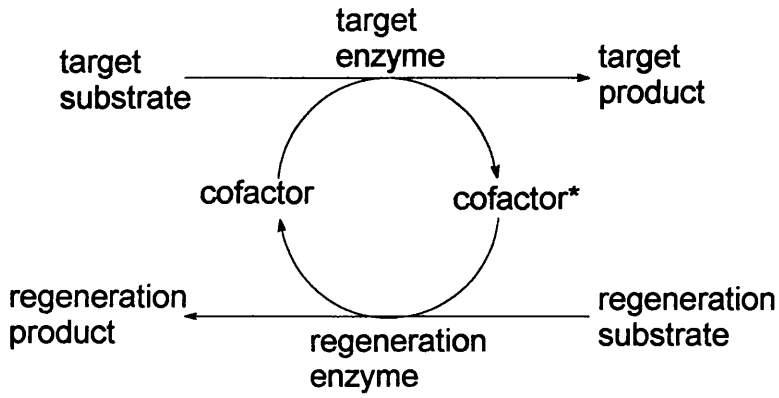
The most prescribed principle of cofactor regeneration is the use of isolated enzymes. Many different systems have been described. Some are used at preparative scale and some are also used in analytical devices such as biosensors (e.g. ethanol analyser (Yamazaki & Maeda, 1987)).

There are two basic ways of using isolated enzymes to regenerate cofactors which can be described as second enzyme and second substrate methods (Figure 1.7). The second enzyme method is more common and involves the use of a second bioconversion, in addition to the target bioconversion, which uses the opposite species of the cofactor couple. If, for example, the target reaction uses NAD, the regeneration reaction will use NADH. Thus by coupling these two reactions there is constant cycling of the cofactor. The second bioconversion requires an additional substrate to be added in stoichiometric amounts and an additional enzyme and will also produce an additional product.

The second substrate method also involves the use of a second bioconversion, but in this case only one enzyme catalyses the target and regeneration reactions. There is a need for an additional enzyme to be added and an additional product will be produced.

The advantage enzymatic systems is that the cofactor is regenerated with a very high efficiency. The enzyme catalysed reactions are very specific about where the hydride ion is added or removed from the cofactor and so there is little chance of an inactive cofactor being regenerated. However, it may be difficult to optimise a process for two enzymes and compromises may have to be made to the rate of both processes. Some work has been done on optimising the rate of both the target and

Second Enzyme



Second Substrate

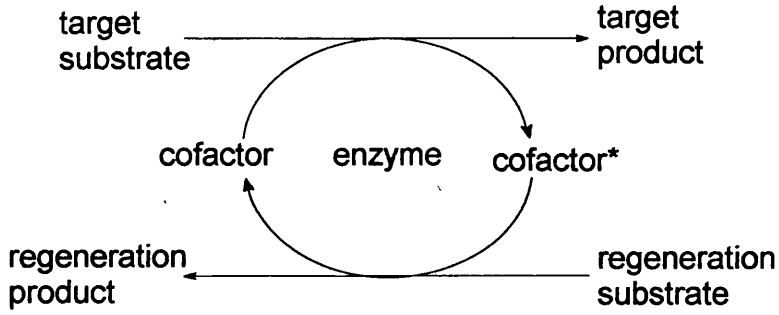


Figure 1.7: Principle of enzymatic regeneration.
*' * ' indicates changed cofactor, whether reduced or oxidised*

regeneration reactions (Hogan & Woodley, 1999 and Ikemi *et al*, 1990). The second substrate has to be added in stoichiometric amounts and so has to be inexpensive and the second product is also produced in stoichiometric amounts and its removal will add to the cost of downstream processing. A standard method for the regeneration of NADH at a preparative scale uses the enzyme formate dehydrogenase with formate as the substrate which produces CO_2 as a gaseous product, thus making the work up easier (Ward & Young, 1990).

1.5.2.3 Chemical methods

One of the biggest disadvantages of enzymatic methods is the need to add a second enzyme in most cases. Not only will this make a significant contribution to the total cost of the cofactor, but it will also add to the difficulty of process optimisation as there will be two enzymes to optimise for. For this reason, chemical oxidising or reducing agents which regenerate the cofactor may be an advantage.

Figure 1.8 shows the principle behind the use of chemical reagents to regenerate a cofactor. In theory, the cofactor is regenerated by the chemical agent alone which is then either recycled itself or added in stoichiometric amounts. In practice, the rate of reduction or oxidation by the chemical is too slow to be of benefit and often an additional enzyme must be added to allow for a favourable rate. For example, FMN reductase can be used to catalyse the regeneration of NAD(P) (Drueckhammer *et al*, 1985). This of course will invalidate one of the advantages of the chemical methods over enzymatic methods, in that there will be the additional cost and optimisation of a second enzyme. A few methods also describe the addition of more than one chemical agent, so that the hydride ions transferred in the regeneration process are passed along a chain of several electron mediators to the cofactor which improves the rate (Montaine *et al*, 1987).

There are few examples of the use of chemical reagents to regenerate NAD(P)H. Jones and co-workers (1972) describe a method for preparative-scale reduction of cyclic ketones and aldehydes using horse liver alcohol dehydrogenase with catalytic amounts of NADH regenerated by sodium dithionite. There is a risk with this method

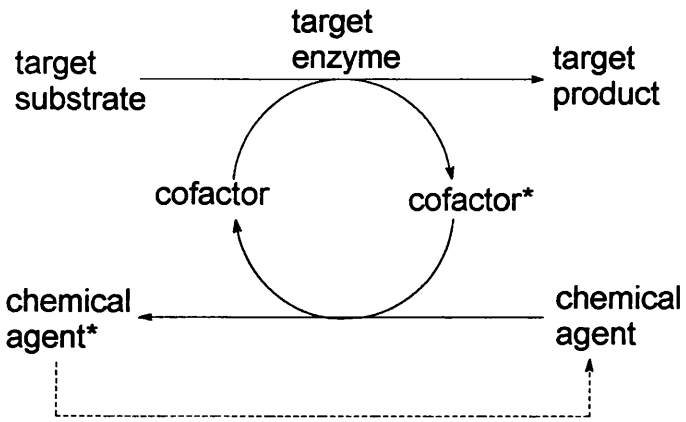


Figure 1.8: Principle of chemical methods of regeneration.
*' * ' indicates changed cofactor or chemical agent, whether reduced or oxidised*

of the reductive inactivation of the enzyme and the disadvantage of the instability in aqueous solution of the reducing agent.

There are more examples for the use of chemical reagents to regenerate NAD(P). Many different oxidising agents have been surveyed for this purpose including methylene blue, FMN, FAD, acriflavin and riboflavin, with the best of these being flavin mononucleotide (FMN) (Jones & Taylor, 1976 and Mansson *et al*, 1976). Polymerisable and immobilised forms of these oxidising agents have also been used to some effect (Spetnagel & Koltz, 1978 and Mansson *et al*, 1976). Lee & Whitesides (1985) have also surveyed both chemical and enzymatic methods of regeneration of NAD(P)H but found that enzymatic methods were superior as they were more efficient at regenerating active cofactor. Although oxidising agents such as FMN can autooxidise in the presence of air and so are themselves regenerated, the rate of this is also slow and so near to stoichiometric amounts of the oxidising agent often have to be added.

1.5.2.4 Photochemical methods

Photoexcitation by visible light can accelerate the oxidation of NAD(P)H by several electron transport dyes (Chambers *et al*, 1974). For example, the regeneration of NAD is achieved photochemically by immobilised acriflavin (Mansson *et al*, 1976).

More specifically, methods to mimic the light reactions of photosynthesis have been examined. These methods are similar to chemical methods in that the cofactor is regenerated by reaction with an oxidising or reducing agent, but the energy for the production of the reducing agent comes from the excitation of the electrons of a photosensitiser by visible light. NAD(P)H can be regenerated from NAD(P) and has been used at a preparative scale for several different reductions (Mandler & Willner, 1986).

Figure 1.9 shows the general principles involved. Light falls on the photosensitiser and elevates an electron in its outer shell to an excited state. This electron is then

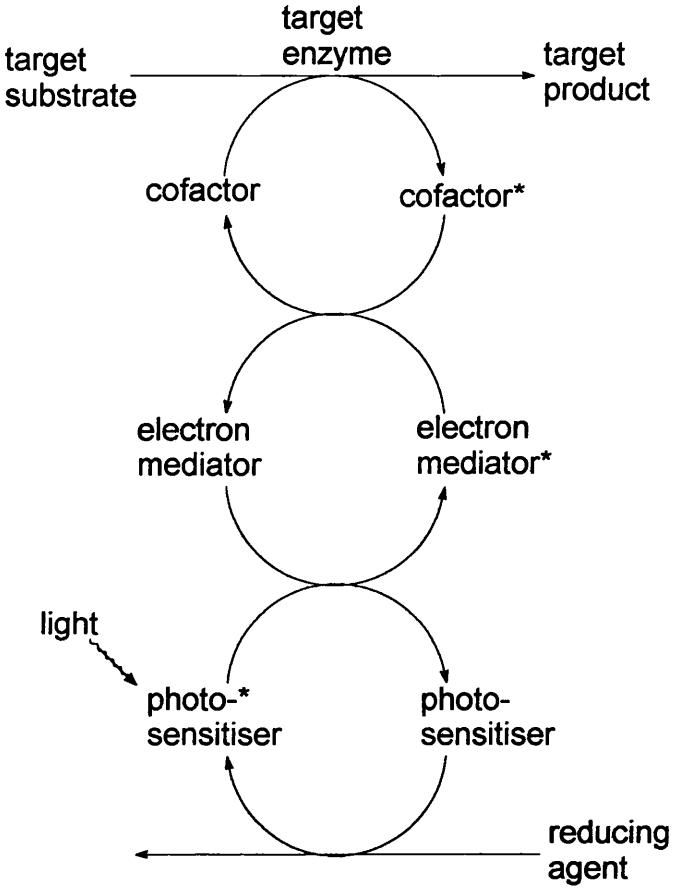


Figure 1.9: Principle of photochemical methods of regeneration.

transferred via electron transport dyes to the NAD(P) for regeneration. As with electron transport systems in cells, electrons are passed along the chain to the cofactor, while the proton to form the hydride ion is acquired from the solution. The photosensitiser is itself regenerated by chemical reduction with a reducing agent.

This method has many of the disadvantages of chemical methods, including the need for an additional enzyme to produce a favourable rate of regeneration. In addition the method is complex and requires the addition of light to the reactor. The light provided does, however, provide the energy to drive what would otherwise be an unfavourable reaction.

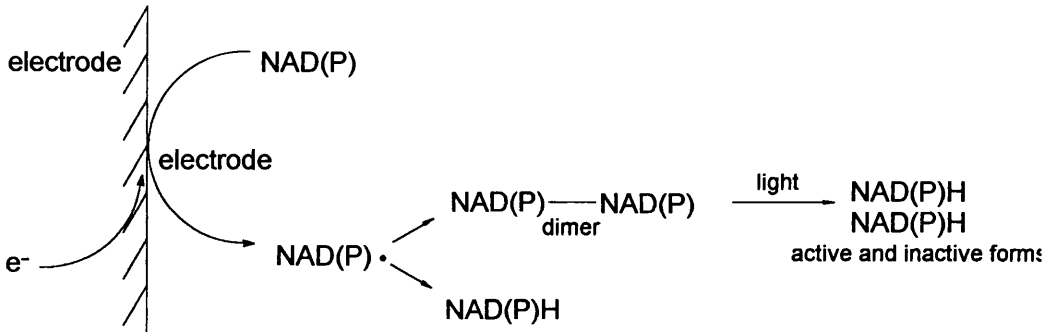
1.5.2.5 Electrochemical methods

Electrochemical methods are applicable to the regeneration of NAD(P)(H) cofactors because the electron transfers which can occur result in the oxidation (loss of electrons) or reduction (gain of electrons) of the cofactor. This area has been reviewed by Nakamura *et al* (1988).

Regeneration of NAD(P)H occurs at the cathode in two steps (Figure 1.10). The first step is the formation of an NAD(P) radical by the transfer of an electron from the cathode to the cofactor. The radical can then acquire a hydride ion from solution to form active cofactor, or it can react with another radical to form an enzymatically inactive dimer. This dimer is easily oxidised either in the presence of molecular oxygen (Careli *et al*, 1980) or photochemically in the absence of oxygen (Czocharalska *et al*, 1980) and the product functions as an active cofactor.

The energy potential of the reduced cofactors is higher than for the oxidised state, therefore regeneration of NAD(P)H from NAD(P) is more difficult than the opposite way round. This results in the need for a high overpotential for electrochemical reduction of the cofactor and frequently results in a loss in enzymatic activity. This overpotential may also lead to interference from other reactions on the electrode, which may be reduced by the use of electron mediators such as methylviologen (DiCosmo *et al*, 1981 and Maeda & Kajiwara, 1987) and bipyridine-rhodium

Reduction



Oxidation

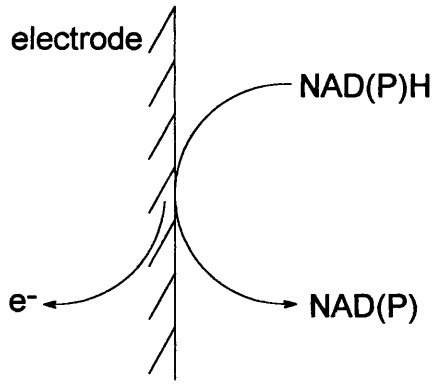


Figure 1.10: Principle of electrochemical methods of regeneration.

complex (Careli *et al*, 1980). The NAD radical produced as an intermediate during electrolysis can dimerise to form an enzymatically inactive form. This has been overcome by using a mercury electrode coated with a thin liquid crystal membrane of cholesterol oleate which electrolytically reduced NAD+ (Aizawa *et al*, 1976 and Yun *et al*, 1996). Although this resulted in enzymatically active cofactor being regenerated, it is not suitable for use in a continuous reactor (Nakamura *et al*, 1988). Another measure to reduce dimerisation is to immobilise the NAD+. This prevents the intermolecular interaction of the NAD+ necessary for dimerisation. For instance NAD+ has been immobilised on alginic acid (Aizawa *et al*, 1976).

Oxidation is easier to do electrochemically. Regeneration of NAD(P) is thought to happen by a one step transfer of two electrons from NAD(P)H to the anode (Aizawa *et al*, 1975). This can be done directly, although a high overpotential is again necessary: 1.1V at a carbon electrode (Bladel & Jenkins, 1975) and 1.3V at a platinum electrode (Wallace & Coughlin, 1977) for NADH. In an attempt to reduce the requirement for this overpotential an electron transport mediator can be added (Tse & Kuwana, 1978) or immobilised on the electrode (Gorton, 1986).

1.5.3 Engineering options

1.5.3.1 General

In addition to the process of regeneration, cofactor recycling also requires a separate process of retention of the cofactor within the reactor so that it can then be used over many cycles. This is not an obstacle if a batch stirred tank reactor is used as all the reaction components are added to the reactor and are not moved from there. However, if continuous or plug flow reactors are used or if strategies such as *in situ* product removal are adopted, there may be a need to actively retain the cofactor within the reaction environment by some means. Many strategies have been described for this, including:

- microcapsules / artificial cells (Chang, 1987)
- coimmobilisation of enzyme and cofactor (Mansson *et al*, 1983; Yamazaki & Maeda, 1987)
- reversed micelles (Hilhorst *et al*, 1983)

- immobilisation on various supports
- dialysis
- enzyme membrane reactor (stirred tank / hollow fibre)
- charged membrane reactor

Of these, the continuous stirred tank membrane reactors have proved most successful. The nicotine adenine cofactors are very small molecules with molecular weights of around 700. Therefore retention can be difficult, as they will not be retained by a normal membrane as enzymes are. For this reason, many of the methods described rely on the cofactor being immobilised onto soluble or insoluble polymer matrixes, such as dextran or PEG (polyethylene glycol) (Mansson & Mosbach, 1987).

In addition to these consideration, the synthetic and regeneration reactions could be carried out in separate environments, with the cofactors and reactants cycled between the two environments. Again, this leads to further engineering choices.

1.5.3.2 Enzyme membrane reactor

The principle of the enzyme membrane reactor is that enzymes have high molecular weights and so are retained by a semi-permeable ultrafiltration membrane, where as most substrates and product are of lower molecular weight and so can pass through such a membrane. Cofactors are also of low molecular weight and so they are not usually retained by. However, it is possible to derivatise a cofactor by various methods, including linking to PEG or dextran, so that the effective molecular weight is increased to such an extent that it will be retained. The activity of these derivatised cofactors is not adversely affected in most cases (Mansson & Mosbach 1987). Once both the enzymes and cofactors are retained in the reactor, the products and unreacted substrates can be easily removed to allow for a continuous system, incorporating product removal systems. Much work has been done on the enzyme membrane reactor with cofactor recycling, including scale up to preparative scale.

The enzyme membrane reactor can take two forms. It can either be a stirred tank or a hollow fibre. Intensive stirring in the stirred tank and flow of medium through the hollow fibre prevents concentration polarisation of the membrane in each case (Kragl *et al*, 1993). In addition the enzyme can be retained by the membrane or immobilised on the membrane, each with either diffusive or convective transport. Kragl and co-workers (1993) describe the use of an enzyme membrane reactor in the industrial production of l-tert leucine.

1.5.3.3 Charged membrane reactor

The enzyme membrane reactor described previously has certain disadvantages, one of which is that it requires the derivatisation of the cofactor so that it will be retained by the ultrafiltration membrane. Although many enzymes are still able to function well with the derivatised form of the cofactor, there are some which have a much reduced activity with a derivatised compared to a native cofactor. Ikemi and co-workers (1990) describe a modification to the enzyme membrane reactor, where the ultrafiltration membrane used is negatively charged, thus it retains both large molecules and smaller ones with a negative charge. The nicotinamide adenine cofactors are negatively charged and so are retained effectively by this type of membrane, although the degree of retention depends on conditions such as the pH, ionic strength and the presence of inorganic salts which may shield the electrostatic repulsion. This method has been used in the continuous production of sorbitol, with cofactor regeneration using an enzymatic system providing a turnover number of 106,000 (Ikemi *et al*, 1990).

1.5.4 Choice of conditions

When considering a cofactor recycling system in a bioconversion process, there are many different options to consider. The basic principle of cofactor regeneration must be considered, as well as the specific method. This will be determined both by the cofactor to be regenerated and the target reaction. Factors to examine include whether the target reaction occurs in organic or aqueous phase, the optimum pH and temperature and the rate of the target reaction, weighing up these factors should allow certain choices to be disregarded at the outset while others may need further

investigation. For example, if choosing an enzymatic regeneration method, the second enzyme chosen should have a similar optimum of pH and temperature to the target enzyme and whichever method of regeneration is chosen, the rate of regeneration should be similar to or faster than the target reaction, otherwise, higher concentrations of cofactor will need to be added.

The reactor design and configuration also has to be considered. The simplest method is to use a batch stirred tank reactor with the regeneration process occurring *in situ*. More commonly, a continuous process is preferred when a method of retaining the cofactor is a necessary part of its recycle. The choice of reactor will depend both on the target reaction and the regeneration reaction, although one type of regeneration adopted may rule out certain retention methods. If a method is not ruled out completely, one method of retention may be a more suitable combination with a particular type of regeneration process. For instance, the enzyme membrane reactor is well suited to work with enzymatic methods of regeneration and a charged membrane may not be suitable for use with electrochemical methods.

1.5.5 Scale-up

Most published work on cofactor recycling has been done on an analytical or practical scale (Coughlin *et al*, 1988). There is little that has been described for developing such processes to a scale suitable for an industrial process. Ikemi and co-workers (1990) describe an enzymatic method of NAD regeneration in a charged membrane reactor for the industrial production of sorbitol, but this was at a relatively small volume and low concentration Others have mentioned the implications of scaling-up a particular method, for instance the scale-up of enzymatic methods using a second dehydrogenase enzyme will encounter problems associated with product inhibition (Lee & Whitesides, 1985).

Many factors will determine whether a particular process is suitable for industrial scale. The cost of the process may be prohibitive, even when recycling is adopted. For this reason, when a cofactor recycling system is investigated, the whole cost of the process including the cost of extra equipment and consumables must be

considered. The process must also be robust enough to work well at a larger scale. For instance, the membranes used in enzyme membrane reactors must be strong enough to cope with the conditions of the process. Likewise, any immobilisation support must be structurally sound enough to cope with any stresses applied to it.

Mixing may become significant, especially if the regeneration process has many constituents, like a second enzyme or many electron mediators. This problem may be overcome by immobilising these constituents together or retaining them in microcapsules so that they are always in close proximity. However, this may bring new constraints from mass transfer. If a two phase system is adopted, mass transfer between phases can be improved by adjusting the interfacial area (Brookes *et al*, 1986). This can be done by decreasing the bubble, droplet or solid size of a gas/liquid, liquid/liquid or solid/liquid interface respectively. There must be a compromise in these situations. If the interfacial area is high, making particles small, there will be difficulties during downstream processing with removing them from the products. Small solids are difficult to remove from a liquid, small liquid droplet size leads to emulsions and small bubbles can damage an enzyme or cause foaming (Prins & vant Reit, 1987). To maintain an increased interfacial area, extra energy is required to be added via the agitator (Dararíswamy & Sharma, 1984). There may also be problems in areas of poor mixing where phase separation can occur and areas of high mixing where stable emulsions may result.

Some enzymatic methods of regeneration require the addition of hydrogen or oxygen gas as the terminal electron source or sink respectively. Oxygen transfer, and therefore probably hydrogen transfer rates decrease with scale, therefore the supply of these gases could become a limiting factor on a large scale.

An increase in volume when scaling-up has to be accompanied by an increase in substrate and product concentration so that the space-time yield remains high. This may introduce problems of substrate and product inhibition or toxicity. Therefore, at larger scales options such as ISPR and substrate feeding may have to be adopted or considered. Product inhibition is known to be a problem with many dehydrogenase

enzymes (Lee & Whitesides, 1985) and so may be a limiting factor when using some enzymatic methods of regeneration.

1.6 Tetralone - tetralol bioreduction

The bioconversion chosen as a model system in this project involves the asymmetric reduction of a ketone to an alcohol, with the introduction of a chiral centre (Figure 1.11). This reaction forms one step in a series of chemical steps to produce the drug candidate MK-0499. MK-0499 is a potent potassium channel blocker which mediates repolarization of cardiac tissue (Tschaen *et al*, 1995). The chemical reduction proved inefficient and so a microbial method was developed. The yeast *Trichosporon capitatum* was the best catalyst found as it produced the S enantiomer of the product with an enantiomeric excess of between 71% and 99% depending on the growth conditions of the yeast and the bioconversion conditions. The yeast was grown to a cell density of 32g dry cell weight per litre on a specially devised medium with glycerol as a carbon source, rather than glucose, as this supported a higher enantiomeric excess (Reddy *et al*, 1996).

The substrate (6-bromo-β-tetralone) is poorly water soluble and so is dissolved in ethanol before addition to the reactor with the cells in mid exponential phase. This resulted in up to 1.42g/L of the product ((S)-6-bromo-β-tetralol) being produced in 8 hours representing a conversion of 71%.

In this case, the cofactor recycle was performed by the cell in which the target bioconversion occurred. To study other methods of cofactor recycle, it will be necessary to isolate the enzyme which catalyses this reduction.

This model system was chosen because it is representative of many industrially useful redox reactions. The substrate and product are both fairly insoluble in water, which is typical of many industrial reactions. The conversion of substrate to product is fairly high with whole cells, but it could possibly be improved by using an isolated enzyme so that side reactions would not occur. The reaction is also a reduction which produced a chiral centre which is the type of reaction which is industrially useful.

6-bromo-β-tetralone (s)-6-bromo-β-tetralol

Figure 1.11: Tetralone to tetralol bioconversion.

This makes the regeneration of NAD(P)H necessary. Therefore, many of the problems which will have to be considered with this model reaction are also likely to be applicable in industrial situations.

2 A structured approach to the selection of cofactor recycle systems for redox biocatalysis

2.1 Summary

A decisional tool is proposed to assist the choice of the optimal enzymatic cofactor regeneration system for redox biocatalysis. The tool comprises a series of questions addressing a potential process involving an enzymatic cofactor regeneration system and offers possible solutions to guide an engineer to an economically feasible process. The questions are weighted according to the economic scenario of the process, taking into account the relative costs of the synthetic and regenerative enzymes and the synthetic substrate.

2.2 Introduction

In recent years biocatalytic methods have become an established tool in the chemical synthesis of complex agrochemical and, in particular, pharmaceutical molecules. Indeed in 1994 fifteen of the 25 top-selling drugs were single isomers and this development is set to continue with biocatalysis playing a vital role (Cannarsa, 1996 and Davies & Reider, 1996) To date the majority of implemented reactions are hydrolytic, in particular for racemic resolution. However future application of the enzyme-based methods must capitalise on their exquisite ability to deliver optically pure molecules with multi-chiral centres and regioselective functionality. Hence application will move beyond the use of hydrolytic reactions to asymmetric carbon-carbon bond synthesis and redox reactions (Faber, 1997). In the case of redox reactions current implementation is frequently limited by the need to supply expensive cofactors essential for enzyme activity. This can be achieved in a whole cell by supply of co-substrate (Kometani *et al,* 1994) or by cofactor recycle using a second reaction system. For reactions involving large complex molecules (limited by cellular access) (Angelova & Schumauder, 1999) and those where side reactions may occur this isolated enzyme with cofactor recycle should be considered and this is the subject of the decision-making tool presented in this work.

Cofactor recycle can be separated into the processes of regeneration of the active cofactor, which can be brought about by enzymatic, chemical or electrochemical methods (Chenault *et al,* 1988), and retention of the cofactor within the reactor, to allow reuse and therefore recycling of the cofactor. This chapter will exclusively address the regeneration of the cofactor by enzymatic methods.

The need for a rapid decision making procedure to help define the process flowsheet is twofold. First there is increasing pressure, especially in the pharmaceutical sector, to develop processes rapidly since speed to market is essential. Secondly process research which had been established in recent years to overcome productivity limitations for biocatalytic processes provide an ever increasing number of decisions to be taken in order to provide the optimal flowsheet. One area in which this decision making can render a process feasible or otherwise is in the decisions surrounding cofactor recycle. This chapter provides a framework for making such decisions dependent upon the particular economic scenario of a given reaction.

2.3 Economic scenarios

Within any industrial enzyme catalysed process different economic scenarios can arise depending on the relative costs of various components of the process. The product value in these situations is assumed to be high enough to make the process economically feasible. The cost of the synthetic enzyme (which produces the desired product) and regenerative enzyme (which regenerates the cofactor) along with the synthetic substrate, relative to each other, are taken here as the variables in each economic scenario (Table 2.1). The absolute value of the costs are ignored, it is the costs of each variable relative to each other which is considered. The enzyme costs should be considered in cost per unit of activity, thus removing the need to consider how much of the enzyme is needed. The activities of the two enzymes should be closely matched so that the production and consumption of the cofactor occurs at similar rates. The cost of the regenerative substrate is ignored here for the sake of clarity but it is accounted for later in this analysis. In any case a regeneration system which requires a high cost regenerative substrate will not be economically viable. The relative costs of these factors influence the decisions to be made when choosing

Table 2.1. Economic scenarios, comparing the relative costs of the synthetic and regenerative enzymes and the synthetic substrate.

	Economic scenario	synthetic enzyme cost	regenerative enzyme cost	synthetic substrate cost
1	All costs high	high	high	high
2	Low cost synthetic enzyme	low	high	high
3	Low cost regenerative enzyme	high	low	high
4	Low cost synthetic substrate	high	high	low
5	High cost synthetic enzyme	high	low	low
6	High cost regenerative enzyme	low	high	low
7	High cost synthetic substrate	low	low	high
8	All costs low	low	low	low

an enzyme regeneration system, making some questions more important to consider and find solutions for, than others. For instance, if the cost of the regenerative enzyme is low, then it can be used at an operational pH which is not its own pH optimum. This will mean that more of the enzyme has to be added to obtain its optimum activity, but as the cost is low, this will still not contribute greatly to the overall cost of the process. Strategies such as enzyme immobilisation should not be considered in this scenario as this will unnecessarily add to the cost of the overall process without giving any extra advantage. Conversely, if the regenerative enzyme cost is high, then the process will have to be optimised in the direction of the regenerative enzyme, and strategies such as immobilisation, allowing for reuse of the enzyme should be implemented.

2.4 Selection strategy

2.4.1 Decision chart

In order to make a decision on the process conditions to be used, certain experimental investigations must be carried out, so that the decision can be justified on the basis of these results. Figure 2.1 shows the 'master' decision chart which shows all of the possible decisions and questions to be considered when approaching an enzymatic cofactor recycle method.

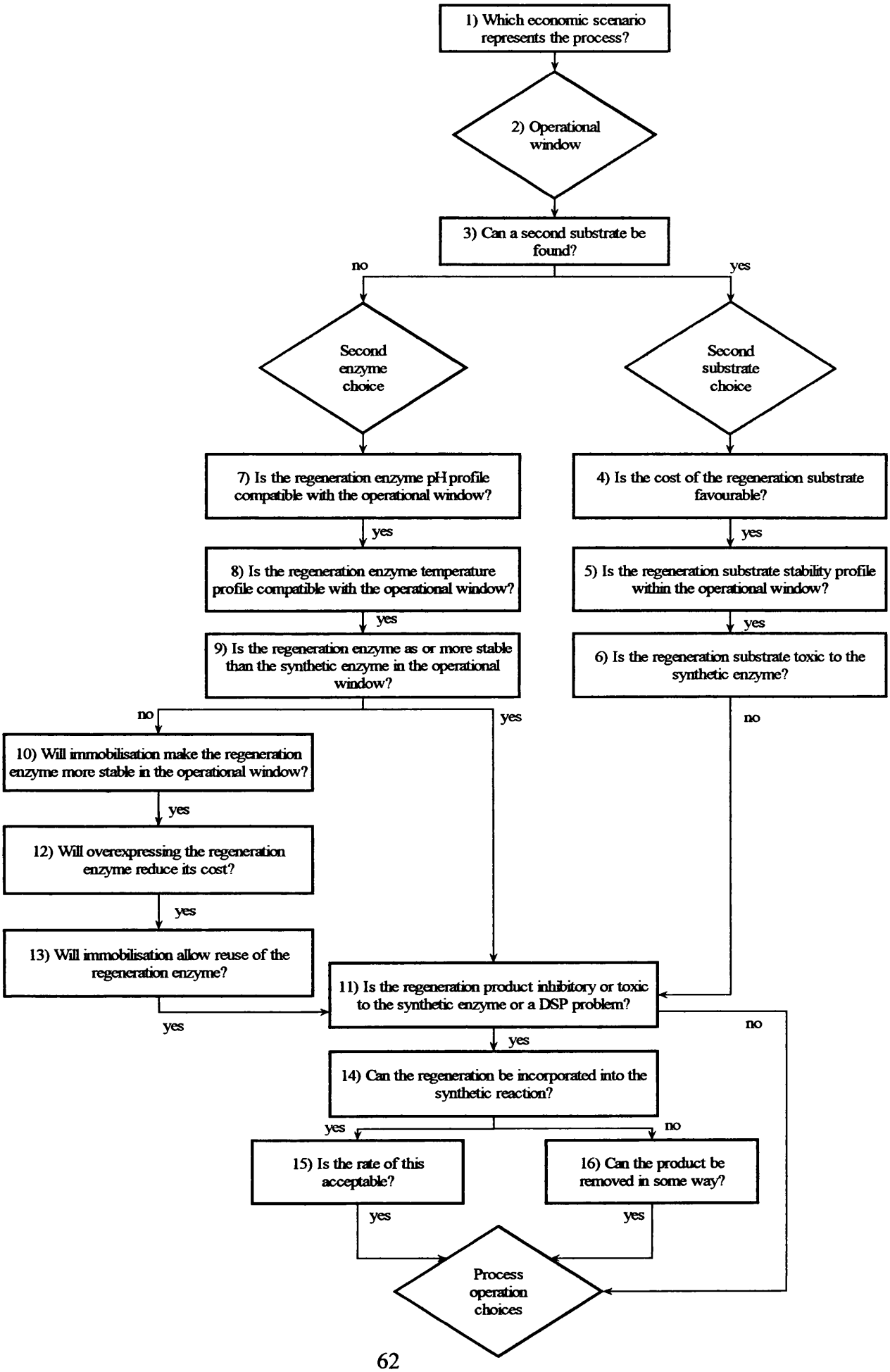
2.4.2 Economic scenario

The relative costs of synthetic and possible recycle enzymes, and also the synthetic substrate will give weight to certain questions depending on which of the components contributes the most to the cost of the process (question 1 in figure 2.1). Certain decisions can be given more or less weight depending on the scenario arising, i.e. product inhibition becomes less important with decreasing enzyme cost. This is discussed fully in section 2.5.

2.4.3 Synthetic reaction

After determining which economic scenario the process fits, the next consideration when choosing the regeneration system for a cofactor dependent reaction is to

Figure 2.1, following page:
Decision chart for enzymatic cofactor regeneration. Diamond boxes represent a fixed decision, for example whether the cofactor is to be regenerated by a second enzyme or a second substrate method. Rectangular boxes represent questions to be asked about the proposed process. For each question there is a possible 'yes' or 'no' answer. Where an arrow for an answer is not shown, that answer leads to another choice of enzyme or process. For example, question 7: is the regeneration enzyme pH profile compatible with the operational window?, if the answer is yes, follow the arrow to question 8, if the answer is no the regeneration enzyme is not suitable for this synthetic reaction and a different enzyme should be found.
DSP: Down Stream Processing.


1) Which economic scenario represents the process?
2) Operational window
3) Can a second substrate be found?
no
yes
Second enzyme choice
Second substrate choice
7) Is the regeneration enzyme pH profile compatible with the operational window?
4) Is the cost of the regeneration substrate favourable?
yes
yes
8) Is the regeneration enzyme temperature profile compatible with the operational window?
5) Is the regeneration substrate stability profile within the operational window?
yes
yes
9) Is the regeneration enzyme as or more stable than the synthetic enzyme in the operational window?
6) Is the regeneration substrate toxic to the synthetic enzyme?
no
yes
no
10) Will immobilisation make the regeneration enzyme more stable in the operational window?
yes
12) Will overexpressing the regeneration enzyme reduce its cost?
yes
13) Will immobilisation allow reuse of the regeneration enzyme?
yes
11) Is the regeneration product inhibitory or toxic to the synthetic enzyme or a DSP problem?
no
yes
14) Can the regeneration be incorporated into the synthetic reaction?
yes
no
15) Is the rate of this acceptable?
16) Can the product be removed in some way?
yes
yes
Process operation choices

establish the operating window of the synthetic system (question 2). That is, the stability of the substrates, products and cofactors and the enzyme's stability and activity over a range of pH and temperatures. This defines the region of useful activity for the enzyme and required productivity; giving a good indication as to the possible reactor options (Woodley & Lilly, 1996 and Lilly & Woodley, 1996). The window will depend on the amount of acceptable activity loss the process can tolerate. A low cost process can have a more flexible operating window, with a wider range of pH and temperature, as more activity or stability can afford to be lost. The flexibility or rigidity of the operating window will be directed by the most expensive component of the process. Any regeneration system must work within these parameters otherwise a loss in productivity will arise.

2.4.4 Second Substrate

The synthetic reaction may be reversible; this can be demonstrated by adding an excess of product to the enzyme and assaying for the appearance of substrate (question 3). If reversibility is seen, it is possible to screen for more effective regenerative substrates. Certain redox enzymes can simultaneously run both the synthetic and recycle reactions with the addition of a thermodynamically favourable quantity of regenerative substrate, such as Alcohol dehydrogenase from *Thermoanaerobium brockii.* (Peretz *et al,* 1993 and Keinan *et al,* 1986). This situation is often preferable, as the window of operation requires little adjustment as the process only needs to be optimised for one enzyme, therefore the likelihood of conflicting requirements is much reduced. If a second substrate can be found, this is the generally the preferred option to using a second enzyme, but once the second substrate choice is made, there are further questions which must be made of the process regarding this regenerative substrate.

2.4.5 Regenerative Substrate

The regenerative substrate should be as cheap as possible, and preferably much less than the synthetic enzyme and substrate, otherwise the regeneration costs too much (question 4). The regenerative substrate should be stable within the operating window, otherwise a compromise may need to be found between this substrate and

the enzyme, which lends less weight to the use of one enzyme running in both directions (question 5). If the regenerative substrate is toxic to the synthetic enzyme, the enzyme will lose activity and therefore more will have to be added to achieve a reasonable rate (question 6).

2.4.6 Regenerative Enzyme

There must be a pH window within which both enzymes have satisfactory activity (question 7). Ideally there should be a good overlap of the pH optimum for the regenerative and synthetic enzyme, although if the relative cost of the regenerative enzyme is low, a pH could be chosen away from its maximum activity. However in all cases if there is no overlap, the enzyme cannot be considered further.

The same principles apply to temperature compatibility as with pH compatibility, in that some drop in activity can be tolerated for the regenerative enzyme if its cost is low and the two temperature profiles do not overlap exactly (question 8). Care must be taken to ensure sufficient activity is seen at the defined operating temperature: for example novel enzymes from extremophiles are becoming commercially available, which are often quoted in terms of their activity at above average temperatures for "normal" biocatalysts such as the activity of Glucose dehydrogenase from *Thermoplasma acidophilum* which is quoted at 55°C (Smith *et al*, 1989). When these enzymes are used at a temperature more suitable for most enzymes, their activity may be too low and therefore, price per unit of activity too high.

The residual activity of a potential regeneration enzyme over time must be investigated within the operating window (question 9). The regenerative enzyme may have limited stability, such as Alcohol dehydrogenase from baker's yeast which is unstable even at 25°C, *c.f.* Glucose dehydrogenase from *Bacillus cereus* which is stable at 55°C for 7 days (Wong *et al*, 1985). Ideally the regenerative enzyme should be at least as stable as the synthetic enzyme, to reduce its contribution to the total cost.

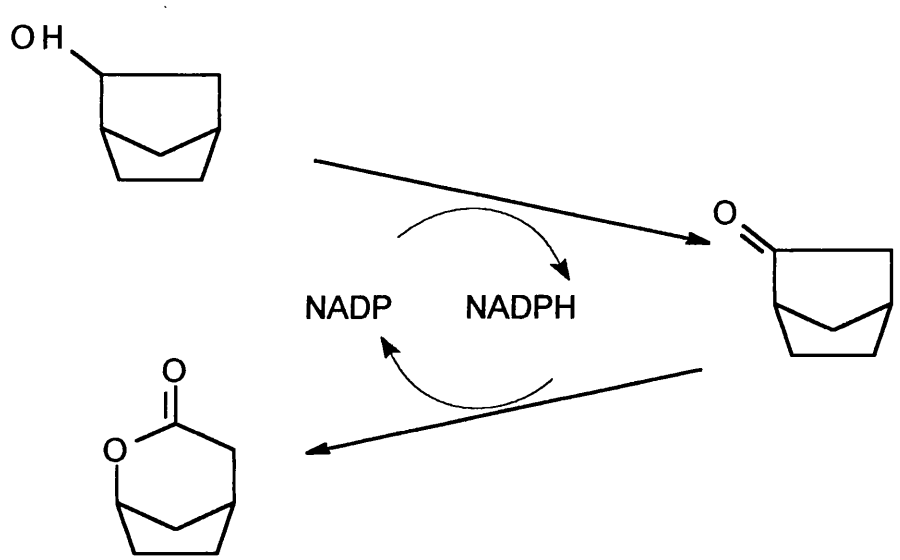
Immobilisation can lead to the enzyme being reused and in some cases an increase in the enzyme's stability is also observed, such as increasing the enzyme's resistance to denaturation by protection against interfaces and steric resistance (Gerhartz, 1990). Increasing the stability of the regenerative enzyme may allow the process to be run under conditions of temperature and pH which are more favourable to the synthetic enzyme (question 10). Downstream processing costs will be reduced and product recovery made easier if the catalyst can be separated from the reaction media efficiently (question 13). Immobilisation will only allow reuse if the stability of the enzyme allows it to retain sufficient activity over the period it is intended to be used. It is likely that regenerative enzyme costs will be high unless a recombinant is available (question 12), e.g. Glucose-6-Phosphate dehydrogenase from *Leuconostoc mesenteroides* is relatively cheap due to the cloning and overexpression of it's gene into *E.coli.*

2.4.7 Regenerative product

Many useful NAD(P)H generating dehydrogenases suffer from product inhibition at relatively low concentrations (Chenault & Whitesides, 1987) (question 11). Also gluconate (6-phosphate), the product of the glucose(-6-P) dehydrogenase catalysed reaction can hamper synthetic product recovery, as well as potentially degrading NAD(P)(H) by phosphate leaching (Chenault *et al,* 1988). Inhibition or toxicity to the synthetic enzyme will reduce the yield on the enzyme and lower conversion of the regenerative substrate will have to be tolerated.

In some cases the regenerative enzyme can produce a product which is the substrate for the synthetic reaction (question 14), i.e. produce a ketone used as a substrate by Baeyer-Villigerases by dehydrogenation of the alcohol precursor (figure 2.2) (Gagnon *et al,* 1994). This can potentially remove problems caused by either the regenerative product or synthetic substrate, as they are the same compound. The concentration of this intermediate will be kept low by consumption to form the synthetic product. This type of combined regenerative and synthetic reaction will only be successful if the rate of the overall reaction is acceptable (question 15). The regenerative enzyme will probably have to process a more complex molecule than the

Figure 2.2.
The recycling of NADPH using a two step conversion on a alcohol derivative of a bicyclic ketone to produce lactones.



simple regenerative substrates generally used as it is a precursor of the synthetic product and as the reaction rate may be much lower than expected.

It may be possible to actively remove the regenerative product as it is formed, removing inhibition/toxicity against the enzyme and increasing the synthetic product concentration (question 16). Various options for ISPR (*in situ* product removal) have been discussed elsewhere (Freeman *et al*, 1993 and Chauhan & Woodley, 1997).

2.5 Modifications to selection strategy

2.5.1 Variation to decisional chart

Once the economic scenario for a particular process has been short cuts can be made to the 'master' decision chart proposed here. Some questions will become important and should be given a greater weighting than other questions which may not be critical to making the process economically viable, depending on which are the most significant costs in the process. Many of the questions proposed in this scheme may have a flexible answer, rather than a 'yes' or 'no' answer. Taking question 2 as an example: the operational window for the synthetic reaction. The lower cut off point for the activity of the synthetic enzyme, below which the enzyme's activity is considered too low, is decided by the operator of the scheme. Thus for an expensive synthetic enzyme the lower cut off point chosen will be higher and the operational window more rigid than for a cheaper synthetic enzyme which will have a more flexible operational window.

Table 2.2 shows a summary of the variations which can be made to the decision chart in figure 2.1 which are also discussed more fully below. An example of the modified chart (figure 2.3) for a low cost regenerative enzyme scenario is given and discussed in section 2.5.4.

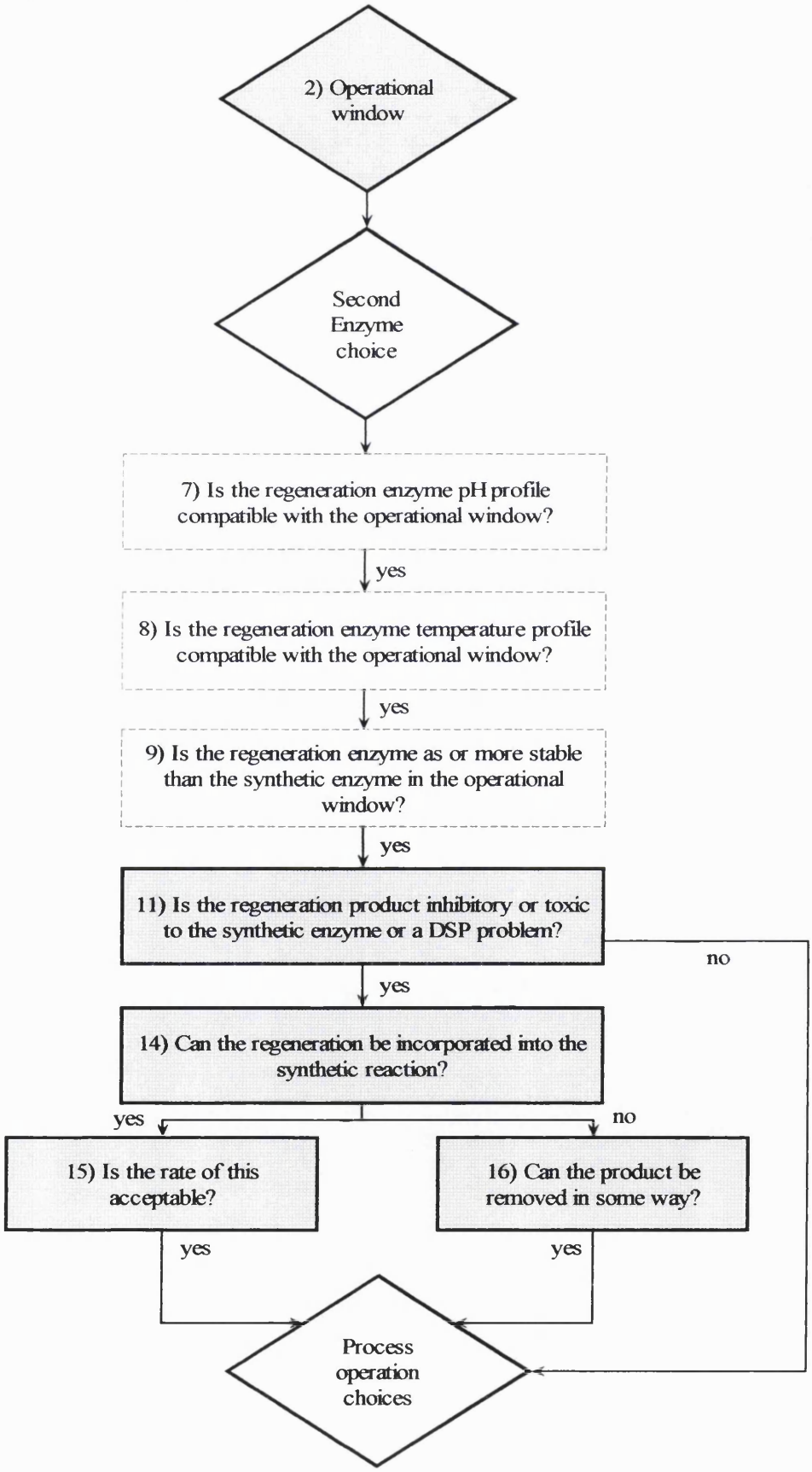
2.5.2 All costs high

When all the costs considered in these economic scenarios are high both enzymes and the substrate have to be used efficiently. The pH profiles of the two enzymes

Table 2.2. (following page)
Modifications to the decision chart depending on the economic scenario for the process. The question numbers refer to the numbering of the boxed questions in figures 1 and 3.

● important questions, O questions which can be ignored, ◑ questions which have more flexible answers.

Figure 2.3.
Decision chart for low cost regenerative enzyme scenario as an example of how the chart can be modified for different economic scenarios. Shaded boxes represent important questions which should be given more weight. Boxes bordered with broken lines represent less significant questions which can be more flexible.



must be compatible so that each has its highest activity under the process conditions. Measures to reduce the enzyme cost or spread the cost of the enzymes over several uses, such as overexpression or immobilisation are useful and worth investigating. High product concentrations are needed to get good use of the substrate and so product inhibition and toxicity should be eliminated where possible.

2.5.3 Low cost synthetic enzyme

When the synthetic enzyme is low cost the operational window can be more flexible to allow for conditions which result in slightly lower activities and stability's for the synthetic enzyme. Question 3 is important as using a second substrate method would reduce the cost as the enzyme is cheap. It is important that the regeneration enzyme is optimised, therefore questions 7 and 8 are significant: a regenerative enzyme which has optima of pH and temperature outside the synthetic enzyme's operational window will have a low activity and therefore more of the enzyme will be required.

2.5.4 Low cost regenerative enzyme

The process should be optimised for the more expensive synthetic enzyme, even if this is to the detriment of the regenerative enzyme. The use of a second substrate is not considered as this would require the use of more of the expensive synthetic enzyme. In this situation, the synthetic substrate cost is also high and so strategies to optimise the use of the substrate must be employed. For example, conversion of the substrate to the product must be optimised therefore questions dealing with the regenerative product are important. Some of the questions concerned with the regenerative enzyme can be ignored completely, i.e. issues of immobilisation and overexpression. Other questions concerned with the regenerative enzyme still have to be considered (i.e. issues of pH and temperature) but the outcome of these questions can be more flexible and therefore not such a strict overlap with the synthetic enzyme is necessary.

2.5.5 Low cost synthetic substrate

The process must be optimised for the two enzymes, possibly at the expense of the substrate, therefore any strategy which reduces the cost of the enzymes should be investigated. Again, as the synthetic enzyme is expensive, the second substrate option is not considered. Questions concerning the regenerative product and conversion of the synthetic substrate to product are not so important as the substrate can be sacrificed to some extent if necessary.

2.5.6 High cost synthetic enzyme

The process must be optimised for the synthetic enzyme as this contributes most to the cost of the overall process. Some sacrifice of the regenerative enzyme's activity and stability can be tolerated as more of this enzyme can be added without a great increase in cost, therefore, the pH and temperature of the process does not have to coincide exactly with the optimum of the regenerative enzyme. However, questions 7 and 8 still have to be addressed as the regenerative enzyme must have some activity and stability under the operational conditions. The second substrate is not a viable option as the more of the high cost synthetic enzyme will be needed and using the cheaper second enzyme is likely to reduce the cost of the process as a whole. Questions 10, 12 and 13 are ignored as the additional cost of immobilisation or overexpression cannot be justified when the regenerative enzyme is cheap.

2.5.7 High cost regenerative enzyme

The process must be optimised for the regenerative enzyme. The pH and temperature compatibility of the synthetic and regenerative enzymes is important as the regenerative enzyme must be used at its maximum activity and stability. Strategies such as immobilisation and over expression are important if they result in an overall decrease in the cost of the regenerative enzyme. The second substrate option is a good one in this scenario as it eliminates the need for the expensive regenerative enzyme.

2.5.8 High cost synthetic substrate

Both enzymes are cheap and therefore can be sacrificed, but the yield on the synthetic substrate is important. The operational window must allow for the stability of the substrate. Questions 14 and 15 are ignored as more of the synthetic substrate is likely to be needed.

2.5.9 All costs low

The solution to each of the questions is more flexible than with other scenarios as some loss can be tolerated for each of the enzymes and the substrate. Each question still has to be considered as the two enzymes must be compatible to some extent and there cannot be unlimited inhibition or toxicity. The extra cost of strategies such as overexpression, immobilisation and ISPR cannot be justified when the cost of each of these enzymes is low already.

2.6 Discussion

A rational selection strategy can be drawn up for the selection of an enzymatic cofactor regeneration process as detailed in this paper. If the economic scenario is considered for a process, including the relative costs of the synthetic substrate and enzyme and the regenerative enzyme, the decisional chart can be modified. In some circumstances modification can lead to the amount of experiments used to define the process being reduced, thus potentially cutting the cost of process synthesis and time to market.

The decisional tool proposed in this paper is not exhaustive and does not account for methods of cofactor regeneration other the enzymatic, although enzymatic regeneration tends to be the method of choice in most cases (Chenault *et al,* 1988). This selection strategy also does not address the issue of reactor design and retaining the cofactor and regeneration system to allow for recycling of the cofactor. Considering these extra factors will lead to more selection strategies including the type of reactor to choose and whether the synthetic and regeneration reactions occur in the same or different vessels.

As recombinant technology increasingly allows for production of large quantities of enzymes at relatively low price and the substrates used are becoming more complex, scenario 7 will be the most common.

3 Isolation of an oxidoreductase enzyme from the yeast *Trichosporon capitatum*

3.1 Summary

An NADH-specific reductase enzyme from the yeast *Trichosporon capitatum* MY1890 was isolated to apparent homogeneity which reduced 6-bromo-β-tetralone to its corresponding (S)-alcohol, ((S)-6-bromo-β-tetralol) with an enantiomeric excess of 99.54%. Purification involved protamine sulphate precipitation, anion exchange chromatography on Q Sepharose FF, affinity chromatography on Amicon Affinity Dyematrex Red A and gel filtration on Superdex 200 HR. The final purification stage resulted in a 54-fold increase in specific activity, to 430U/mg with a yield of 1.34%.

3.2 Introduction

Dehydrogenase enzymes have been widely used for the reduction of carbonyl groups of aldehydes or ketones and of carbon-carbon double bonds, as well as in the recycle of nicotine adenine cofactors (Davies and Reider, 1996; Cannarsa, 1996). Both of these reactions offer potential asymmetric syntheses of a pro-chiral substrate leading to a chiral product. This reaction is potentially very useful in the production of optically pure pharmaceutical intermediates and other fine chemicals (Faber, 1997).

The yeast *Trichosporon capitatum* was identified as a useful biocatalyst in the asymmetric bioreduction of 6-bromo-β-tetralone to its corresponding (S)-alcohol, ((S)-6-bromo-β-tetralol) (Reddy *et al*, 1996). This work showed that whole cells of the yeast produced a product titre of up to 1.42g/L (6.3mM) after 4hrs (at an initial reaction rate of 1g/hour) representing a conversion of 71% and an enantiomeric excess of up to 99%. This whole cell catalysed reaction offered a route to an optically enhanced intermediate in the production of an experimental drug difficult to carry out chemically (Tschaen *et al*, 1995).

Bioconversions catalysed by whole cells encounter various difficulties, including diffusion limitations for substrate and product to move into and out of the cell which

can lead to a low conversion (Angelova and Schmauder, 1999). There is also the possibility of interference from other enzymes within the cell, including side reactions of the substrate or product which decrease the product yield and enantio-complementary enzymes which reduce the enantiomeric purity of the product. Despite these drawbacks, whole cell biocatalysts are frequently favoured when more than one enzyme step is required, or where the enzyme is membrane bound or unstable upon isolation.

Previous industrial examples involving reductase bioconversions have used whole cells (Chartrain *et al*, 1996), mostly due to the high cost of adding cofactors in stoichiometric amounts to isolated enzyme systems. However, many cofactor recycle options are now available and in this chapter the reductase enzyme is isolated both to overcome the problems associated with whole cell operation and to allow the isolated system to be studied.

3.3 Materials and Methods

3.3.1 Yeast cultivation

Trichosporon capitatum MY1890 (Merck & Co. culture collection) was stored as a 1mL frozen cell suspension at -70°C in 25% glycerol. This suspension was thawed to room temperature and used to inoculate a 250mL Erlenmeyer flask containing 50mL of medium (30g/L glycerol, 25g/L hysoy peptone, 20g/L yeast extract (Sigma)). The flask was aerobically incubated on an orbital shaker (250rpm) at 29°C for 24h. Subsequently, a 2L Erlenmeyer flask containing 500mL of medium was inoculated with 10mL of the 24h. seed and incubated in the same conditions as before for a further 48h.

3.3.2 Analytical Methods

3.3.2.1 Spectrophotometer microassay

An assay mixture of 5% of a 10mg/mL tetralone in ethanol solution was mixed with 95% of 0.5mg/mL cofactor (NADH or NADPH) in water. This mixture was stored in a -20°C freezer for up to 1 week and thawed when needed. 500μL of the assay mixture was mixed with 500μL of the enzyme sample to be assayed in a cuvette

and the disappearance of the reduced cofactor was monitored by spectrophotometer at 340nm and 22°C for 10 minutes. The rate of disappearance of the reduced cofactor in a control sample which contained no tetralone was found to be negligible over the 10 minutes assay duration. Enzyme activity is expressed as standard enzyme units per mL (UmL^{-1}), defined as the transformation of 1 μmol/min of substrate (in this case cofactor) per mL.

3.3.2.2 Bioconversion assay

The production of tetralol was measured in fractions which showed a change in cofactor concentration from the spectrophotometer assay. Cuvettes from the spectrophotometer assay were incubated for 24 hours at 22°C and then the contents assayed by reverse phase HPLC to look for tetralol production.

3.3.2.3 Reverse phase HPLC assay

A Zorbax RX-C8 column (250 x 4.6mm) (Mac-Mod Analytical) was used for the separation of the tetralone from the tetralol. The mobile phase was a 50% by volume solution of acetonitrile and acidified water (0.1% phosphoric acid). Detection was by UV at 220nm and 22°C. Tetralone and tetralol eluted at 6.5 and 4.7 minutes respectively, at a flow rate of 1.5mL/min.

3.3.2.4 Total protein assay

Total protein was assayed by the Bradford Coomassie Brilliant blue dye (G250) binding method (Bradford, 1976) using the Bio-rad protein assay kit (Bio-rad, Hercules, CA, USA). Bovine Serum Albumin (BSA) fraction V (Sigma, Poole, Dorset, UK) was used as a standard to calibrate in the range of 0 - 1mg/mL and the absorbance of the assay mixture measured by spectrophotometer at 595nm.

3.3.2.5 SDS-PAGE Gel electrophoresis

Protein purity was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Mattice *et al*, 1976). A 12.5% polyacrylamide resolving gel with a 3% polyacrylamide stacking gel in 0.25M

Tris/glycine buffer at pH 8.3 was employed to separate the proteins. The enzyme samples were pre-incubated in sodium phosphate buffer, pH7 with 1% SDS at 100°C for 5 minutes. Low range SDS-PAGE molecular weight standards from Bio-rad were used for molecular weight determination. The protein bands were stained with coomassie blue G-250 stain for 1 hour, followed by destaining in 30% methanol and 10% acetic acid in water (v/v) for 2 hours.

3.3.2.6 Chiral HPLC assay

The two tetralol enantiomers were separated with a normal phase Chiracel OD column (250 x 4.6mm) (Chiral Technologies) using a mobile phase of 98% hexane and 2% isopropanol. Detection was by UV at 220nm and 22°C. The (S) and (R) enantiomers eluted at 25.37 and 29.31 minutes respectively (Reddy *et al*, 1996) at a flow rate of 0.6mL/min.

3.3.3 Purification Techniques

3.3.3.1 Cell harvest and washing

Cells grown in 2L Erlenmeyer flasks for 24hrs were harvested by centrifugation in a Beckman J2-MI centrifuge (Beckman, High Wycombe, Bucks., UK) at 11,300 x g for 25 minutes. The cell pellet was resuspended to the original volume in 10mM Tris buffer at pH 7 containing protease inhibitor (complete protease inhibitor tablet (Boehringer Mannheim, Lewes, E.Sussex, UK) or phenylmethylsulfonyl fluoride). The resuspended cells were centrifuged for 25 minutes at 11,300 x g, the supernatant discarded and the cells resuspended in buffer as before.

3.3.3.2 Cell disruption

One litre of the washed cells were broken using a microfluidizer M-100EH (Microfluidics Corporation) by 4 passes at 15000bar, which gave at least 99% cell disruption, as seen by light microscopy. Alternatively the washed cells were broken using an APV Lab 60 Homogeniser by 5 passes at 500bar, which also gave 99% cell disruption.

3.3.3.3 DNA removal

At this stage, the DNA can be removed by treatment with protamine sulphate (grade X) from Salmon (Sigma). This causes the negatively charged DNA to precipitate so that it can be removed and then will not adversely bind to the groups on the anion exchange column used subsequently. A 1% protamine sulphate solution in water was prepared (0.01g / ml); 500μL of this solution is added per 10mL of protein sample which was stirred on ice for 30 minutes. The precipitate and cell debris were removed by centrifugation at 39, 200 x g for 30 minutes.

3.3.3.4 Anion exchange chromatography

The clarified homogenate was applied to an anion exchange column. Initially 200mL of DEAE Sepharose CL-6B (Pharmacia) resin was used in a Pharmacia XK26 column, but as the flow rate was very low (2mls/min) this was changed to using 500mls of Q Sepharose Fast Flow resin (Pharmacia) at a flow rate of 20mL/min, which gave a very similar protein separation. About 1L of the clarified homogenate could be applied to the column before the protein was seen to breakthrough. The column was then washed with 2 column volumes (1L) of start buffer which consists of 10mM Tris at pH7 with protease inhibitor added. The protein was eluted between the start buffer and high salt buffer which consisted of start buffer with 1M NaCl added. This was either done with a Pharmacia Biopilot creating a linear gradient between the two buffers over 5 column volumes or by using a step-wise elution of one column volume each of start buffer with 0.2M NaCl, 0.3M NaCl, 0.4M NaCl and 0.5M NaCl, then a 2 column volume wash with the high salt buffer.

Fractions of about 15mL were collected in a Pharmacia LKB FRAC-100 fraction collector and assayed by the spectrophotometer microassay for enzyme activity and also by the Bradford method for total protein content. Active fractions were tested by the bioconversion assay to verify that the tetralol product was produced.

For all the chromatography steps the buffers were kept on ice and the column cooled to 4°C by an external water jacket and a Grant LTD6G cooling water bath. The fractions in the fraction collector were also kept on ice.

3.3.3.5 Affinity chromatography

Two affinity resins were tested; Cibacron Blue 3GA Type 3000 (Sigma) and Amicon Affinity Dyematrex Red A (Millipore).

A column of 50mL of the Cibacron Blue resin was loaded with the pooled, dialysed fractions from the anion exchange column at a flow rate of 0.5mL/min, using a Pharmacia BioPilot. The column was washed with 2 column volumes of the start buffer (low salt), then eluted with a linear gradient over 5 column volumes between the start buffer and high salt buffer, as with the anion exchange column, using the Pharmacia Biopilot. No protein was removed by washing with the start buffer and although protein was eluted with the salt gradient, no enzyme activity was found in the eluent. The column was also washed with the Tris buffer containing 2M NaCl and also with 20mM NADH and 20mM NAD. No enzyme activity was eluted with each of these washes and therefore it was concluded that the protein of interest had bound permanently to the column.

A column of 100mL of the Amicon Red A gel was loaded with the pooled, dialysed fractions from the anion exchange column at a flow rate of 3.5mL/min using a Pharmacia BioPilot. The column was washed with 2 column volumes of start buffer and then a gradient elution was performed between the start buffer and start buffer with 1M NaCl added over one column volume followed by 2 further column volumes of start buffer with 1M NaCl. 10mls fractions were collected and assayed as before.

3.3.3.6 Gel filtration

Active fractions from the affinity column were concentrated with Amicon Centricon-30 centrifugal concentrators (Millipore) and 200μL applied to a Superdex 200 HR 10/30 gel filtration column (Pharmacia) and eluted with a flow rate of 0.75mL/min, 0.1M Tris buffer pH7, using a Pharmacia Biopilot. BioRad gel filtration standards were run under the same conditions on the same column.

3.3.4 Enzyme storage

It was necessary to have a method of storing the enzyme for a few days in between some of the purification steps. Ammonium sulphate precipitation proved to be a reliable method of stabilising the enzyme and was particularly useful in storing the partially purified enzyme after the anion exchange step. The enzyme was found to precipitate at 80% ammonium sulphate and to retain 90% of its activity as a precipitate at 4°C after 21 days. Therefore this method was used for storage of the enzyme.

3.4 Results

3.4.1 Cell homogenate production

Figure 3.1 shows the growth curve for *T. capitatum* in the 2L Erlenmeyer flask. These cells were harvested after about 48 hours, when the cells had reached the end of the exponential phase of growth and a dry cell weight of about $18gL^{-1}$ was routinely obtained.

After harvest, the cells were broken open by microfluidisation or homogenisation. Disrupted cells showed up dark, as opposed to bright, under light microscopy. This showed that microfluidisation resulted in over 95% of cells disrupted and homogenisation resulted in over 98% of cells disrupted.

3.4.2 Anion exchange chromatography

Figure 3.2 shows the chromatogram of a DEAE Sepharose column run with a gradient elution. The protein of interest elutes around fractions 22-30, at a NaCl concentration of about 0.4M. Although this separation is successful, it is lengthy due to the low flow rate ($2mLmin^{-1}$) required by the resin. Therefore, Q Sepharose Fast Flow resin was also investigated as an appropriate separation technique instead of the DEAE Sepharose.

Figure 3.3 shows the chromatogram of a Q Sepharose column run with a straight gradient elution. The enzyme of interest elutes at a salt concentration of

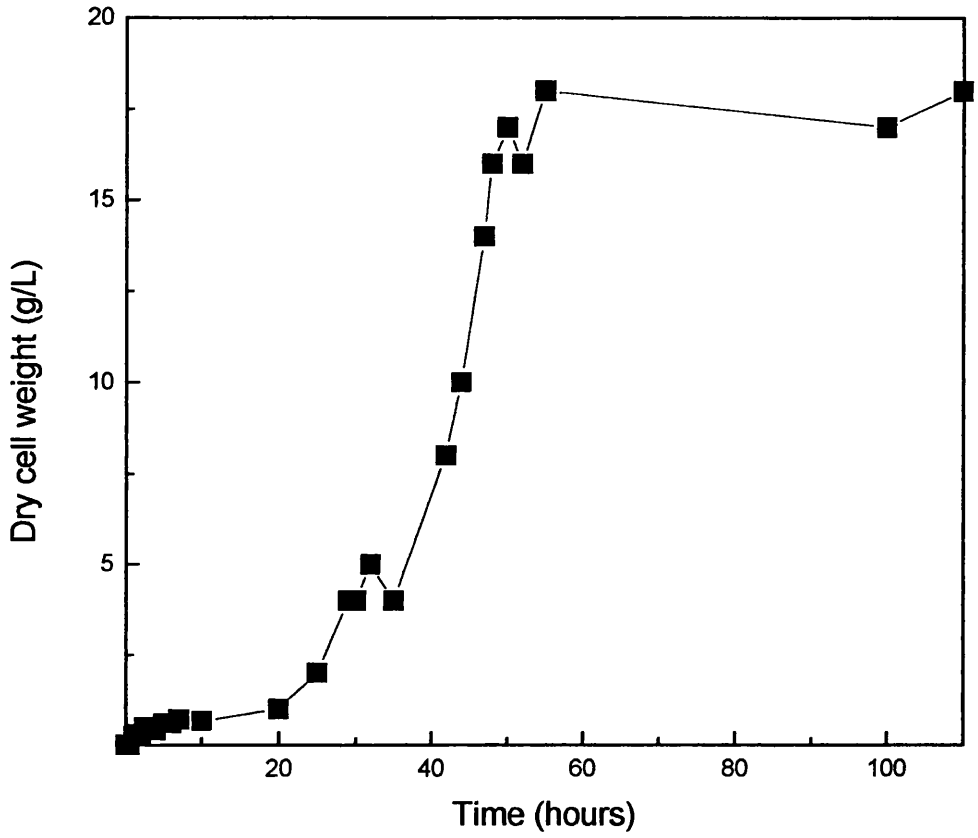


Figure 3.1:
Growth curve for *T. capitatum* grown in a 2L Erlenmeyer flask aerobically incubated on an orbital shaker (250rpm) at 29°C.

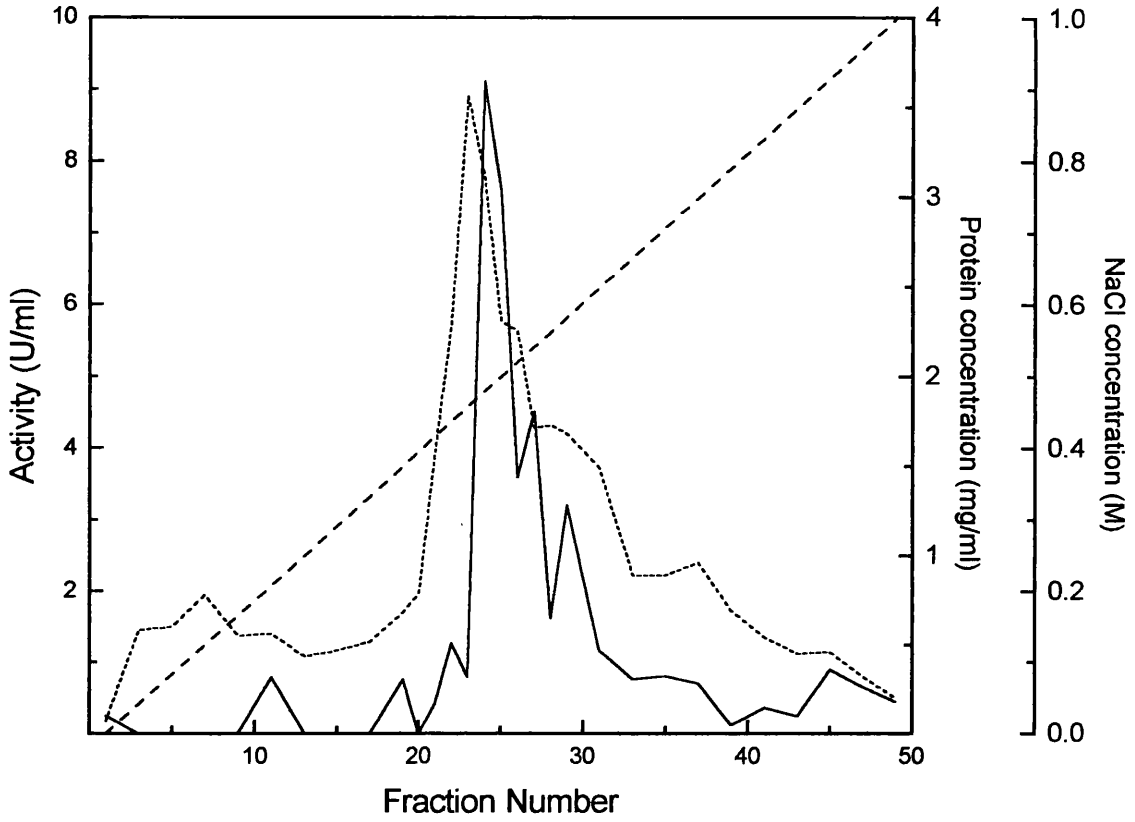


Figure 3.2:
200ml DEAE Sepharose column, loaded with clarified homogenate from *T. capitatum* and run at 2mLmin^{-1} with a gradient NaCl elution as shown (------). Protein concentration determined by Bradford assay (.....), enzyme activity determined by spectrophotometer assay (———).

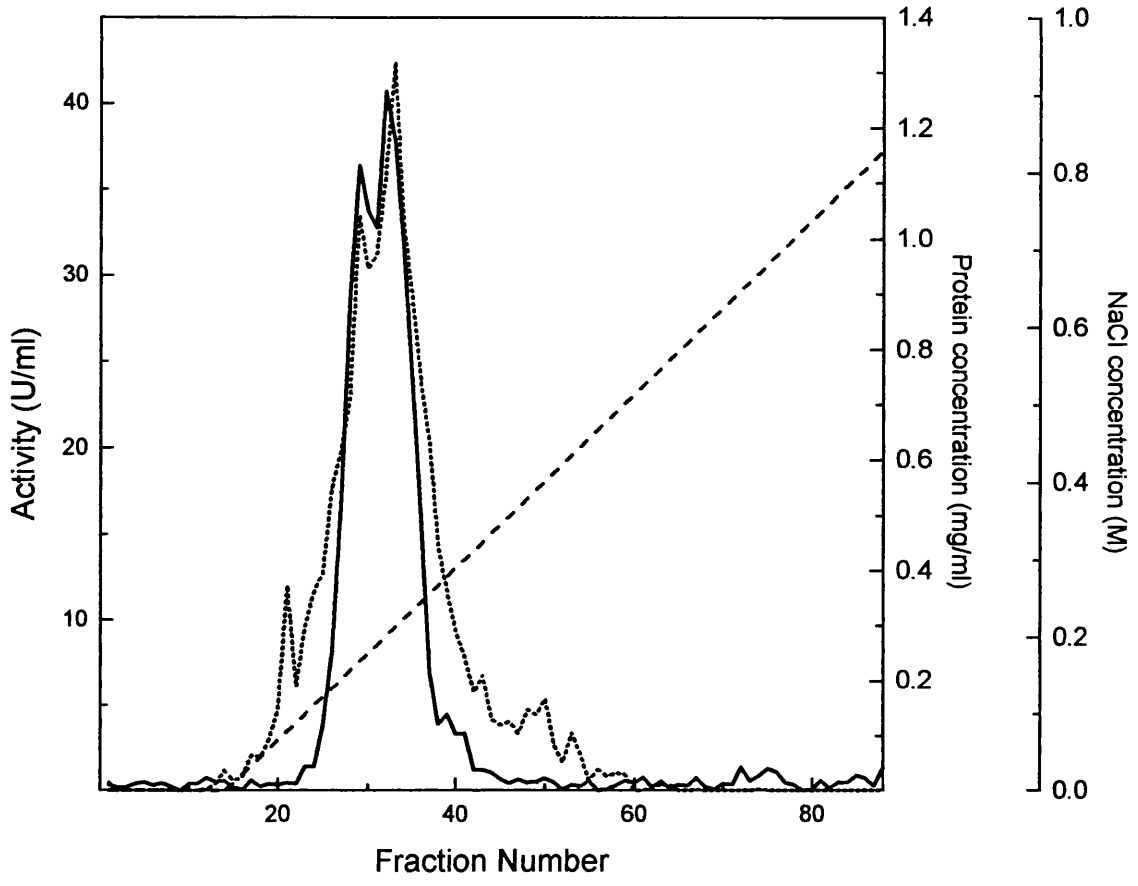


Figure 3.3:
500ml Q Sepharose Fast Flow column, loaded with clarified homogenate from *T. capitatum* and run at 20mLmin^{-1} with a gradient NaCl elution as shown (----). Protein concentration determined by Bradford assay (.....), enzyme activity determined by spectrophotometer assay (——). Fractions 23-42 pooled and dialysed for use in the next stage of purification.

approximately 0.2M NaCl. A similar separation is obtained to the DEAE Sepharose column, although a higher enzyme activity is attained in the Q Sepharose column. Fractions 24 to 36 produced 0.4mM of tetralol in the biotransformation assay, and so these fractions were pooled. These pooled fractions were dialysed against water to remove any salt before being used in the next stage of purification.

3.4.3 Affinity chromatography

Figure 3.4 shows a chromatogram for an Amicon Red A column run with the pooled fractions from figure 3.3, and also includes the result of the bioconversion assay. The protein flows through the column and does not appear to bind, however, many other proteins do bind and a satisfactory purification is obtained.

3.4.4 Gel filtration

The active protein eluted at 18.3mls which was compared with the elution of standard proteins and found to correspond to 80,000MW. These active fractions were run on a native PAGE gel with which showed that fraction 19 contained one major protein band (figure 3.5).

3.4.5 Cofactor preference

The homogenate and active fractions from the anion column were assayed with both the spectrophotometer and bioconversion assays using the phosphorylated (NADPH) and non-phosphorylated (NADH) forms of the cofactor. The activity of the sample with the phosphorylated form was negligible compared with the non-phosphorylated cofactor for both the homogenate and the purified fractions. Subsequently the enzyme activity was only measured with NADH.

3.4.6 Enantiomeric excess

The enantiomeric excess which the purified enzyme gives rise to was assessed by running the microassay sample from fraction 19 of the gel filtration fractions on the chiral HPLC assay. This gave rise to concentrations of 0.87mM (S)-6-bromo-β-

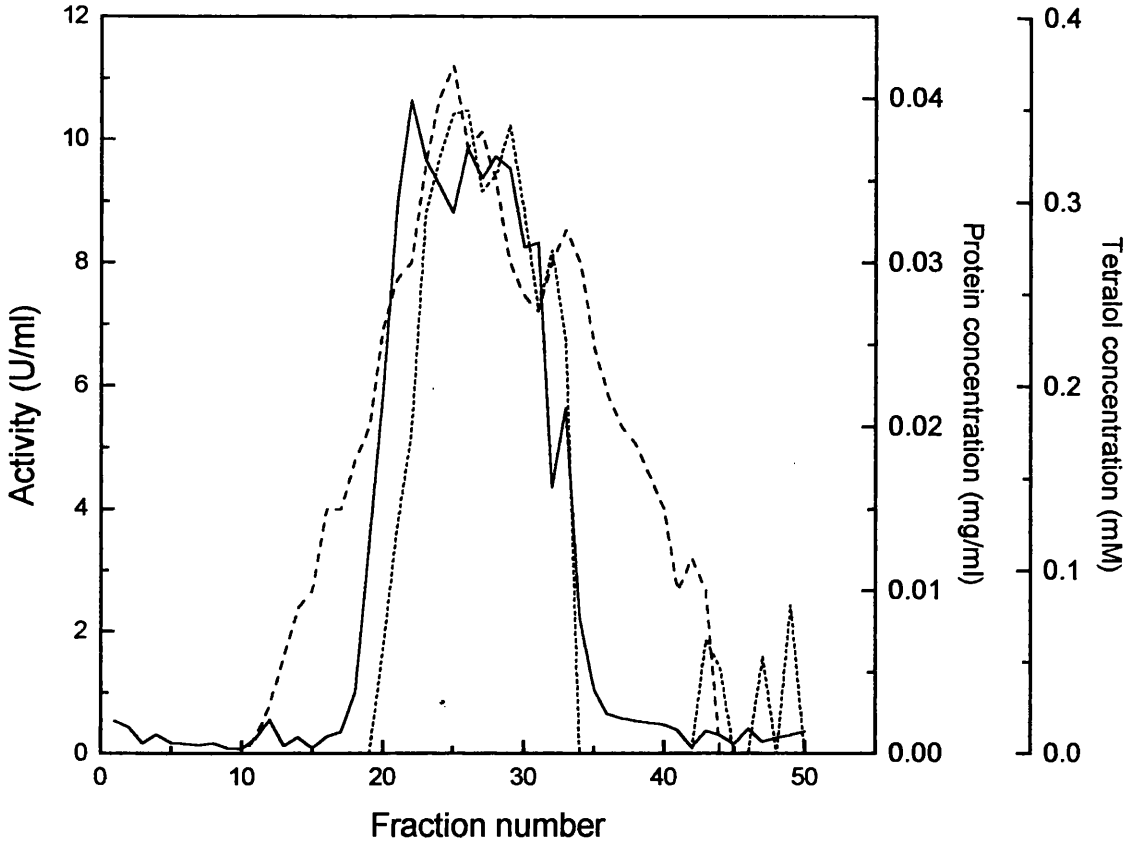


Figure 3.4:
100ml Amicon Red A column, loaded with pooled fractions from figure 1 (Anion exchange column) and run at 3.5mLmin^{-1} with a gradient NaCl elution. Protein concentration determined by Bradford assay (-------), enzyme activity determined by spectrophotometer assay (——)and tetralol production determined by HPLC assay (.....). Fractions 19-34 run on gel filtration column.

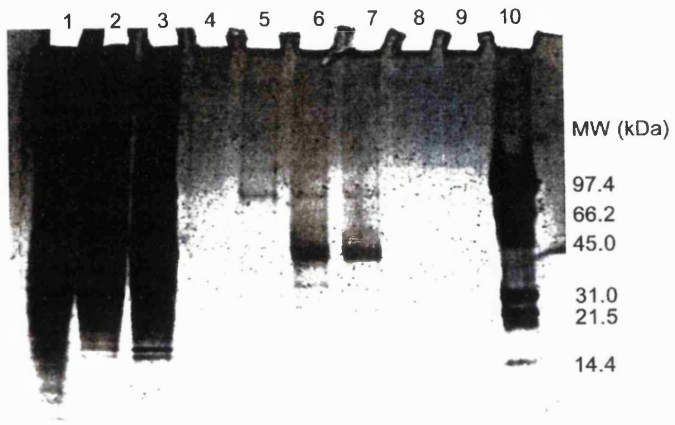


Figure 3.5:
Native PAGE gel (8-16% Tris-Glycine) stained with silver stain showing: lane 1, clarified homogenate; lane 2, pooled anion exchange fractions; lane 3, pooled affinity fractions; lane 5, gel filtration fraction 19; lane 10, low range molecular weight markers.

tetralol and 0.002mM (R)-6-bromo-β-tetralol. The enantiomeric excess of 99.54% was calculated from:

$$\%ee = \frac{[(S)-(R)]}{[(S)+(R)]} \times 100$$

3.4.7 Kinetics

The kinetics for NADH and tetralone were determined by varying the concentration of these substrates in the spectrophotometer assay (figures 3.6 and 3.7 respectively), using enzyme purified up to the affinity column stage. The Km for both was determined by fitting a Michaelis-Menton kinetic equation using a rectangular hyperbola curve to the substrate concentration versus rate graph. Apparent Km values for NADH and tetralone were found to be 0.13mM and 0.40mM respectively. The NADH was seen to inhibit the enzyme at a concentration of 0.6mM, so such inhibition was seen for similar levels of tetralone.

3.5 Discussion

Table 3.1 shows the overall results for the purification of the reductase enzyme from *Trichosporon capitatum*. The enzyme was purified by a factor of 54 from its initial state with a yield of 1.34% to give a final specific activity of 430 U/mg.

This yield is fairly low although the rational in purifying the protein to this level was to identify the enzyme responsible for the reduction of tetralone to tetralol. A partially purified form of the enzyme could be used to develop a cell free process for the production of tetralol from tetralone. In addition, the purification process (shown in figure 3.8) could be streamlined further. For example, at a larger scale the DNA removal step could be omitted. There are also non-column based techniques which may be more suitable to larger scale applications, such as affinity precipitation, for example with Eudragit bound Cibacron blue used for the separation of an alcohol dehydrogenase from *Saccharomyces cerevisiae* (Guoqiang *et al*, 1995). However, most published methods for the isolation of oxidoreductases involve a similar process of sequential column chromatography steps, often involving both anion exchange and affinity steps.

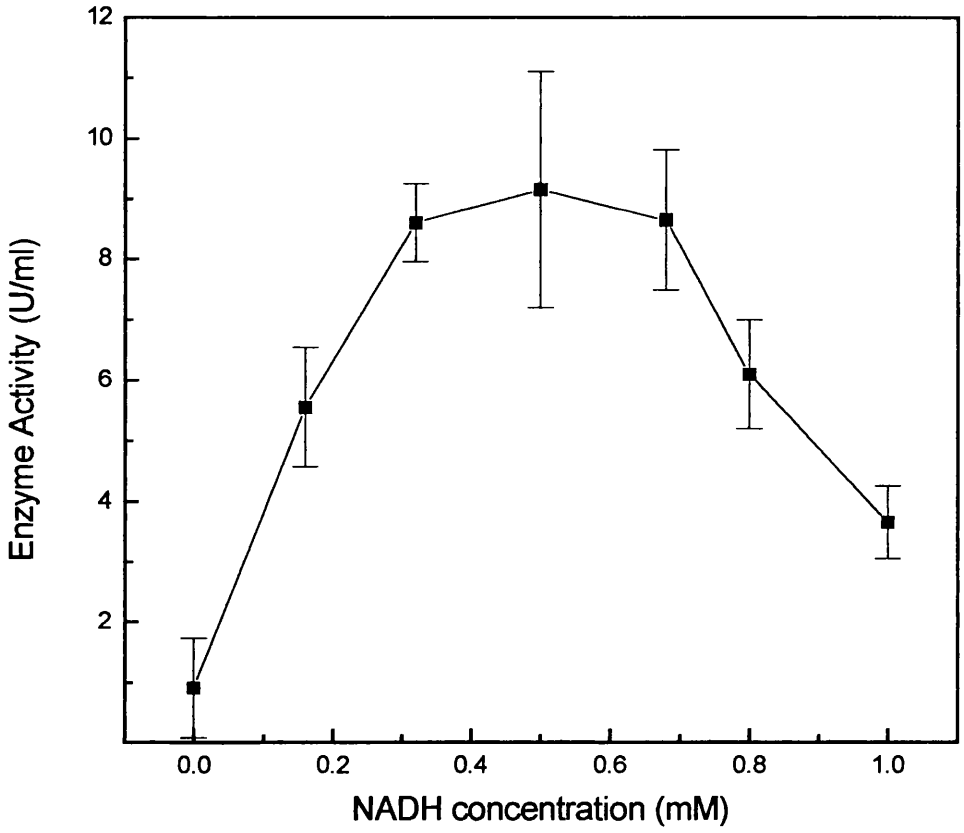


Figure 3.6:
Kinetic studies of the enzyme isolated to the stage of the affinity column, determined at pH7 and 22°C, tetralone concentration maintained at 1mM throughout.

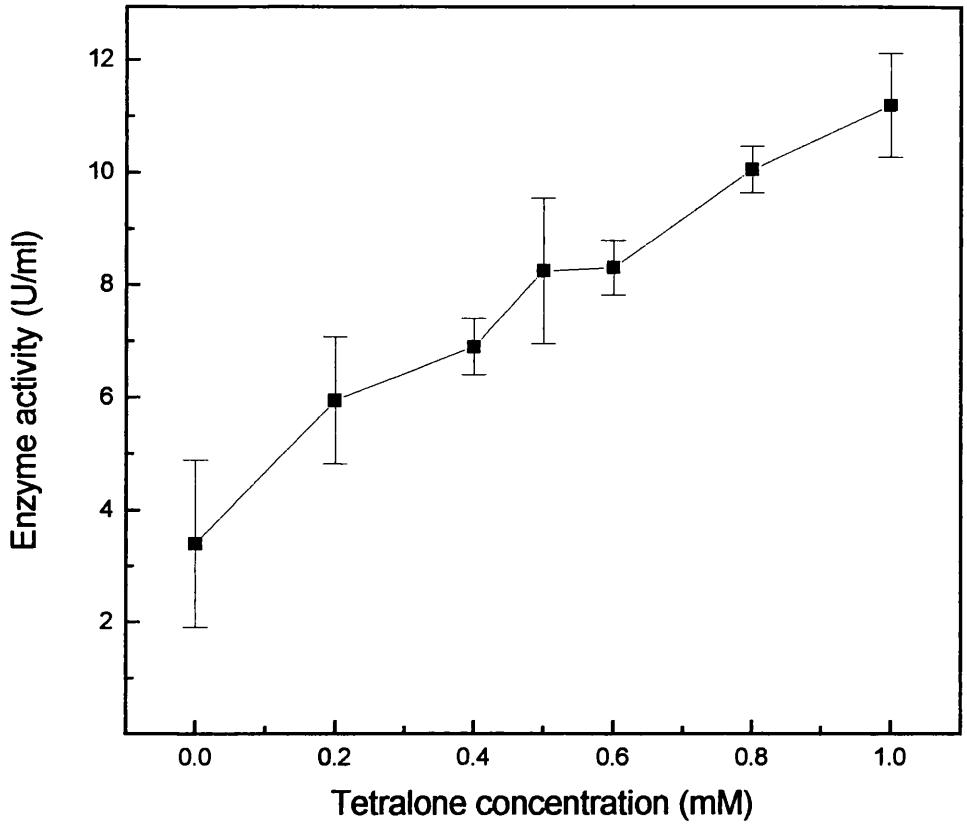
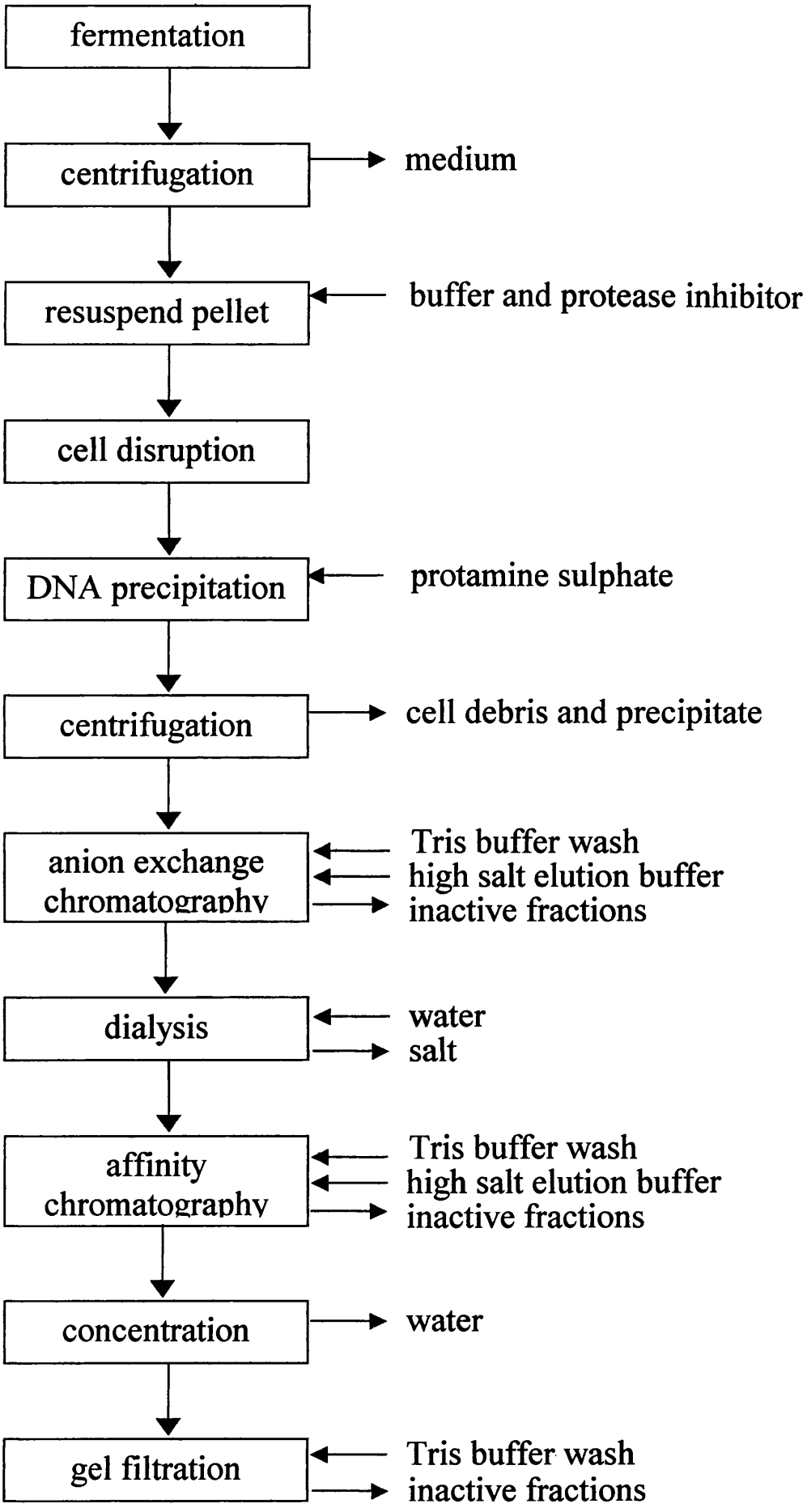


Figure 3.7:
Kinetic studies of the enzyme isolated to the stage of the affinity column, determined at pH7 and 22°C, NADH concentration maintained at 0.6mM throughout.

Table 3.1:
Purification table.

Step	Total activity (U)	Specific activity (U/mg)	Yield (%)	Overall purification
Homogenate	1605	7.89	100	1
Q Sepharose FF	850	33.3	53	4.2
Amicon Red A	467	181.8	29	23
Superdex 200 HR	22	430	1.34	54

Figure 3.8: A process flowsheet for the purification protocol described.



The cofactor preference results showed that the enzyme naturally accepts NADH as its cofactor and not the phosphorylated form of the enzyme. This supports the role of the enzyme in its natural *in vivo* state as a dehydrogenase involved in the reactions of respiration rather than one involved in anabolic reactions. The enzyme is also specific for producing the (S) form of the bromo-β-tetralol, as shown by the enantiomeric excess obtained.

The Km values are within the range expected for this type of enzyme. For example, an NAD^+-dependent formate dehydrogenase from *Agrobacterium sp.* has apparent Km of 0.13mM for formate and 0.17mM for NAD^+ (Iida *et al*, 1993) and NADPH-dependent oxidoreductases isolated from Baker's yeast have a Km values for several ester substrates of between 0.9 and 17.1mM (Heidlas *et al*, 1988).

The characterisation of the enzyme as that with an apparent molecular weight of 80,000, is in agreement with the results of several other oxidoreductase type enzymes, which have molecular weights between about 60,000 and 160,000. For example an alcohol dehydrogenase isolated from *Rhodococcus sp.* strain GK1 was found to be an NAD^+ dependent, 60,000 molecular weight enzyme (Krier *et al*, 1998). Many other oxidoreductases have been isolated from *Rhodococcus sp.* including an NAD^+ dependent phenylalanine dehydrogenase (Hummel *et al*, 1987) and an NADH dependent carbonyl reductase (Zelinski *et al*, 1994). Indeed, oxidoreductase have been isolated from all groups of microorganisms including bakers yeast (Heidlas *et al*, 1988), *Agrobacterium sp.* (Iida *et al*, 1993), *Pichia pastoris* (Hou *et al*, 1982) and *Pseudomonas fluorescens* (Hou *et al*, 1983) among others. However, no other enzymes have been isolated from *Trichosporon capitatum* for comparison and this group of enzymes display a wide range of characteristics.

4 Characterisation of the asymmetric reduction of a ketone by an oxidoreductase isolated from Trichosporon capitatum.

4.1 Summary

The optimum conditions for the operation of a bioconversion of 6-bromo-β-tetralone to (S)-6-bromo-β-tetralol with an isolated reductase enzyme from the yeast *Trichosporon capitatum* have been investigated. The enzyme was immobilised onto Eupergit C in the presence of 0.5M phosphate buffer at pH7 with an optimum enzyme challenge of 0.5U/g beads; both the free and immobilised form of the enzyme were investigated under process conditions. The optimum pH for activity of the free enzyme was in the range 6-7 and 6-8.5 for the immobilised enzyme. Both forms of the enzyme were at their most stable over this range. The temperature optima, a trade off between optimum activity and stability were found to be 21°C and 31°C for the free and immobilised forms respectively. Optimum solvent regime was 50% hexane for the free enzyme and 50% octanol for the immobilised enzyme.

4.2 Introduction

Chapter 3 has described the isolation of the enzyme responsible for the conversion of tetralone to the (S) form of tetralol. Also described previously (section 1.6), Reddy and co-workers (1996) experienced problems with running the reaction as a whole cell system. For example mass balance was not achieved, possibly due to diffusion limitation of the substrate into and the product out of the cells, and a detrimental effect on the cells of the ethanol solvent necessary to dissolve the substrate (Angelova & Schumauder, 1999).

In order to select the ideal process conditions for a cell-free tetralone reduction the conversion must be characterised fully. Many process choices are available for this conversion with an isolated enzyme, including free or immobilised enzymes, reactor type, pH, temperature and solvent regime. All of these are considered in this chapter.

The choice of cofactor recycle system is not discussed here, but will be considered in subsequent chapters.

4.3 Materials and Methods

4.3.1 Reactant and product solubility

The solubility of the tetralone and tetralol in water was determined by making a saturated solution of each and monitoring the level in solution by HPLC over a period of a week, with the temperature maintained at 22°C.

The solubility in other solvents (MES buffer at pH6, Tris buffer at pH7 and 8, DMSO, methanol, ethanol, octanol and hexane) was assayed by trying to dissolve either 0.5, 1 or 10g/L of the alcohol or the ketone over a period of 24 hours, also at 22°C. In these cases solubility was determined by seeing if the solid matter had disappeared at each concentration.

The solubility of the cofactors was not determined as both are highly soluble in water and insoluble in non-aqueous solvents (Adlercreutz, 1996).

4.3.2 Reactant and product stability

The stability of 2mM tetralone or tetralol in aqueous solution at 22°C (pH7) or in the presence of a 50:50 aqueous:solvent mixture was assayed over a period of 24 hours by measuring their concentration by HPLC analysis.

The stability of NADH and NAD^+ in aqueous solution at 22°C (pH7) was assayed by measuring the absorbance at 340nm and 260nm respectively. The same solvent mixtures were used as for the tetralone and tetralol determination.

4.3.3 Enzyme isolation

The yeast *Trichosporon capitatum* MY1890 was grown on production medium (30g/L glycerol, 20g/L yeast extract, 25g/L hysoy peptone) in shake flasks for 48 hours. The protocol involved the homogenisation of washed cells (5 continuous

passes at 500bar in an APV Lab 60 homogeniser), anion exchange chromatography (Q Sepharose Fast Flow, Pharmacia) and affinity chromatography (Amicon Red A, Millipore), as detailed in section 3.3.

4.3.4 Free enzyme assay

An assay mixture of 5% of a 10mg/mL tetralone in ethanol solution was mixed with 95% of a 0.5mg/mL cofactor (NADH) in water. This mixture was stored in a freezer for up to 1 week and thawed when needed. This assay mixture was also modified for various investigations. The assay mixture and free enzyme solution were added to either a cuvette or a well of a 96-well plate. The disappearance of the reduced cofactor was monitored by spectrophotometer at 340nm and 22°C for 10 minutes.

4.3.5 Enzyme immobilisation

Eupergit C (Rohm) was used as an immobilisation matrix. 1g of dry beads were mixed with 5mls of enzyme solution in 1M potassium phosphate buffer at pH 7.5 and left at room temperature for 72 hours, as described in the manufacturer's literature.

4.3.6 Immobilised enzyme assay

100µL of a 250mg/mL immobilised bead slurry was added to each well of a Microscreen 96-well plate (Millipore), which has 5µm filters in the bottom of each 200µl well. The same assay mixture as above was prepared and added to each well to start the reaction. At appropriate intervals the liquid contents of each well were vacuum filtered into a fresh 96-well plate using the Millipore Microscreen vacuum manifold and then the absorbance at 340nm measured in a plate reader. The liquid from each well was then transferred back into the filter plate with a multi-channel pipette for further incubation. The filter plate was incubated on an Eppendorf themomixer (Eppendorf) at 27°C with mixing by shaking between filter steps.

4.3.7 Reverse phase HPLC analysis

A Zorbax RX-C8 column (4.6mm x 25cm) (Mac-Mod Analytical, Chadds Ford, PA, USA) was used for the separation of the tetralone from the tetralol. The mobile phase was acetonitrile and acidified water (0.1% phosphoric acid) (50/50 v/v) at a flow rate of 1.5 mL/min. Detection was by UV at 220nm and 22°C. Tetralone and tetralol eluted at 6.5 and 4.7 minutes respectively.

4.3.8 Enzyme kinetics

The enzyme assay was performed in a 96-well format (200µl wells), with the concentration of the two substrates varied independently. The NADH concentration was varied between 0 and 1.4mM, with the tetralone concentration constant at 3mM. The tetralone concentration was varied between 0 and 3mM, with the NADH concentration constant at 0.6mM.

The behaviour of the enzyme in the presence of the potential inhibitors, NAD and tetralol was investigated. Reaction rate was measured varying the NADH and tetralone concentrations as above, in the presence of 0.5, 1 and 2mM tetralol and 0.2, 0.8 and 1.5mM NAD.

4.3.9 pH optimum and stability

50µl of buffer solutions at pH values between 5 and 9.5 were added to each well; column A on the 96-well plate contained all reactions at pH 5, column B contained all reaction at pH5.5 etc. 50µL of the assay mixture was also added to each well. The reaction was started by adding 100µL of enzyme solution to each well, for the free enzyme, or adding the buffer and assay mixture to the immobilised enzyme.

Stability, normalised to the initial incubation conditions, was measured by incubating the free and immobilised enzymes in the appropriate buffer, at 22°C for up to 24 hours. Then the activity was assayed at intervals within this period. The free enzyme activity was measured at the incubation pH simply by adding assay mixture to each well of enzyme and buffer mixture. The immobilised enzyme activity was

assayed by filtering off the incubation buffer and adding the assay mixture to each well, so that all assays were performed at pH7.

4.3.10Temperature optimum and stability

The temperature of the plate reader was adjusted to the appropriate level for the duration of the 96-well plate assay for the free enzyme temperature optimum. For the immobilised enzyme, the temperature of the Eppendorf shaker was adjusted to the appropriate level also.

To measure temperature stability the free and immobilised enzymes were incubated between 10 and 70°C for up to 24 hours and at intervals used in the standard activity assay at 22°C.

4.3.11Solvent choice

The effect of some of the different solvents used to dissolve the tetralone substrate on the enzyme were studied. A small range of solvents with a range of LogP values were chosen to be tested as a sample. These were dimethyl sulfoxide (DMSO), methanol, ethanol, octanol and hexane (LogP values of –1.3, -0.76, -0.24, 2.9 and 3.5 respectively (Sangster, 1989).

The activity of the free and immobilised enzymes with each solvent was investigated using 96-well plates with each well containing solvent (ethanol, methanol, dimethyl sulfoxide (DMSO), octanol or hexane) with the tetralone dissolved in it, water with NADH dissolved in it and enzyme solution (in the case of free enzyme). The aqueous:solvent ratio was 50:50.

The stability of both the free and immobilised enzymes was studied by incubating the enzyme in the same solvent mixtures as for the activity experiments, at 22°C for up to 24 hours and assaying the activity at intervals within this period. The free enzyme activity was measured by removing a sample (from the aqueous layer in a 2-phase example) and mixing with the assay mixture. The immobilised enzyme activity

was assayed by filtering off the solvent mixture and then adding the assay mixture to the beads.

4.4 Results

4.4.1 Reactant and product characteristics

4.4.1.1 Solubility of tetralone and tetralol

The solubility of tetralone and tetralol (table 4.1) was measured at 9.4g/L (0.042M) and 313.1g/L (1.38M) respectively. Solubility in organic solvents (ethanol, methanol, DMSO, toluene, octanol) is much higher (>10g/L) for both the alcohol and the ketone, with a few exceptions, including hexane and dodecane which have lower solubilities.

4.4.1.2 Stability of reactants and products

Table 4.1 shows the results for the stability of the ketone, alcohol and both forms of the cofactor in a range of solvent mixtures. The tetralone and tetralol retain almost all their activity over the 24 hour period the investigation was run over.

4.4.2 Immobilisation conditions

The standard conditions quoted by the manufacturer for the immobilisation of enzymes onto Eupergit C were not found to produce immobilisation in the case of the tetralone reductase enzyme and was characterised by both significant loss of activity during immobilisation and no binding of the protein to the beads. The immobilisation conditions investigated were pH (6, 7 and 8) and molarity of the phosphate buffer (0.1, 0.5, 1M). It was found that the enzyme's stability was greatly affected by the molarity of the phosphate buffer, being most stable at the lowest concentration and to a lesser extent at higher pHs (figure 4.1). The modified conditions chosen for immobilisation were 0.5M phosphate buffer at pH6, the compromise molarity of the phosphate buffer due to its essential role in the immobilisation process.

Table 4.1:
Stability and solubility of reactants and products.
Solubility in water measured in a saturated solution at 22°C by HPLC analysis. Solubility in other solvents measured at 22°C at three levels, 0.5, 1 and 10g/L: the value stated is the maximum level at which the substances dissolved.
Stability of tetralone and tetralol assayed at 22°C over a period of 24 hours by HPLC analysis. Stability of NADH and NAD^+ assayed under the same conditions by measuring absorbance at 340nm and 260nm respectively.

Solvent	Solubility (g/L)		Stability (% after 12hrs)			
	Tetralone	Tetralol	Tetralone	Tetralol	NADH	NAD
Water	9.4	313.1	>99	>99	58	99
pH6	0.5	0.5	>99	>99	>99	98
pH7	0.5	0.5	>99	>99	>99	99
pH8	0.5	0.5	>99	>99	>99	97
DMSO	1	1	>99	>99	22	99
Methanol	10	10	89	90	11	99
Ethanol	10	5	>99	>99	13	>99
Octanol	10	10	>99	>99	16	>99
Hexane	1	1	>99	>99	15	>99

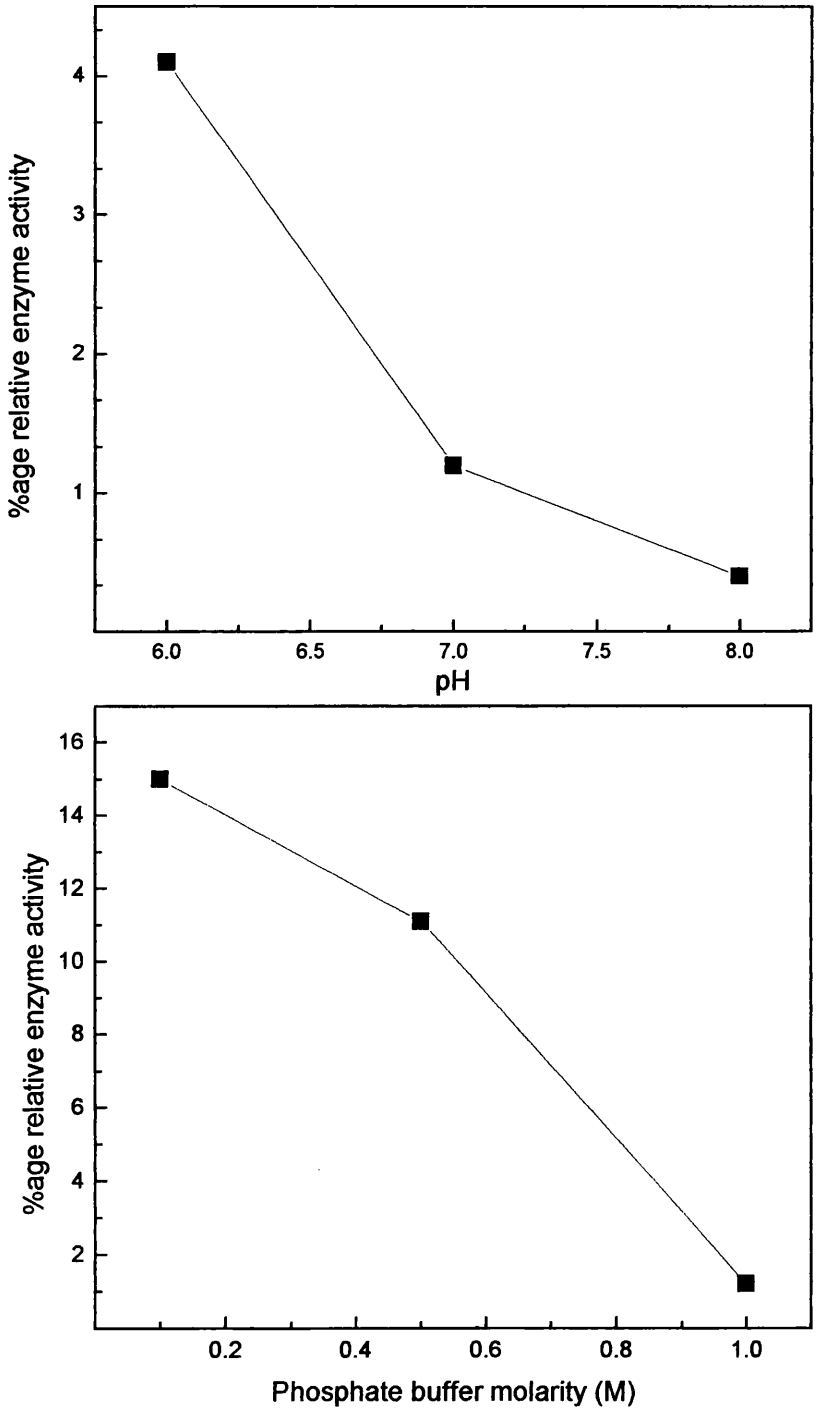


Figure 4.1:
Relative activity of the enzyme after 24hours dialysis with phosphate buffer as a percentage of the initial enzyme activity (10U/mg). *Top panel:* the effect of pH of 1M potassium phosphate buffer on enzyme activity after dialysis. *Bottom panel:* the effect of potassium phosphate buffer molarity on enzyme activity, with a constant buffer pH of 7.

The optimum loading of the enzyme onto the beads was also investigated by challenging the beads with different concentrations of enzyme. Figure 4.2 shows the graph of specific activity of the beads and retained activity on the beads against enzyme challenge. The area of the graph where the two lines intersect corresponds to the optimum enzyme challenge, in this case around 4U/g beads. This is 4mg protein/g beads challenge, which is within the 2-40mg protein/g beads which the manufacturer suggests and leads to a 2.18mg protein/g beads loading.

4.4.3 Enzyme characteristics

4.4.3.1 Immobilised enzyme kinetics

The kinetics for tetralone and NADH were determined by varying the concentration of these substrates in the assay (figures 4.3 and 4.4). The apparent Km for both was determined by fitting a Michaelis-Menten kinetic equation using a rectangular hyperbola curve to the substrate concentration versus rate graph. Apparent Km for NADH and tetralone were found to be 0.63mM and 2.7mM respectively. The Km for NADH and tetralone for the free enzyme were previously found to be 0.13mM and 0.4mM respectively (see section 3.4.7). The free enzyme also experienced substrate inhibition by NADH at concentrations of 0.6mM, which was not seen with the immobilised enzyme.

4.4.3.2 pH optimum and stability

Figure 4.5 shows the pH optimum for both the free and immobilised enzymes, measured as the initial activity over 10 minutes. The free enzyme shows an optimum activity in the range of pH6-7, whereas the immobilisation shows a wider optimum pH range, pH6-8.

Figure 4.6 shows enzyme stability as a function of in both the free and immobilised enzymes. The stability of the enzyme was improved by up to 3 fold on immobilisation, although the optimum still remained at neutral pH.

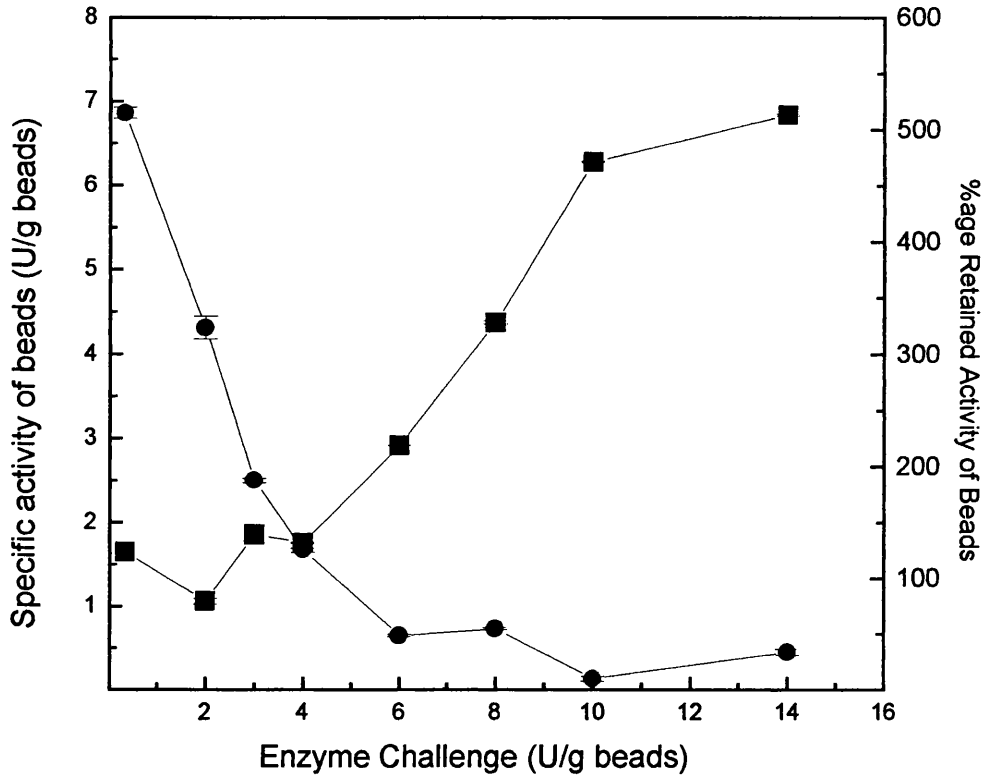


Figure 4.2:
Enzyme loading of the Eupergit C beads with immobilisation conditions of 0.5M phosphate buffer at pH6, incubated at room temperature (22°C) for 72 hours. The specific activity of the beads (■) is calculated as detailed in the text, while the percentage retained activity of the beads (●) is the total bead activity at the end of immobilisation as a percentage of the total activity challenged to the beads at the start of the immobilisation.

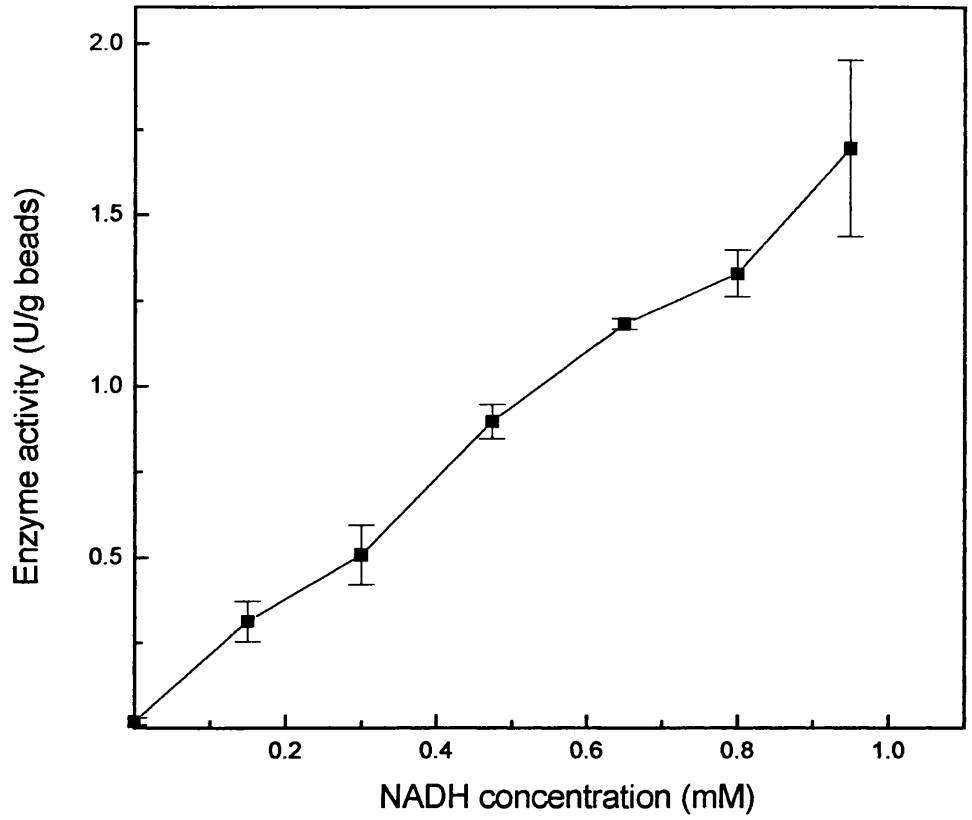


Figure 4.3:

Kinetic studies of the immobilised enzyme determined at pH7 and 22°C, tetralone concentration maintained at 3mM throughout.

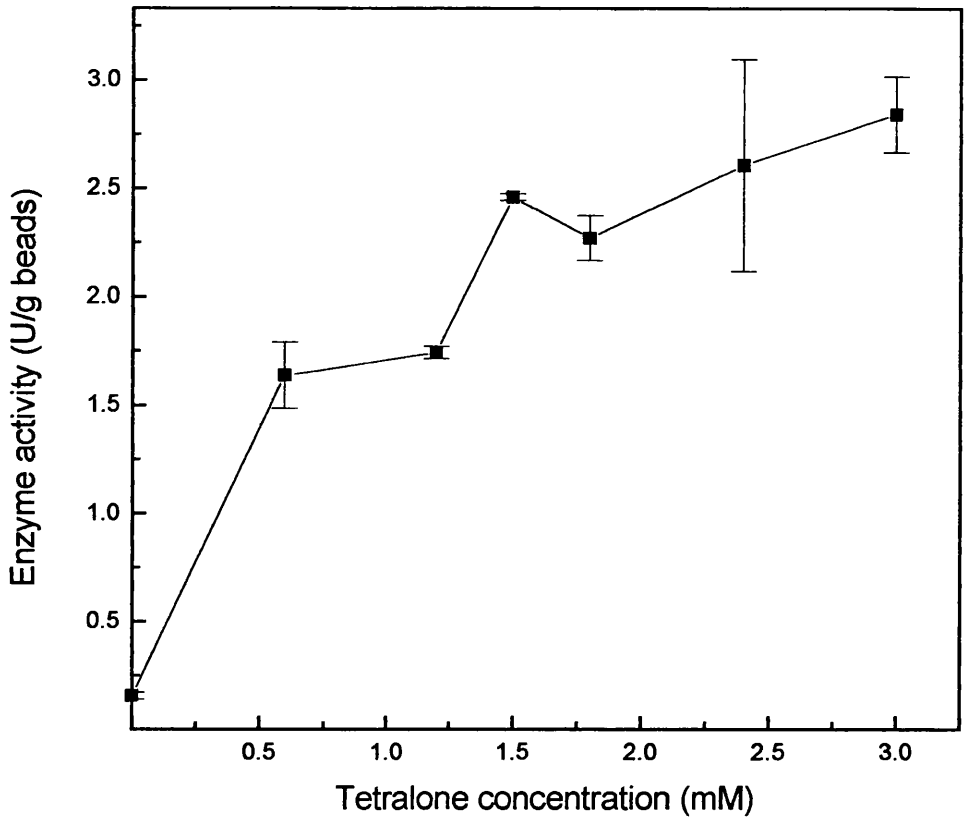


Figure 4.4:

Kinetic studies of the immobilised enzyme determined at pH7 and 22°C, NADH concentration maintained at 0.6mM throughout.

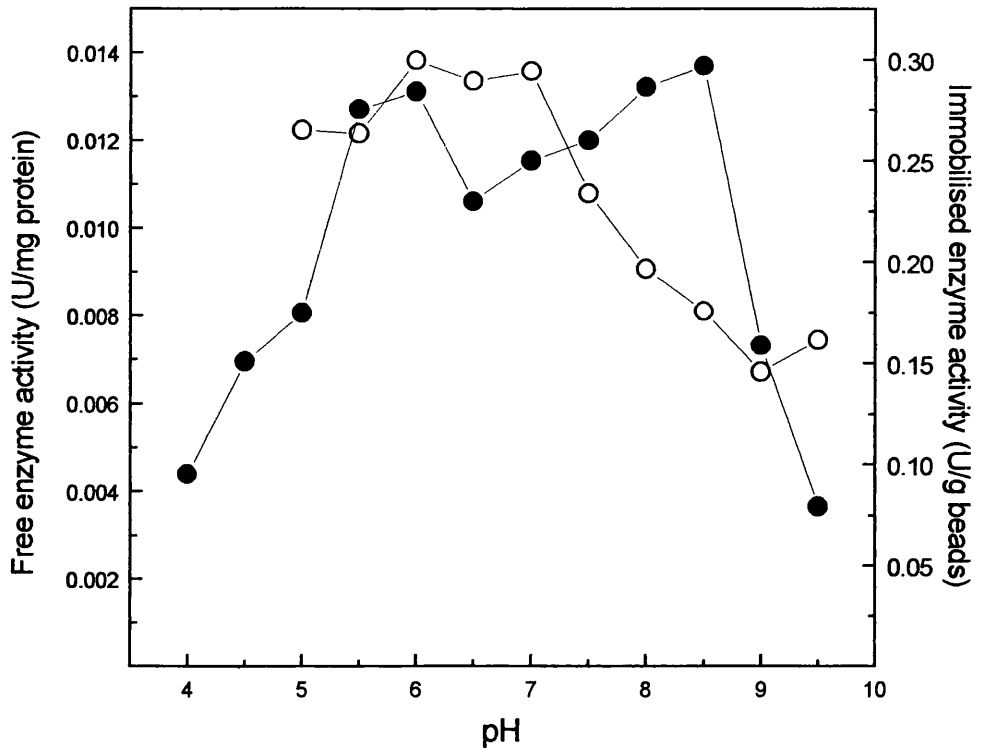


Figure 4.5:
Activity of both forms of the enzyme as a function of pH. (○) free enzyme, (●) immobilised enzyme.

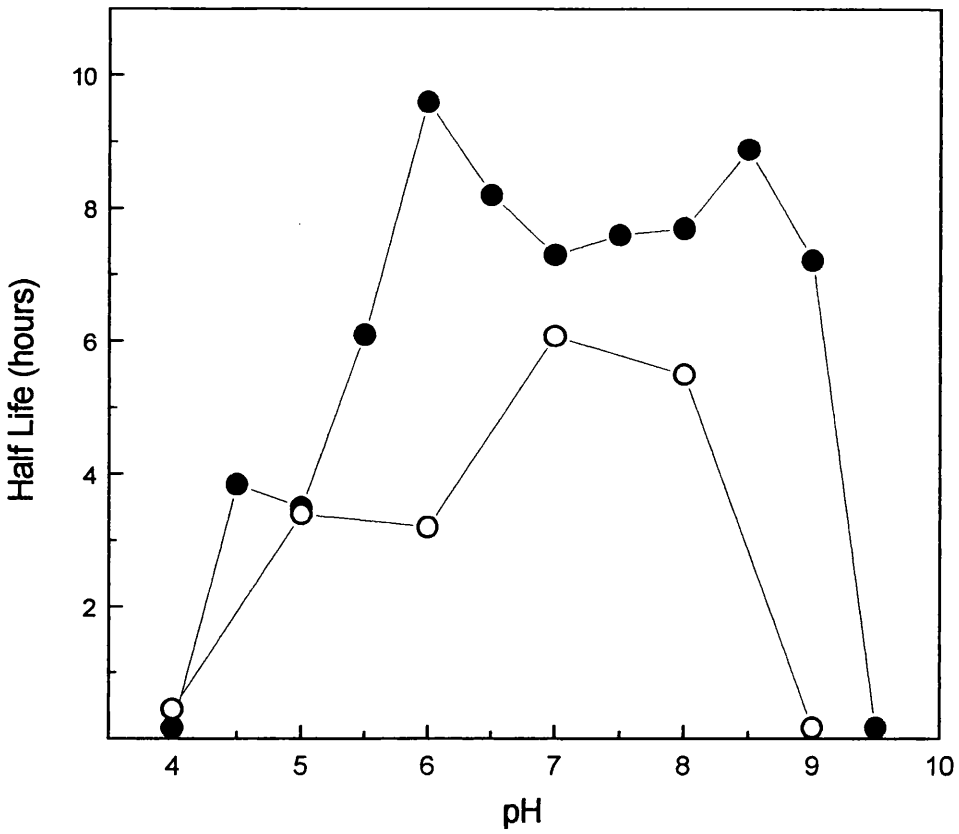


Figure 4.6:
Stability, expressed as half life, of both forms of the enzymes as a function of pH, at 22°C. (○) free enzyme, (●) immobilised enzyme.

4.4.3.3 Temperature optimum and stability

Free and immobilised enzyme both show temperature optimum for activity at 60°C, as seen in figure 4.7. The temperature optimum for stability shows an opposite trend (figure 4.8) with the optimum at around 10°C for both. Immobilisation causes an increase in stability of about 2-fold. The optimised temperature for the process with the immobilised enzyme is 30°C, where the half life for the immobilised enzyme is 8 hours and the activity is at 50% of its maximum. At 40°C, although the activity is at 70% of the maximum, the half life is only 4 hours.

4.4.3.4 Solvent optimum and stability

Figure 4.9 shows the response of both forms of the enzyme to different solvent regimes. All the 50:50 solvent:water mixtures exhibited lower activities than the control (containing only 5:95 ethanol:water) for both forms of the enzyme. With the small solvent sample size which was used in this investigation it is difficult to predict how the enzyme's response to solvents changes with immobilisation, which ranged from a 92% increase (octanol) to an 81% decrease (methanol) in activity compared to the control on immobilisation.

The stability data (figure 4.10) shows a similar lack of predictability to the activity data. In the case of hexane, immobilisation causes a decrease in stability and with methanol there is no change in stability on immobilisation. Both forms of the enzyme show an increase in stability it the presence of octanol, to a maximum of a half life of 21 hours. From this data the optimum solvent conditions for this enzyme are to use an octanol 2 phase system. However, only a 50:50 mix of solvent:water were investigated, the activity and stability could be further optimised by altering this ratio.

4.5 Discussion

The immobilisation of tetralone reductase onto Eupergit C resulted in a specific activity of around 2U/g beads, representing a retained activity of over 90%.. Previously published data for similar immobilisations show wide variations in specific activity and retained activity, from 8.7U/mg glutaryl-aminocephalosporaic

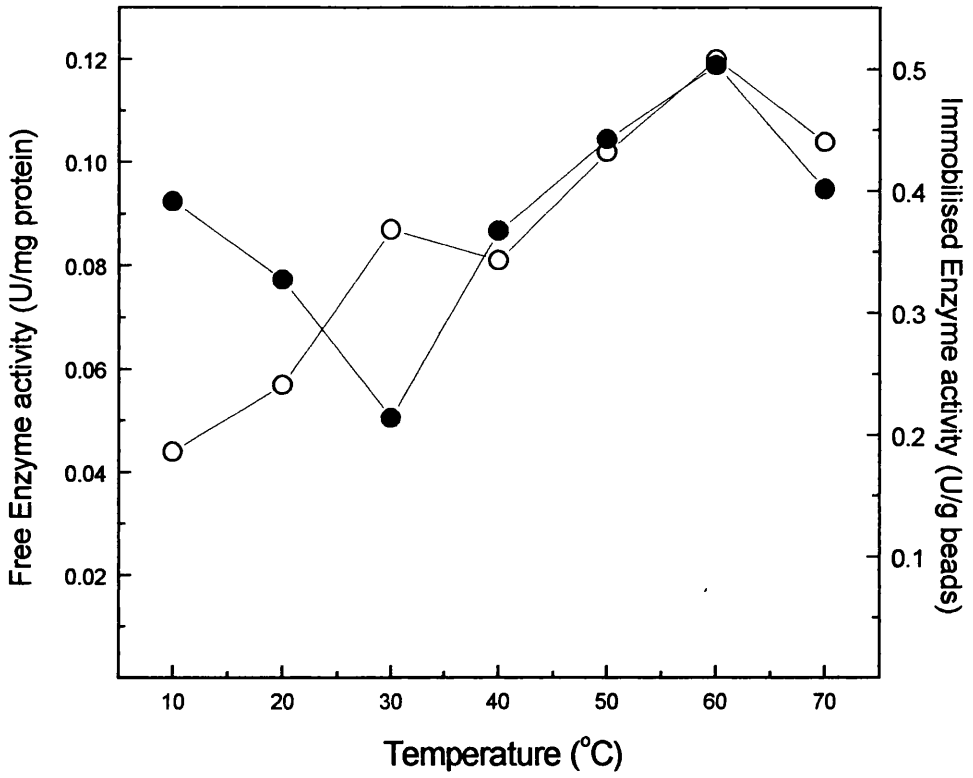


Figure 4.7:
Activity of both forms of the enzyme as a function of temperature. (○) free enzyme, (●) immobilised enzyme.

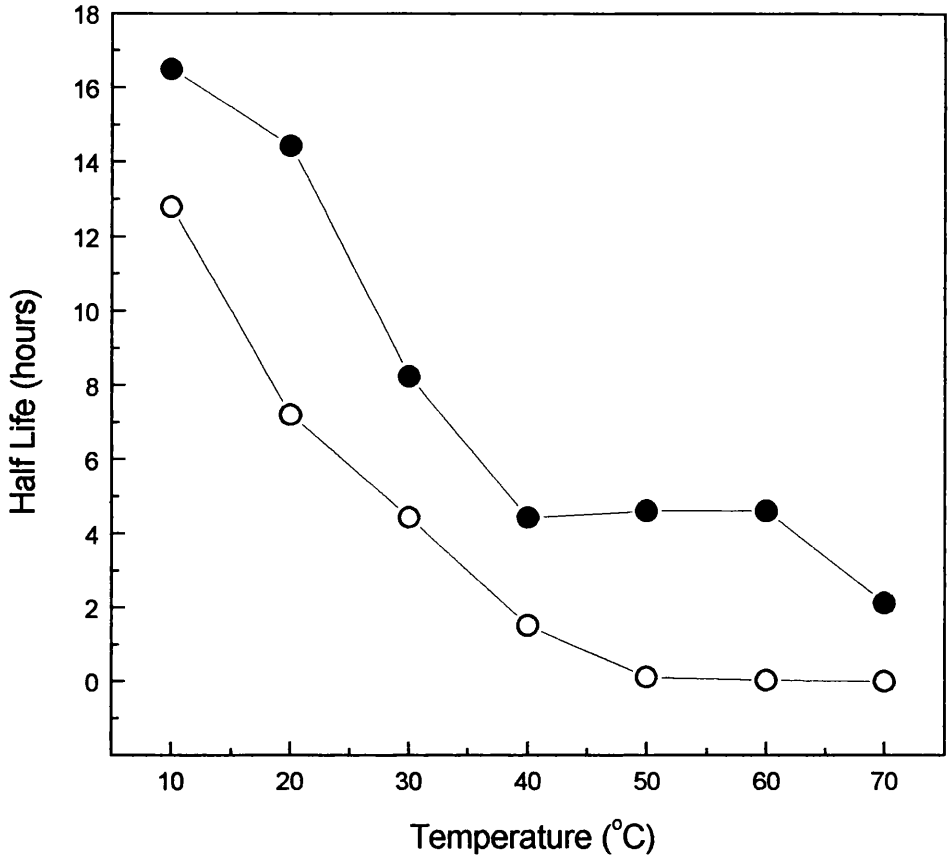


Figure 4.8:
Stability, expressed as half life, of both forms of the enzyme as a function of temperature, at pH7. (○) free enzyme, (●) immobilised enzyme.

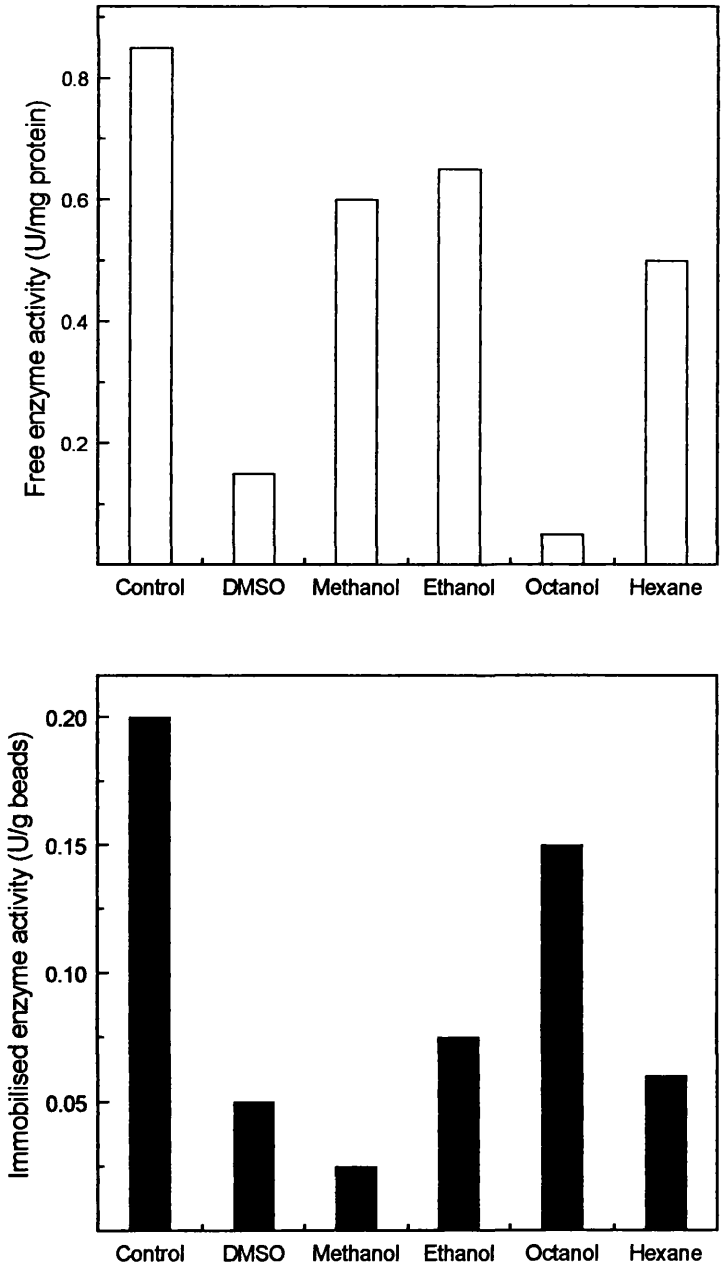


Figure 4.9:
Activity of both forms of the enzyme in the presence of a 50:50 solvent:water mixture with a sample of different solvents. *Top panel*: □ free enzyme; *Bottom panel*: ■ immobilised enzyme. Control is the standard assay conditions with 95:5 water:ethanol ratio.

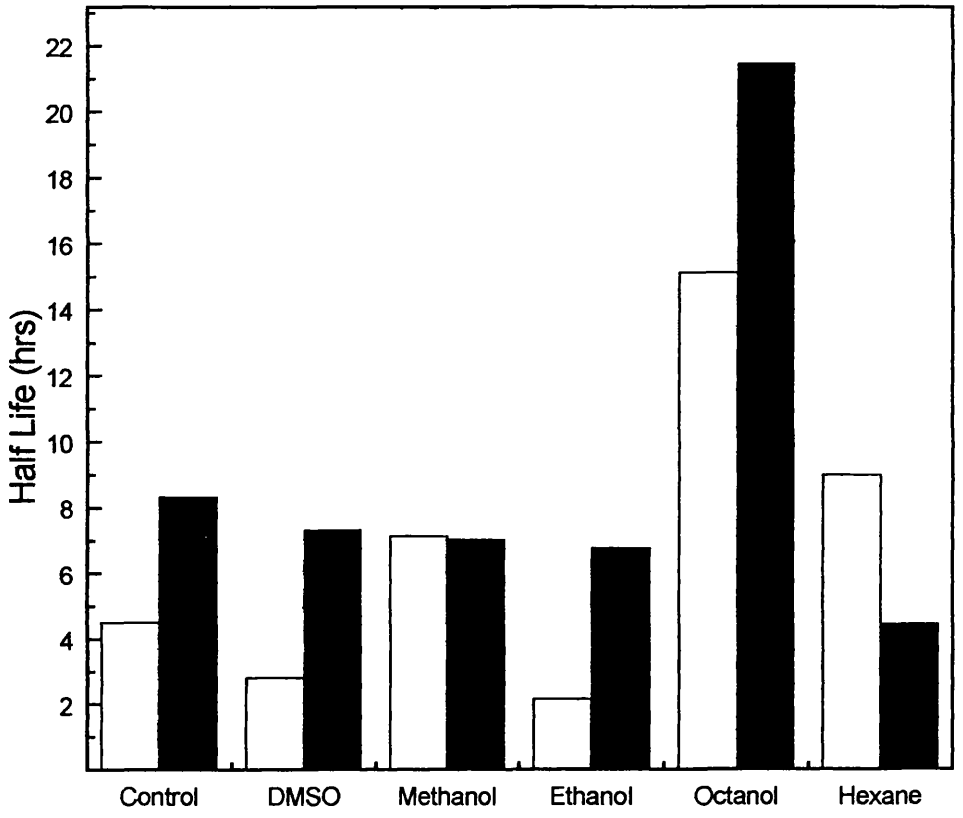


Figure 4.10:
Stability, expressed as half life, of both forms of the enzyme as a function of solvent regime, using a sample of different solvents. (□) free enzyme, (■) immobilised enzyme. Control is the standard assay conditions with 95:5 water:ethanol ratio.

acid-acylase activity (63% retained) (Binder *et al,* 1994) to 421U/g glucose oxidase activity (Wehnert *et al,* 1986) and an 80% retained activity for cytosine deaminase (Katsuragi *et al,*, 1989). The stability of these enzymes on immobilisation was higher than when not immobilised and half lives ranged from 10 days (cytosine deaminase) to 3 weeks (glucose oxidase). These half lives are significantly higher than those for tetralone reductase, probably due to the fact that the free tetralone reductase is also extremely unstable compared to most enzymes.

The optimum conditions for a tetralone to tetralol bioconversion using a reductase from *Trichosporon capitatum* have been investigated. The pH of the reaction influences many aspects of the conversion including biocatalyst activity and stability and the stability of the reactants and products. The optimum activity of the enzyme was around neutral pH (6-7) and immobilisation increased this range up to pH 8.5. The stability of the enzyme followed a similar pattern to activity, with immobilisation increasing the half life of the enzyme by a few hours at most pH values. The pH did not influence the stability of the tetralone and tetralol which remain stable for at least a week in aqueous solution and are therefore the most stable component of the bioconversion. The stability of the cofactors however is known to be influenced by pH with the half life of NADH at 23°C, pH 6 about 32 hours, the stability increasing at high pH and decreasing below pH6. The half life of NAD at 38°C, pH9 of 22 hours, with stability remaining the same at lower pH but decreasing above pH9 (Lowry *et al*, 1961). Therefore a pH around 7 is the optimum for the cofactors also and no trade off needs to be made for pH in this bioconversion.

The optimum temperature for the activity of the biocatalyst was found to be around 60°C for both the free and immobilised enzymes. Temperature stability however showed a different trend, with an increased stability at the lower temperatures. Tetralone and tetralol stability was also higher than the other components and so does not need to be accounted for, however, a higher temperature may increase the solubility of the substrate in water (although there is no experimental evidence to support this). The stability of the cofactors is dependent on temperature. At pH6 the stability of NADH decreases from 32 hours to 1.2 hours

with a temperature change from 23°C to 60°C. The stability of NAD decreases from 6.9 hours to 0.69 hours at pH 10 with a temperature change from 38°C to 60°C (Lowry *et al*, 1961). The stability of both the cofactors at 60°C and pH 7 is 22 hours, which is more stable than the biocatalyst. Therefore the optimum pH for the bioconversion should be determined by the biocatalyst only and will be a trade off between the activity and stability. Figure 4.11 shows this trade off for both forms of the enzyme. The temperature where both the activity and stability are at their highest is where the two lines for activity and stability cross. This is at 21°C for the free enzyme and 31°C for the immobilised enzyme. However, it may be necessary to favour either a high stability or activity, for instance, if the activity of the enzyme is so low that the rate is lowered and therefore the space-time yield is low, then a higher temperature which favours the activity over stability could be chosen. A more flexible approach to the optimum conditions can be obtained by drawing a window of operation, as discussed in section 7.3.

The requirement for a non aqueous environment for the bioconversion is dictated by the low solubility of the substrate (0.042M). The biocatalyst has a lower activity with the solvents chosen for investigation than with the control. There is also no significant change in stability in the presence of most of the solvents, except octanol, or an increase in the stability on immobilisation of the enzyme. However, the immobilised enzyme with an octanol solvent system appears to be the best. For the free enzyme, although stability with octanol is high, the activity is very low and therefore a hexane system would be better.

Kinetic studies of the enzyme have shown that the free enzyme experiences substrate inhibition by the NADH substrate. Although this would limit the reaction if the cofactor was added stoichiometrically, if a cofactor recycle system was operated, the levels of both the oxidised and reduced cofactors should remain low. The tetralone and tetralol substrate and product do not present inhibition problems. However, the tetralol substrate does have a low aqueous solubility which requires that the substrate is fed from a second phase (octanol or hexane). This will mean that the flow regime in the reactor must be stirred, ruling out the option for plug flow

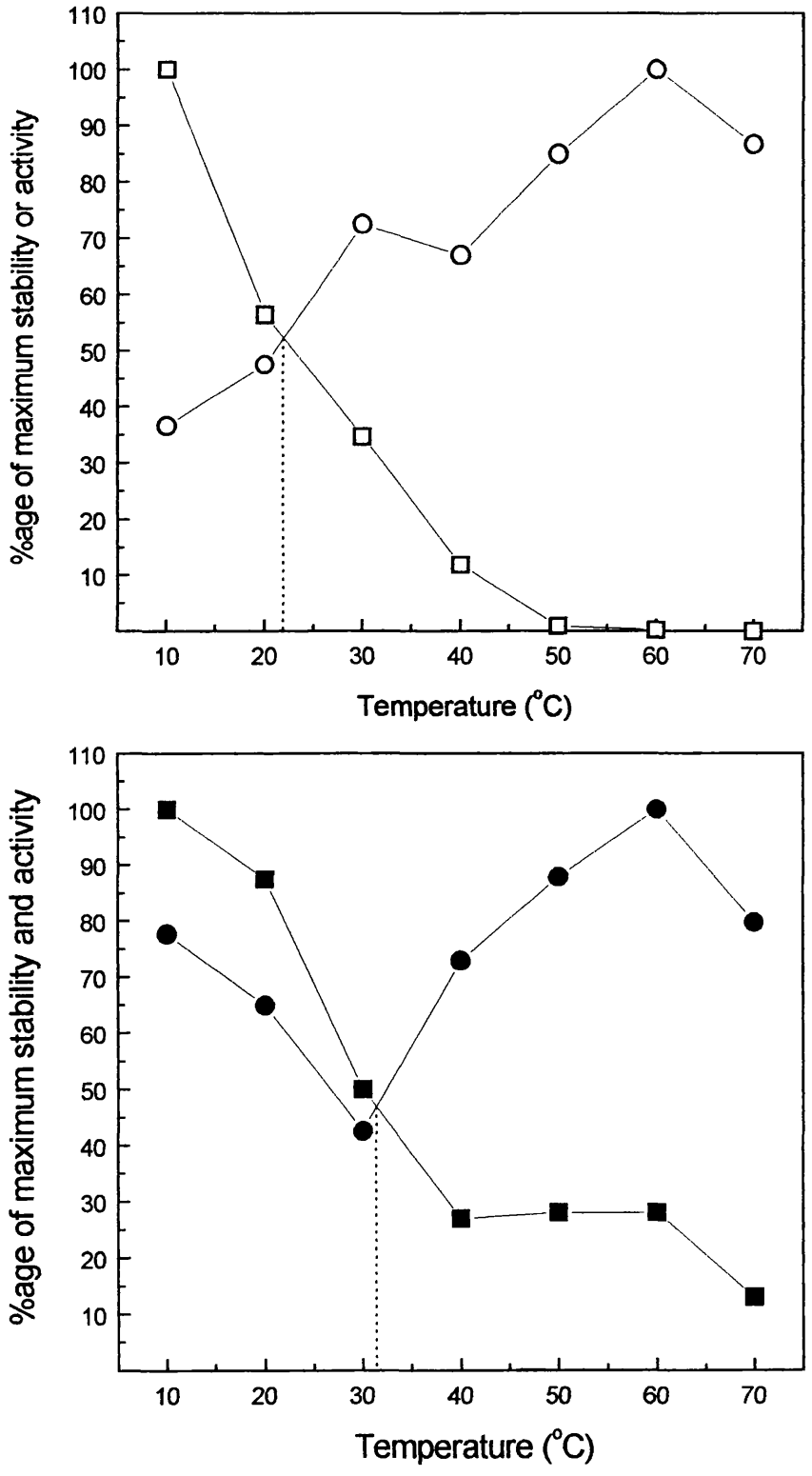


Figure 4.11:
Optimum temperature window.

operation. Plug flow can also be ruled out as more enzyme is required for the equivalent conversion in a continuous stirred tank reactor compared to a continuous plug flow (Lilly & Dunnill, 1976) and the enzyme is not easy to produce (only present at low levels in the yeast cells) and has a fairly low specific activity. Continuous operation of reactor is further eliminated because the biocatalyst half life is quite low, even with immobilisation (a maximum of 22 hours in the presence of octanol at 20°C) which will limit the length of operation of a continuous reactor. Therefore a batch stirred tank reactor would be the reactor of choice for this bioconversion.

The rationale for immobilisation in this case was to increase the stability of the enzyme. This was done to some extent particularly with regard to pH and temperature. However, the increase in stability may not be significantly high enough to make the extra expense of immobilisation worth while.

5 Use of robotics to aid selection of conditions for a second enzyme cofactor regeneration reaction

5.1 Summary

The difficulties associated with operating a robotic system for enzyme reaction characterisation have been investigated with respect to bead handling and accuracy of the system. The main problems identified with bead handling for immobilised enzymes were pipette tips becoming clogged with beads and the bead slurry settling out and therefore not being homogeneous for pipetting. The accuracy of the system is important to investigate for the quantitative use of robotic systems otherwise insufficient data could be collected or too much data could be collected which would lead to wasteful redundancy of the data.

5.2 Introduction

Time to market for new products is becoming an increasingly important priority, especially in the pharmaceutical sector. Additionally, for a pharmaceutical company to remain competitive, it must have an increasingly large product portfolio (Tapolczay et al, 1998). This increasing number of drug leads can be provided by combinatorial techniques. In comparison to traditional chemical techniques, combinatorial techniques cost around two orders of magnitude less and thousands of drug leads can be examined in the same time it took to make one by traditional methods (Persidis, 1998). One of the most critical needs in this strategy is to have a screening process which can keep pace with such high speed synthesis. Advances in high throughput screening (HTS) have therefore been driven largely by the growth in combinatorial chemistry. It is important that process synthesis (i.e. the design of the process flow sheet (Woodley and Lilly, 1994; Woodley and Lilly, 1996)) does not limit the rate of drug development and can keep pace with this large number of drug candidates and therefore techniques to speed process synthesis now need to be developed.

While there are many examples of robotics being used to screen for drugs, this technique had not previously been used for the quantitative investigation of the conditions for an enzyme based bioconversion. This chapter deals with some of the

practical problems identified in developing this technique, the subsequent chapter will look at the technique's application to the model system.

5.3 Methods

5.3.1 Bead Handling

The best method for dispensing equal amounts of the Eupergit C beads (dia. 150μm) into each well was assessed using a Gilson pipetteman pipette (20-250μl) fitted with standard 'yellow' tips or large orifice 'yellow' tips (USA Scientific, Inc.). Previous experiments with immobilised enzymes have successfully used 5ml Gilson pipettes, but these do not dispense quantities small enough for the 96-well plates. Three concentrations of bead suspension were used (25, 50 and 100g dry weight/L) and 50μl were dispensed from each nozzle into pre-weighed eppendorfs and repeated ten times. The wet weight and dry weights of these were then measured.

5.3.2 Accuracy

The inaccuracies in the method of commencing the reaction were quantified by carrying out the tetralone to tetralol bioconversion in a standard 96-well plate. The reaction was either commenced by adding soluble enzyme to the assay mixture (section 3.3.2.1) already in each well with a single channel or 8 channel multi-pipette. In addition a series of 6 identical plates were set up and the reactions in these started with the 8 channel multi-pipette to measure the errors across several plates. The difference between the rates of reaction in each well was taken as the error associated with the assay.

5.4 Results

5.4.1 Bead handling

Figure 5.1 shows the predicted bead concentration versus the dry bead weight for the two types of pipette tip. The large orifice tip stays much closer to the predicted values, although the measured values are consistently higher than those predicted. The standard tip shows a much lower measured compared to predicted value and also does not show a correlation between the bead concentration and measured bead weight. Table 5.1 shows that the large orifice tip is both more precise and accurate

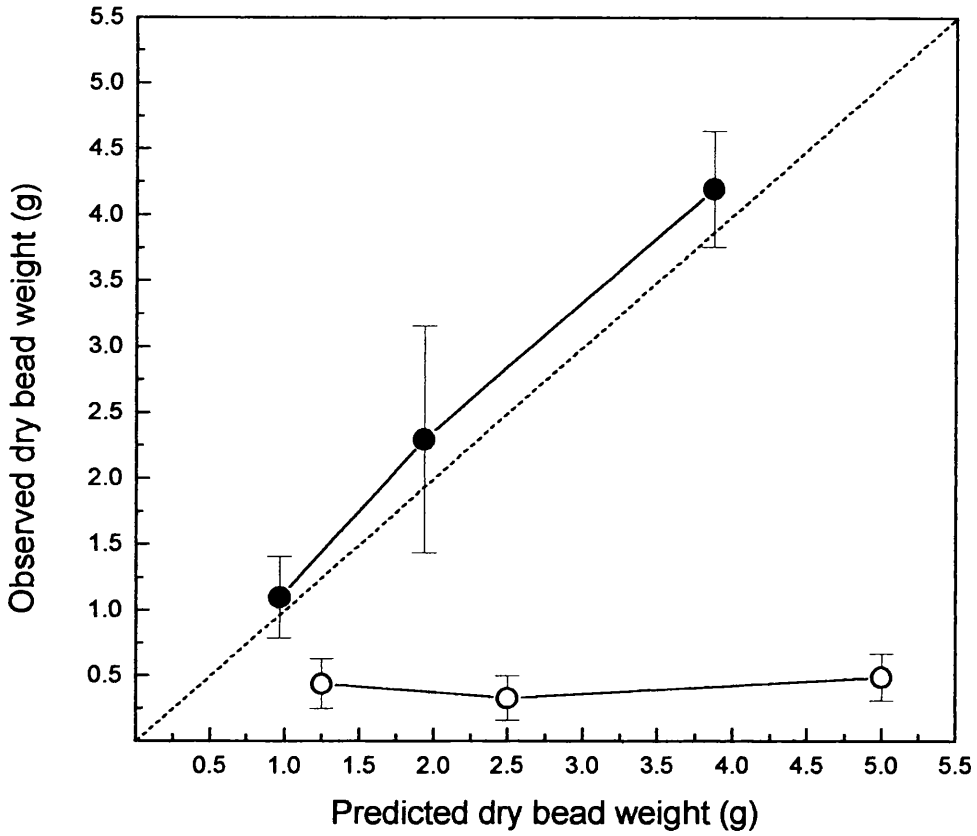


Figure 5.1:

Parity plot showing predicted versus observed values for the dry bead weights dispensed with the large orifice pipette tip (●) and standard pipette tip (O). The errors are the standard error for each treatment, and the result of 10 repeats.

Table 5.1:

Accuracy and precision of standard and large orifice yellow tips for dispensing a 100mg/ml bead slurry. The measured amounts given are the average of 10 readings. SD = standard deviation, CV = coefficient of variation (standard deviation as a percentage of the mean).

		Standard tip	**Large orifice tip**	**Recommended values**
amount predicted		5mg beads	5mg beads	50µl
amount measured		0.49mg beads	5.43mg beads	-
number of readings		10	10	-
accuracy:		+/- 4.51mg	+/- 0.43	+/- 0.4µl
		(90.2%)	(8.5%)	(0.5%)
precision:	SD	0.18mg	0.44mg	0.012µl
	CV	36.7%	10.4%	0.24%

than the standard tip. Compared to the recommended values from Anachem (for dispensing water), the large orifice tip is less accurate (8.5% compared to 0.8% error). The precision of the large orifice tip compared to recommended values is even worse (a coefficient of variation of 10.4% compared to 0.24%). However, the large orifice tip still proves to be the best method to dispense a small amount of bead slurry quickly and reasonably accurately. One reason for the difference in the performance of the tips could be that the smaller aperture of the standard tip has the effect of filtering out some of the beads both when drawing up and dispensing the suspension, thus less beads are delivered into the vessel. The small tip opening also leads to the beads blocking the tip so that nothing can be delivered. The bead suspension had to be constantly agitated by hand for both tips to keep the suspension homogeneous and to prevent the beads from settling and therefore reduce the variation in the number of beads dispensed from time to time. Some settling of beads was also observed within the large orifice tip, which can lead to a concentration of beads near the tip and therefore a higher number of beads will be dispensed.

5.4.2 Accuracy

Table 5.2 shows the results of studies into the inaccuracies associated with starting the reaction in each well of a 96-well plate at a different time. The wells which had enzyme added in earliest with the single channel pipette showed a lower reaction rate than those with the enzyme added last. This was not seen for the multi-channel pipette. The coefficient of variation (standard deviation as a percentage of the mean) was much lower for the multi-channel pipette compared to the single channel and multiple plates, as expected, however this is still quite a high variation around the mean.

From these data it is possible to work out the number of repeats of an individual experiment which are necessary to obtain a mean value with a specified level of certainty and therefore the number of wells in each plate which should have repeats of the same experiment in them. The relationship between number of repeats (N) and the estimated standard deviation from these experiments (σ) is expressed as:

$$N = \frac{4t^2\sigma^2}{L^2}$$

where t is the value of the student's t-test for the specified degree of freedom (95 or 575) and 95% confidence. L is the length of confidence interval, in this case decided

Table 5.2:

Errors associated with commencing identical experiments in 96-wells plates and in 6 sets of 96-well plates. The minimum number of readings necessary for a mean with accuracy of ±0.25% with 95% confidence and 95 degrees of freedom (single channel and multi-channel) or 575 degrees of freedom (6 plates).

	Single channel pipette	**Multi-channel pipette**	**6 plates, multi-channel pipette**
Mean (activity, U/ml)	4.0	5.3	4.8
Standard Deviation	1.0	0.3	0.35
Coefficient of Variation	25.0%	5.7%	7.3%
Number of readings needed	44	4	6

as 0.5%. Table 5.2 shows the number of readings necessary in each case for the specified degree of accuracy. Hence the optimisation experiments in the next chapter are repeated 6 times.

5.5 Discussion

Some of the problems associated with operating a robotic system in the context of enzyme reaction optimisation have been investigated here.

Most of the commercially available robotic liquid handlers are designed to operated with liquids only or a low density solid emulsion (such as a cell suspension). However, many enzymatic systems are operated with the enzyme immobilised on a support and the support may change some of the properties of the enzyme, therefore it is necessary to find a method to dispense accurate quantities of bead slurry in the wells of a plate. The main problems identified in dispensing bead slurry by the pipetting methods investigated are:

- The slurry which the beads are pipetted from must be homogeneous so that equal amounts of beads are picked up each time. The Eupergit C beads tested however, were dense and so quickly settle in solution and so the vessel holding them had to be constantly agitated to maintain a homogeneous state.
- The beads may clog the tips both when drawing the slurry up into the pipette tip and when dispensing, resulting in the slurry being effectively filtered, so that more liquid than solid is drawn or dispensed.

Until the problems of robotic bead handling are addressed, the usefulness of robotic systems when dealing with immobilised enzymes will be limited.

The accuracy of the robotic system is not one that had been discussed in this context before. Previous examinations of robotic systems have been in their use for large scale screening, where large numbers of candidates are assayed for a particular property. This differs significantly from the use of robotic systems described here and in the subsequent chapter. Instead of looking at a very large number of conditions and assaying for a simple positive or negative response, a smaller number of conditions are studied and more quantitative data is sought. With the screening example a false negative or positive response can be allowed for and therefore a degree of inaccuracy

is permitted. However, with the quantitative analysis, the accuracy of the assay system must be much more sensitive, as an inaccurate response could skew the data and give a false impression of the system. With the screening example, only one repeat of each condition is necessary, but with the quantitative example it is necessary to determine the number of repeats which are necessary of the required degree of accuracy. Therefore, the accuracy of the system is determined in section 5.5. Figure 5.2 shows the relationship between the accuracy of a system and the number of repeats necessary. The degrees of freedom and therefore the t value is maintained constant and the standard deviation in this fictional system is varied. The different confidence intervals (L) chosen show that if either the standard deviation increases or the accuracy which is chosen (L) increases the number of repeats necessary has to be increased.

For the real system used in this example the number of repeats necessary were six. It is important to note that, while it is possible to repeat each experiment more than six times, to do so would make those extra repeats redundant. The redundant repeats would not increase the accuracy of the final result nor would they add to the information already gathered with the first six repeats. Redundancy is an important phenomenon in this context as it is tempting to do many repeats of each experiment, just because it is possible to do so. Without first investigating the accuracy of the system it is impossible to ascertain how many repeats are really necessary.

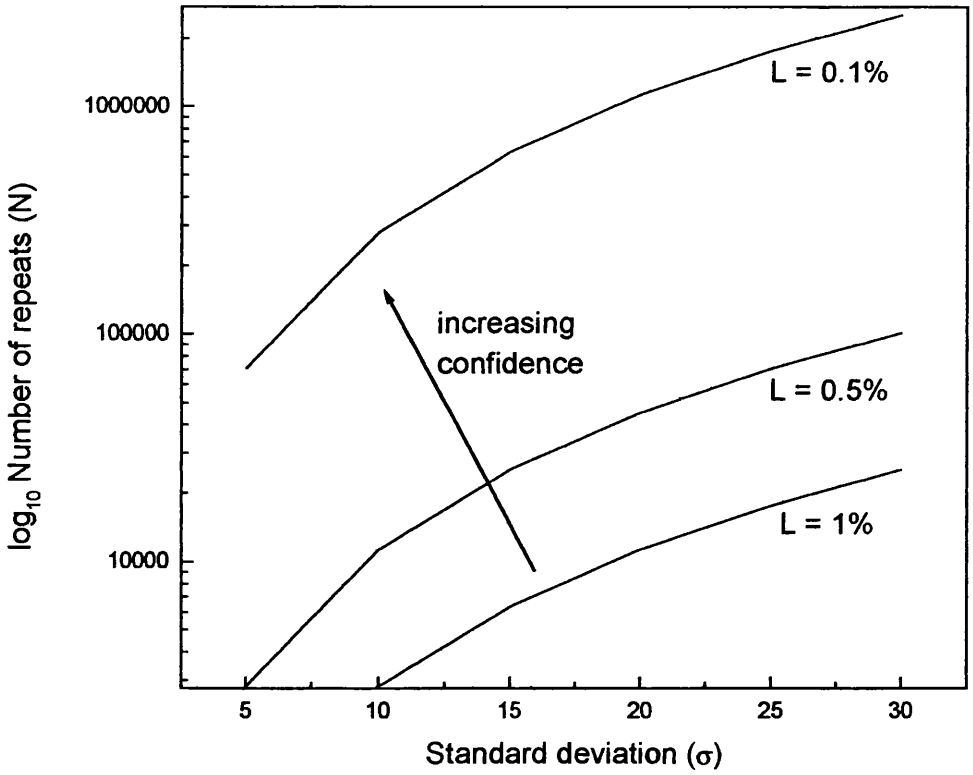


Figure 5.2:

Relationship between number of repeats (N) and standard deviation (σ) for different lengths of confidence intervals (L). This graph represents the equation to calculate the number of repeats necessary for an experiment:

$$N = \frac{4t^2\sigma^2}{L^2}$$

The degrees of freedom and therefore the value of t (Student's t-test) remains constant.

6 Optimisation of conditions for a second enzyme cofactor regeneration reaction

6.1 Summary

A combined, two enzyme system has been optimised using a semi-automated, high throughput screening method. The synthetic enzyme, tetralone reductase, reduces tetralone to tetralol, while a second regenerative enzyme, formate dehydrogenase, converts formate to CO_2 and recycles NADH. The optimum condition of pH (6.8), temperature (26°C) and solvent (30% octanol) were found by factorial experiments using a robotic liquid handling system to produce a semi-automated process.

6.2 Introduction

As described in the previous chapter, time to market for new pharmaceuticals is a priority. Therefore any methods which can speed the progression of process synthesis will be welcome. This chapter demonstrates the use of a semi-automated robotic system for the optimisation of the combined synthetic (tetralone reductase) and regenerative (formate dehydrogenase) reactions. To further streamline the process and to reduce the number of experiments necessary a factorial approach was taken to the optimisation.

Factorial designs are schemes in which the effects of several factors are studied simultaneously. In the analysis of the factorial experiment the effect of each factor on the measured value is considered and also the interaction between the factors can be examined. The outcome is a model which should predict the behaviour of the measured value under any conditions of the factors in the range which they are tested.

The oxidation by formate dehydrogenase was chosen as the NADH regenerative method. This has previously been used successfully by other workers for the regeneration of NADH (Chenault *et al*, 1988). The major advantage of this enzyme is that the product of the reaction is CO_2 when the substrate formate is used. This CO_2 gas will then leave the reaction liquid or can be driven off by gas sparging, resulting in a cleaner system for subsequent downstream processing. Despite the fact that the

enzyme itself is fairly expensive compared to other NADH regenerating enzymes, the substrate is very cheap.

6.3 Methods

6.3.1 Enzyme production

Tetralone reductase was isolated from *Trichosporon capitatum* MY1890 as described in section 4.3.3, as far as the Q Sepharose FF column.

The enzyme was immobilised onto Eupergit C acrylic beads (Rohm). 1g of dry beads were challenged with 5mls of enzyme solution (6U/g beads challenge) in 0.5M potassium phosphate buffer at pH 6 and left at room temperature for 72 hours, before washing the unbound enzyme off the beads with 1M phosphate buffer, pH7. This resulted in a specific activity of 3 U/g beads and 50 % retained activity on the beads.

6.3.2 Reverse phase HPLC assay

A Zorbax RX-C8 column (4.6mm x 25cm) (Mac-Mod Analytical) was used for analytical separation of the tetralone from the tetralol. The mobile phase was acetonitrile and acidified water (0.1% phosphoric acid) (50/50 v/v) at a flow rate of 1.5 mls/min. Detection was by UV at 220nm and 22°C. Tetralone and tetralol eluted at 6.5 and 4.7 minutes respectively.

For automated HPLC analysis with the Gilson Liquid Handler 215 (Gilson) this method was scaled-down to 2.5 minutes total assay time by using a LUNA 5μm C8(2) 30 x 4.6mm column (Phenomenex), using the same elution conditions as before and 1.5ml/min flowrate. Tetralone and tetralol eluted at 1.09 and 0.83 minutes respectively.

6.3.3 Immobilised enzyme assays

The activity of both the synthetic (tetralone reductase) and regenerative (formate dehydrogenase) enzymes were separately assayed by following the respective decrease or increase in NADH concentration at 340nm.

The activity assay mixture for the tetralone reductase was 5% of a 10mg/ml tetralone in ethanol solution mixed with 95% of a 0.5mg/ml NADH in water solution.

This gave final concentrations of 2.2mM and 0.67mM for the tetralone and NADH respectively. The assay mixture for the formate dehydrogenase was 130mM formate and 0.9mM NAD in 0.5M Tris buffer at pH7.

100µl of a 250mg/ml immobilised bead slurry was added to each well of a series of identical Microscreen 96-well plates (Millipore), fitted with 5µm filters in the bottom of each well. The appropriate assay mixture was added to each well to start the reaction. At suitable intervals the liquid contents of each well of one of the plates were vacuum filtered into a fresh 96-well plate using the Millipore Microscreen vacuum manifold and the absorbance at 340nm measured by plate reader. Another of the identical plates was filtered and assayed at the subsequent time interval (i.e. each plate was sacrificed). The filter plates were incubated on an Eppendorf themomixer (Eppendorf) at 27°C with mixing at 400rpm.

6.3.4 Substrate and product adsorption onto Eupergit C beads

It was found that both the tetralone substrate and tetralol product adsorbed to the Eupergit beads during the course of the reaction. This was measured by incubating 5mM each of tetralone and tetralol in an aqueous ethanol solution (50:50) with 0.4g/ml Eupergit C beads and assaying for the presence of the reactants in the solution. In addition it was found that the tetralone and tetralol could be eluted from the Eupergit beads with a range of different solvents. The beads used in the adhesion study were then used to look at tetralone and tetralol elution by washing with successive 250µl aliquots of either methanol, ethanol or acetone and assaying for the reactants in the filtrate.

6.3.5 Cofactor challenge

The total turnover number of the reaction (TTN) is described as the total number of moles of product produced divided by the total number of moles of cofactor present (Chenault *et al,* 1988) and is an indication of the efficiency of the regeneration process. However, the highest potential TTN is determined by the ratio of cofactor to substrate added to the reaction, if an excess of cofactor is added, the TTN will be lower. Therefore the minimum amount of cofactor which can be added and still achieve at least 90% conversion was investigated. Both reactions involving both

enzymes were run together with the amount of tetralone and formate added kept constant at 10mM and 130mM respectively and the concentration of NAD^+ varied between 0 and 10mM. The amount of tetralol produced over a 24 hour period was monitored by HPLC analysis.

6.3.6 Factorial design

The optimum conditions of pH, temperature and solvent were investigated for the 2 enzyme reaction with a factorial experiment. A Central composite orthogonal factorial design was set up with three factors: temperature (varied between 20 and 40°C), pH (varied between 5 and 8) and solvent concentration (varied between 10 and 50%) using JMP software (SAS Institute Inc.). Three solvents were also tested, ethanol, octanol and hexane. These conditions were chosen based on previous experiments which characterised the activity and stability of tetralone reductase (chapter 4).

6.3.7 Automated optimisation reactions

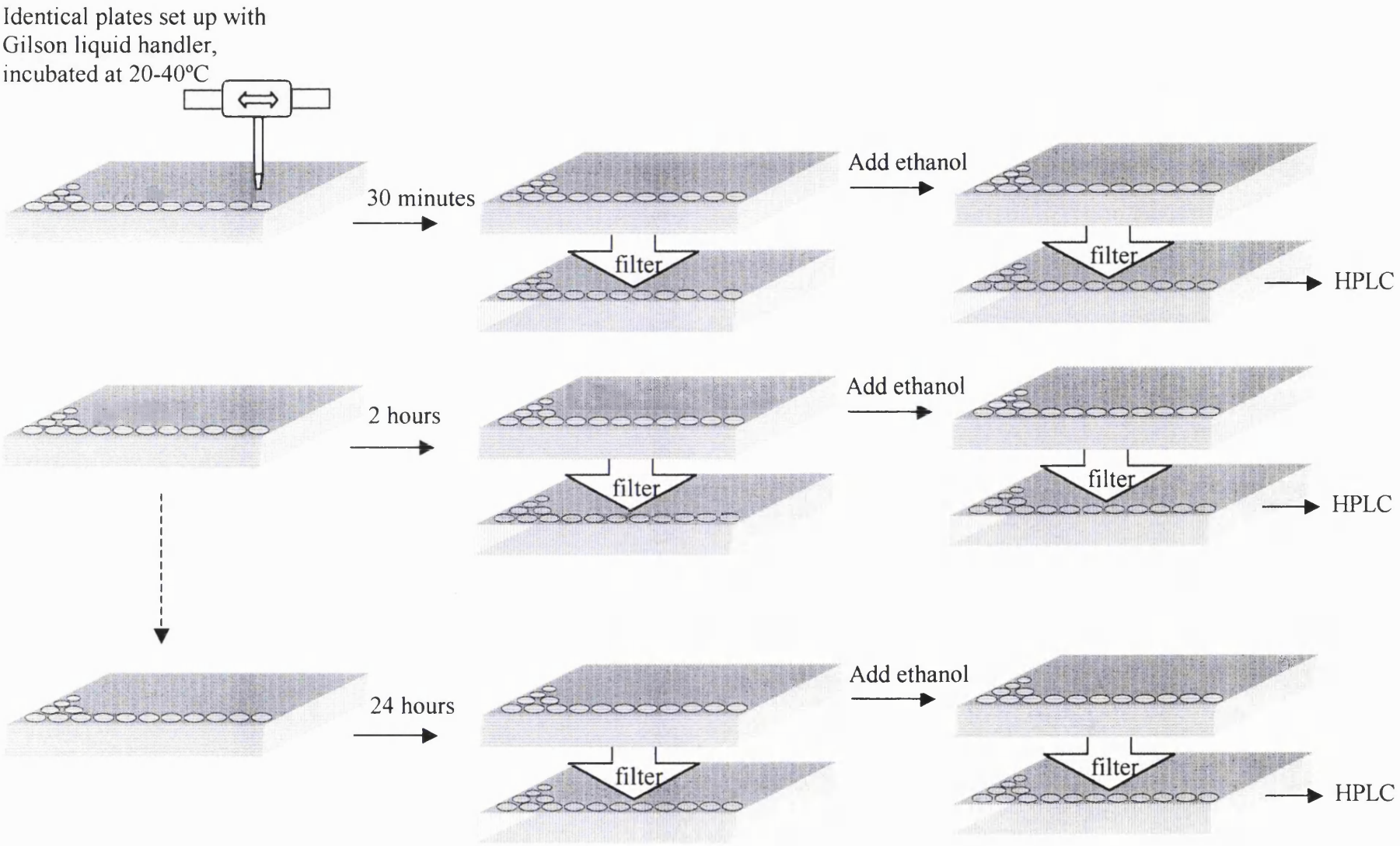
The experiments selected by the factorial design were set up in 96-well filter plates covered with plastic film covers (Fisher Scientific). Each plate contained different conditions of solvent type, solvent concentration and pH and were kept at an appropriate temperature, according to the factorial design. In addition each well contained 5mM of tetralone and 130mM of formate and the reaction was started by adding 100µl of immobilised tetralone reductase and FDH bead slurry. Several identical plates were set up and at suitable time intervals the contents of the plates were filtered into another standard 96-well plate below the filter using a Millipore vacuum filtration manifold (Millipore, Watford, Herts., UK). The filtrate was discarded as the tetralone and tetralol adhere to the Eupergit C beads, which were then eluted by washing with 250µl of ethanol and filtering this into another 96-well plate which was subsequently assayed by HPLC on the Gilson Liquid Handler. Figure 6.1 shows a scheme of the experimental set up.

6.3.8 Scale-up

The fully optimised conditions were used to demonstrate that the process could be scaled-up to 100mls (a factor of 500). A batch stirred tank mode was chosen as the reactor type with an overhead marine turbine impeller (dia. 4cm) at a stirrer speed of 243rpm to agitate the reaction. A final concentration of tetralone of 50mM was used

Figure 6.1, following page:

Semi-automated optimisation experiments.


Identical plates set up with
Gilson liquid handler,
incubated at 20-40°C
30 minutes
filter
Add ethanol
filter
HPLC
2 hours
filter
Add ethanol
filter
HPLC
24 hours
filter
Add ethanol
filter
HPLC

with 200mM formate and 0.5mM NAD^+. The pH of the system was initially adjusted by adding acid and alkali, but no buffer was used. A total of 23g of immobilised Eupergit C beads was added with equal specific activities of the tetralone reductase and the formate dehydrogenase, for a total activity of 28U of each enzyme.

The amount of tetralone and tetralol in the aqueous and organic layers was assayed by HPLC at appropriate intervals. The amount of tetralone and tetralol associated with the Eupergit C beads was also assayed by washing the beads with 200µl of ethanol and assaying the filtrate by HPLC.

6.4 Results

6.4.1 Immobilisation of formate dehydrogenase

The immobilisation of formate dehydrogenase onto Eupergit C was investigated, using both the same conditions as for the immobilisation of tetralone reductase (0.5M phosphate buffer pH6) and the standard conditions recommended by the manufacturers (1M phosphate buffer pH7). The final specific activities of the FDH on the Eupergit beads were 2.01 and 1.09U/g beads for the 1M and 0.5M buffer concentrations respectively. Therefore immobilising both the FDH and tetralone reductase on the same beads was not possible due to the difference in optimum immobilisation conditions and so separate batches of the two immobilised enzymes had to be prepared and then mixed for the combined reactions.

The enzyme challenge for the FDH was also investigated resulting in an optimum enzyme challenge of around 10U/g beads which gave a specific activity on the beads of 2.5U/g and a retained activity of 25%.

6.4.2 Substrate and product adsorption onto Eupergit C beads

Figure 6.2 shows the amount of tetralone and tetralol in the supernatant and that adsorbing to the Eupergit C beads when the beads were incubated with an organic/aqueous solution of the substrate and product. The amount of the tetralone and tetralol in the supernatant falls to low levels (less than 5mM) after 20-30 minutes and a concomitant amount may be detected on the beads. Therefore most of the

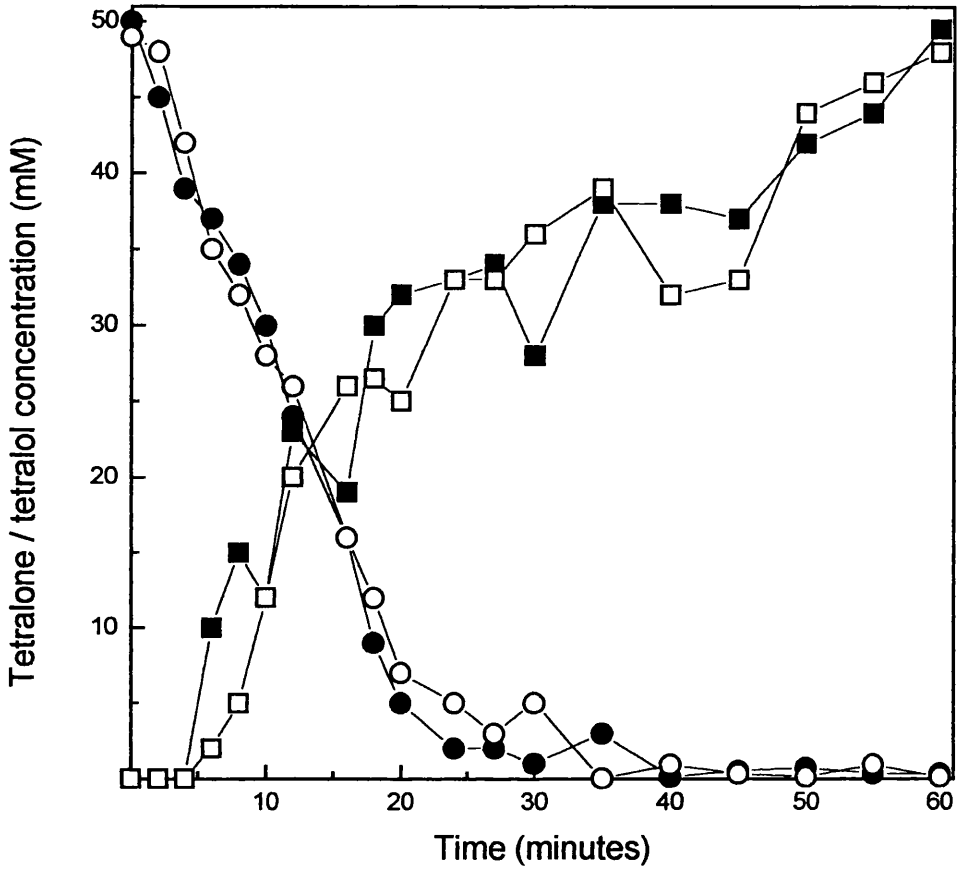


Figure 6.2:

Adhesion of tetralone and tetralol onto Eupergit C beads. 50mM each of tetralone and tetralol were dissolved in a 50:50 mixture of ethanol and water with Eupergit C beads with immobilised FDH added. The levels of both reactants were assayed in the supernatant by HPLC and the amount on the beads was assayed by removing a sample, and washing the beads with ethanol and testing this wash by HPLC. (●) tetralone in supernatant, (O) tetralol in supernatant, (■) tetralone on beads, (□) tetralol on beads.

tetralone and tetralol is assumed to be associated with the Eupergit C beads after 30 minutes.

Ethanol proved to be the most effective elutant for the tetralone and tetralol. 250µl of ethanol was sufficient to elute 98.9% of both reactants from 0.04g of beads. A second wash with 250µl of ethanol resulted in no detectable tetralone or tetralol. Methanol and acetonitrile required three 250µl aliquots to elute 97.2 and 89.1% respectively.

6.4.3 Cofactor challenge

The appropriate amount of cofactor to add at the start of the reaction was investigated and the results are shown in Figure 6.3. The conversion of tetralone to tetralol over a period of 24 hours was investigated. As the amount of NAD^+ added was increased, the conversion after 24 hours incubation also increased, so that at around 1mM NAD^+ the conversion was close to 100%, representing a TTN of 100. At lower initial NAD^+ concentrations the conversion was lower than 90% which would lead to an inefficient process. Therefore a tetralone/NAD^+ molar ratio of 10/1 was chosen for the optimisation experiments.

6.4.4 Optimisation

The factorial data showed that there were no interaction effects between the pH, temperature and solvent concentration factors, therefore each of these factors influences the reaction independently. The quadratic of these factors (eg. temperature x temperature or pH x pH) was important to the model of the data. The three sample solvents were investigated independently, with octanol providing the highest activity and ethanol the lowest. Figure 6.4 shows that there appears to be a relationship between the solvent type and the optimum temperature for the reaction, possibly due to an effect on the stability of the enzymes by the solvents. From this it is also possible to see that octanol gives the highest activity (up to 114U/µl). The predicted response for ethanol appears to be a straight line, whereas the other two solvents show a curve. It is possible than the optimum for ethanol is at a lower temperature than those tested (below 20°C).

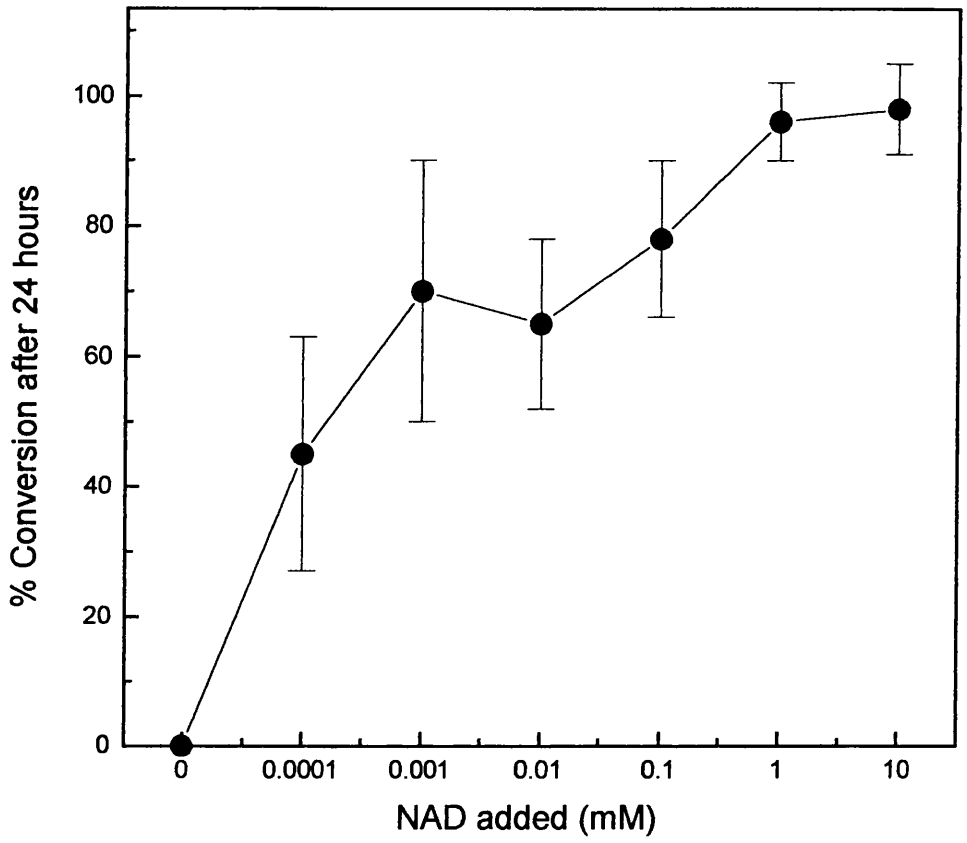


Figure 6.3:

Cofactor challenge. The synthetic and regenerative enzyme reactions were run together with the amount of tetralone added fixed at 10mM while the amount of NAD^+ cofactor added varied between 0 and 10mM, with formate added in excess.

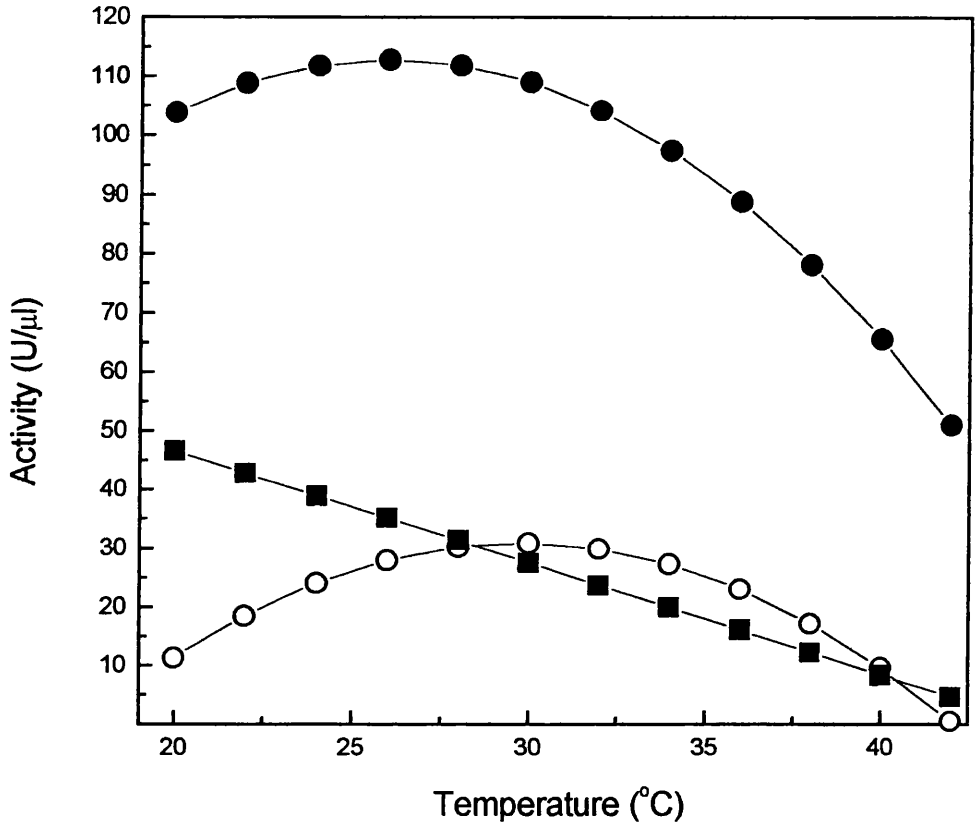


Figure 6.4:

Activity vs. temperature predicted values from the response surface models of the combined two enzyme biotransformation for the three solvents tested. The pH was 6.8, solvent concentration 30%. (●) octanol, (O) hexane, (■) ethanol.

Figure 6.5 shows the optimum conditions when using octanol as solvent. The optimised conditions for the synthetic and regenerative reactions run together is a pH of 6.8, 26°C and 30% octanol as solvent.

6.4.5 Scale-up

Figure 6.6 shows the results of this scaled-up reaction, from this it can be seen that an almost complete mass balance for the tetralone and tetralol is achieved. It is not possible to assess the mass balance for the formate dehydrogenase reaction as this was not measured during the experiment. Table 6.1 shows a comparison of data between this optimised scaled-up reaction with immobilised enzyme and the whole cell reaction as described by Reddy and co-workers (1996).

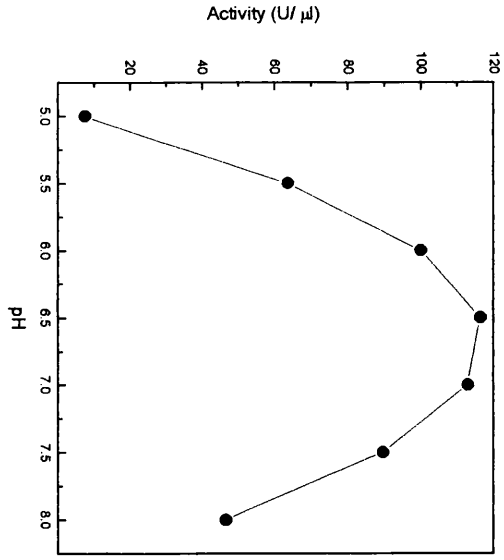
6.5 Discussion

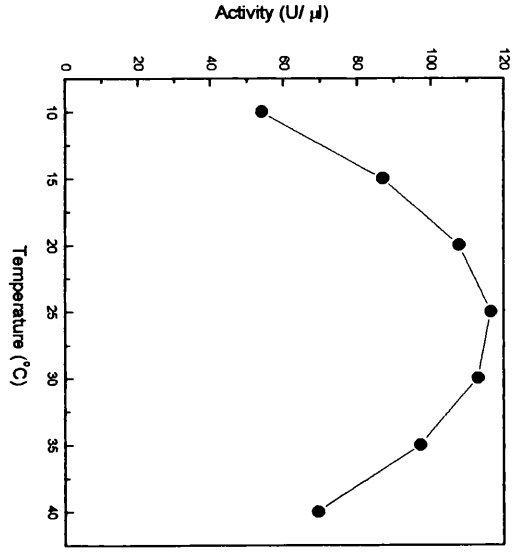
A factorial approach to optimisation has proved useful in this case. Preliminary data had already been established and set the limits within which the factorial was set up. If this had not been done, it may have been necessary to repeat the process until the correct range of conditions was found, thus requiring more experiments to be done. The factorial approach not only reduces the number of experiments necessary, but it can also offer additional information which separately conducted experiments usually do not. For instance, factorial experiments can uncover interactive effects between treatments, for example pH and temperature may have an effect on the enzyme which it is not possible to predict from looking at those two conditions separately. However, in this case, there were no interactive effects found between the treatments which were chosen.

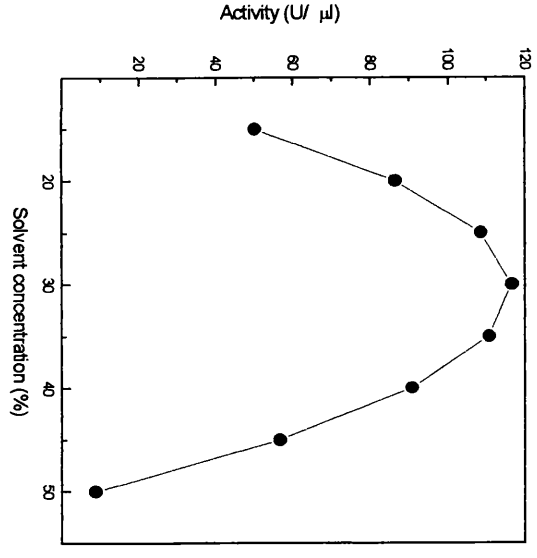
The synthetic (tetralone reductase) and regenerative (formate dehydrogenase) reactions were optimised together, rather than finding the optimum conditions for the regenerative reaction and then trying to fit the two operational windows together, as first proposed in chapter 2. This was because assaying both reactions together took into account any interactive effects between the two reactions, for example, if the product of one reaction was inhibitory to the other (also discussed in chapter 2). However, it is not possible to predict how the regenerative reaction will behave under the conditions chosen on its own. Therefore if this particular immobilised formate dehydrogenase reaction was to be used as a regenerative step in another reaction, it would still have to be optimised again.

Figure 6.5, following page*:*

Predicted values from the response surface model of the combined two enzyme biotransformation for octanol. The predicted optimum pH is 6.8, temperature is 26°C and solvent is 30%.


Activity (U/ µl)
20
40
60
80
100
120
pH
5.0
5.5
6.0
6.5
7.0
7.5
8.0


Activity (U/ µl)
0
20
40
60
80
100
120
Temperature (°C)
10
15
20
25
30
35
40


Activity (U/ µl)
20
40
60
80
100
120
Solvent concentration (%)
20
30
40
50

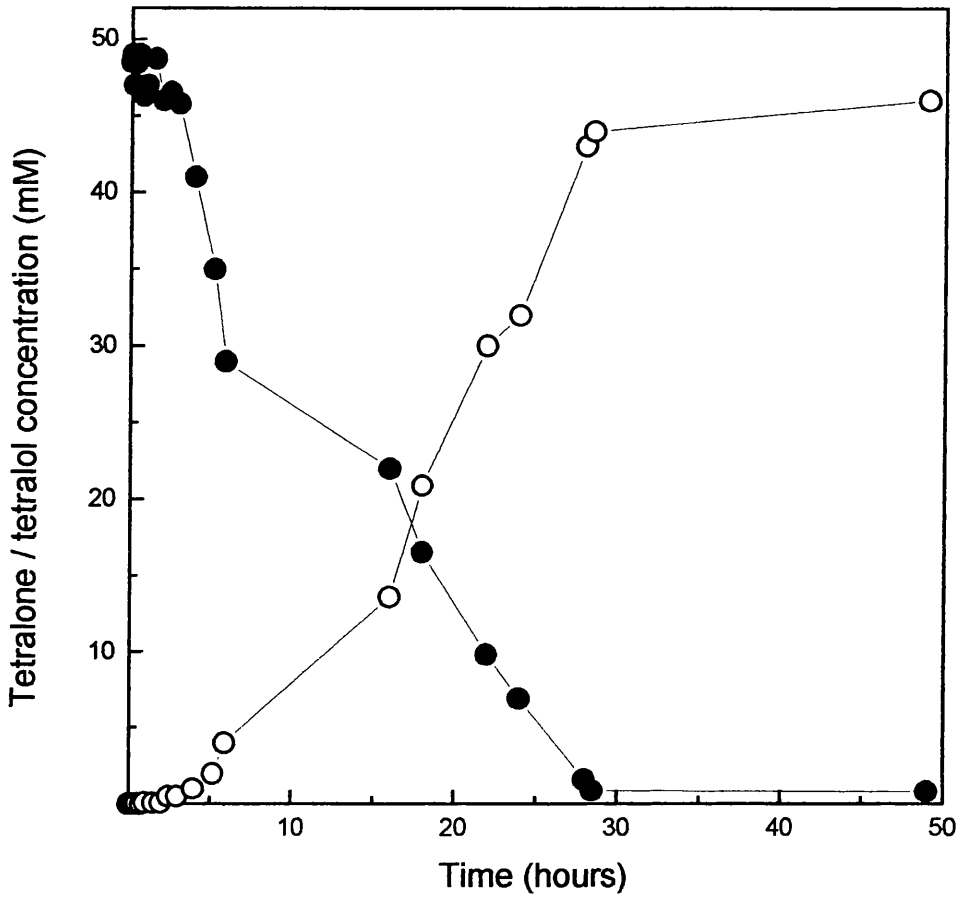


Figure 6.6:

Combined two enzyme biotransformation scaled up to 100ml using the optimised conditions of pH6.8, 26°C and 30% octanol. 50mM tetralone, 200mM formate and 0.5mM NAD^+ added at start of reaction, 28U enzymes.

(●) total tetralone (in aqueous and organic layers and adhering to beads), (○) total tetralol.

Table 6.1
Comparison of tetralone to tetralol reaction catalysed by immobilised tetralone reductase with formate dehydrogenase cofactor regeneration and the same reaction catalysed by whole cells, as described by Reddy and co-workers (1996).

	Immobilised recycle reaction	**Whole cell reaction**
Total volume	100mL	23L
Final product titre	9.76g/L	1.42g/L
Initial reaction rate	3.178g/L/hr	1g/L/hr
Percentage conversion	86%	71%

In a further attempt to reduce the number of experiments necessary to have a useable amount of design data the activity and stability of the combined enzymes were not investigated separately. The experiment was run over 24 hours within which the half life of the tetralone reductase is significant (the half life is around 6 – 16 hours for most conditions, see chapter 4). If the stability of the immobilised formate dehydrogenase is higher than the tetralone reductase then the small drop in activity will be insignificant. If the stability is lower than the tetralone reductase, then this will be seen in a low conversion rate under that condition. However, no data on the stability of the immobilised formate dehydrogenase was collected and so again, if this regenerative enzyme was to be used for another recycle system, it may be helpful to have this data to work from.

The semi-automated method, using a robotic liquid handler, has the potential to accelerate the process further. The potential disadvantages for the development of this technique have been discussed in the previous chapter. The automated approach is of particular merit when repeating many different conditions, for instance testing many different solvents (only three were used in this study). The set-up time for the system can be significant and the experimental time is often comparable with non-robotic methods. However, the robotic system can theoretically work 24 hours a day and although some attention is required during the course of the experiment, the operator is mostly free to do other work.

The optimised conditions were successfully scaled up by a factor of 500. The final product titre, initial reaction rate and percentage conversion are all improved over the whole cell catalysis. However, it is important to point out that the whole cell catalysis was not as fully optimised for pH and temperature as the isolated enzyme, and the stability of the enzyme is assumed to be greatly reduced once it is isolated. The previously unreported phenomenon of the substrate and product adhering to the immobilisation matrix beads could lead to a method of product recovery. The beads could be filtered from the reaction mixture and then washed with a suitable solvent to elute the product. The remaining substrate could then be separated from the product.

7 Discussion

7.1 Limitations to the whole cell process

While a whole cell catalyst is frequently the biocatalyst of first choice, there are certain limitations which do not always make it the most desirable. There can be diffusional limitations due to the cell's membrane so that the substrate's entry into the cell and product's exit from it is restricted (Angelova & Schmauder, 1999).

Organic solvents are frequently required to dissolve the substrate or product, as in the case of tetralone. Whole cells are often sensitive to many of these solvents as they interfere with the cell membrane. This can also lead to the resolution of the previous problem of diffusional limitation as the solvent can make holes in the cell membrane, thus allowing the transmission of larger molecules (Adlercreutz, 1991).

The complex nature of whole cells can lead to other problems derived from the large number of other enzyme catalysed reactions which can also occur. Side reactions by other enzymes present may degrade the substrate or the product, thus reducing the yield. There may also be more than one stereoscopic form of an enzyme, each making a different enantiomer. If only one enantiomer is desired, then the enantiomeric excess will be lower than required. No evidence was found that there was degradation of the substrate or product by *T. capitatum* over the timescales used. The enantiomeric excess in the whole cell was fairly high, at up to 99% and therefore it seems that only one enantiomeric form of the enzyme was operating.

Some enzymes can be present at fairly low levels in cells and therefore high cell densities are necessary for an efficient space time yield. The high volumes which may be necessary to hold such a large quantity of cells could lead to a low substrate and therefore product concentration which will make downstream processing a more difficult and therefore more expensive process. The cell density could be increased by immobilising the cells to a matrix which can be used in a packed bed with a higher catalyst density, but cells treated in this way must be resting and not in a growth phase.

The more complex nature of the reaction solution can also lead to downstream processing difficulties. Cell growth medium contains many different complex substances which will have to be removed from the product. The cells can be washed free of their growth medium before being used as a catalyst, but the cells themselves can still release substances which contribute to a more complex processing requirements.

The model reaction with whole cells was operated with the cells still in a complex growth medium which would contribute to an expensive downstream process. The concentration of the tetralone reductase enzyme within the cells appears to have been low. After purification the tetralone reductase contributed to less than 1% of the total protein in *Trichosporon capitatum*.

However, whole cell catalysts have many advantages. Catalyst production is relatively cheap as it requires the minimum of processing. The enzymes contained within the cell are all fairly stable and the cell may also be able to regenerate the enzyme. Cofactor recycle for redox reactions occurs within the cell, providing the right balance of reductive or oxidative substrates are offered to the cell. Although the cellular concentration of the required enzyme may be low, it is possible to increase it by genetic engineering methods to overexpress it.

7.2 Potential benefits of the isolated enzyme

The major obstacles to running a process with a whole cell catalyst are also the major benefits of using an isolated enzyme catalyst. Diffusional limitations are no longer a problem as the cell membrane is removed. This problem may arise again if the enzyme is immobilised within a matrix and the substrate and product have restricted access to the enzyme due to the matrix (Carleysmith *et al*, 1980).

Side reactions are potentially eliminated from an isolated enzyme process. Most enzymes are not isolated fully to the single enzyme, as this can lead to a very low enzyme stability. Therefore it is important to at least remove any enzymes which can interfere with the reaction. This did not appear to be a problem with the tetralone reductase reaction and even with whole cells there was no significant problem with

side reactions. Again, no significant problem was found with two opposite enantiomeric forms of the tetralone reductase enzyme in the whole cell, but if there was, this could be rectified by separating the desired enantiomeric form in isolating the enzyme.

One of the foremost advantages of the isolated enzyme over the whole cell for the model system described is the increased ease of downstream processing. The isolated enzyme is a much cleaner system than the whole cell, as only the necessary components of the reaction are added. It is not even necessary to add buffer, as it would be for washed whole cells, as any pH requirements can be met by the addition of acid or alkali. The substrate concentration and therefore product concentration formed can also be increased compared to the whole cell method as the enzyme concentration can be increased. The final product titre for the isolated enzyme method was 9.76g/L as opposed to 1.42g/L for the whole cell. The most significant cost in downstream processing can often be attributed to the removal of water or solvent from the product, therefore a more concentrated product is most desirable.

However there are also significant problems with operating a whole cell process. Not least of these in the model reaction is the requirement for cofactor recycle to provide an economically feasible process. In the case of the model reaction a solution was found in using formate dehydrogenase as a second enzyme to regenerate NADH.

The tetralone reductase enzyme was found to be unstable upon isolation, with a half life of up to 16 hours which increased slightly to around 20 hours after immobilisation. Together with the instability of the enzyme, the purification of the enzyme, which is present in a fairly low concentration within the whole cell, will contribute to make this a relatively expensive enzyme to use in a process.

7.3 Isolated enzyme window

It is possible to construct a window of operation for the tetralone reductase enzyme with the results from chapter 3. Figure 7.1 shows such a window with temperature and pH chosen as the axes. The enzyme and cofactor are both affected by pH and temperature, but the tetralone and tetralol were not found to do so under the

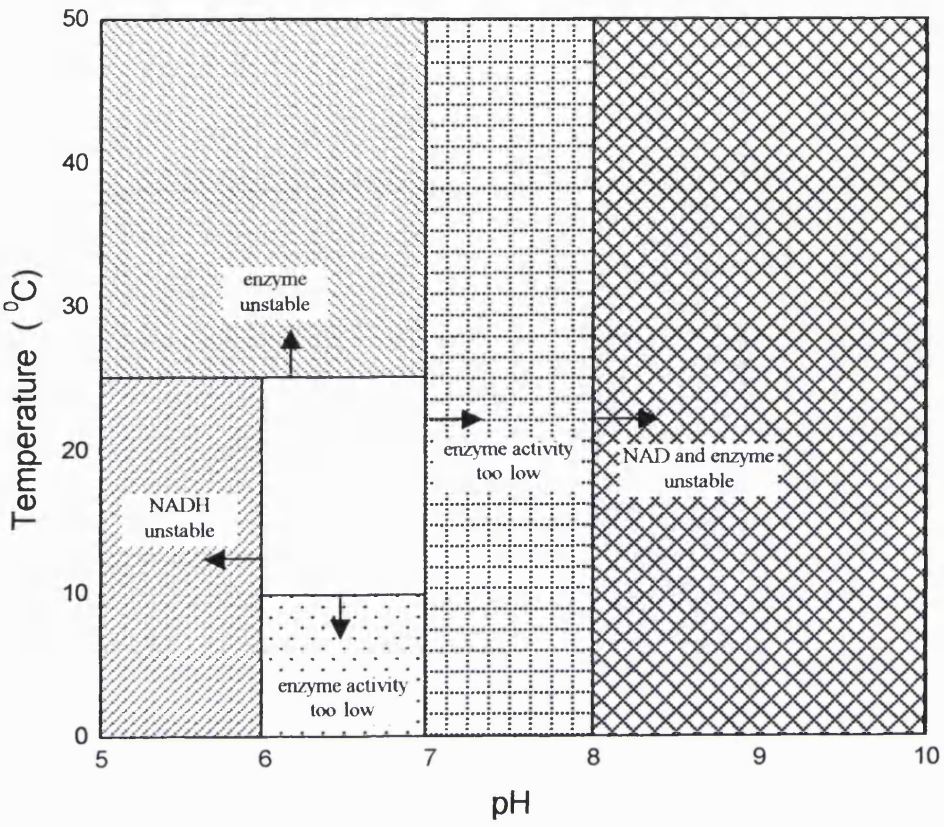


Figure 7.1:

Window of pH vs temperature for free tetralone reductase enzyme. The white box indicates the area with optimum conditions.

conditions tested. The optimum temperature for the enzyme is a compromise between a high activity and high stability (section 4.5). The optimum pH for the enzyme however, is a range where both the activity and stability are high. The oxidised and reduced forms of the cofactors are both most stable in the same range of pH as the enzyme and are not as sensitive to temperature deactivation as the enzyme (Lowry *et al*, 1961 and Wong & Whitesides, 1981). This leaves a window of operation for the temperature and pH between pH6-7 and 10-25°C. The lines drawn on the diagram in figure 7.1 represent threshold values for each of the conditions, for example, the minimum enzyme activity has been set at 0.04U/mg protein. These values can be altered to give a larger or smaller window according to the requirements of the process. Other axes could also be chosen to highlight other aspects of the process.

Figure 7.2 shows the same window of operation diagram for the tetralone reductase enzyme immobilised onto Eupergit C. In this case, the stability of the enzyme is increased slightly in respect to both temperature and pH. Although this increases the size of the window along the temperature axis, is does not increase it along the pH axis as the enzyme activity and cofactor stability are more limiting to the processes at higher pH values. Such information can lead to important design criteria as the diagram shows that attempting to increase the enzyme stability with respect to pH would not improve the process any further.

Figure 7.3 shows the same operational window as figure 7.2 with the factorial experiment results from chapter 6 superimposed over the window. This shows more detailed information about the nature of the tetralol production for the combined synthetic and regenerative processes. The peak optimum conditions are shown within the window, but the contour lines surrounding it also show what happens if the process is moved around within the window. Therefore the contours can indicate the loss of tetralone production if the process is moved away from the peak optimum conditions.

The factorial experiments were actually carried out over a range which was larger than the operational window, which may have led to some redundancy of information. The thresholds for the immobilised formate dehydrogenase are not known as they were not investigated separately from the tetralone reductase and so cannot be added

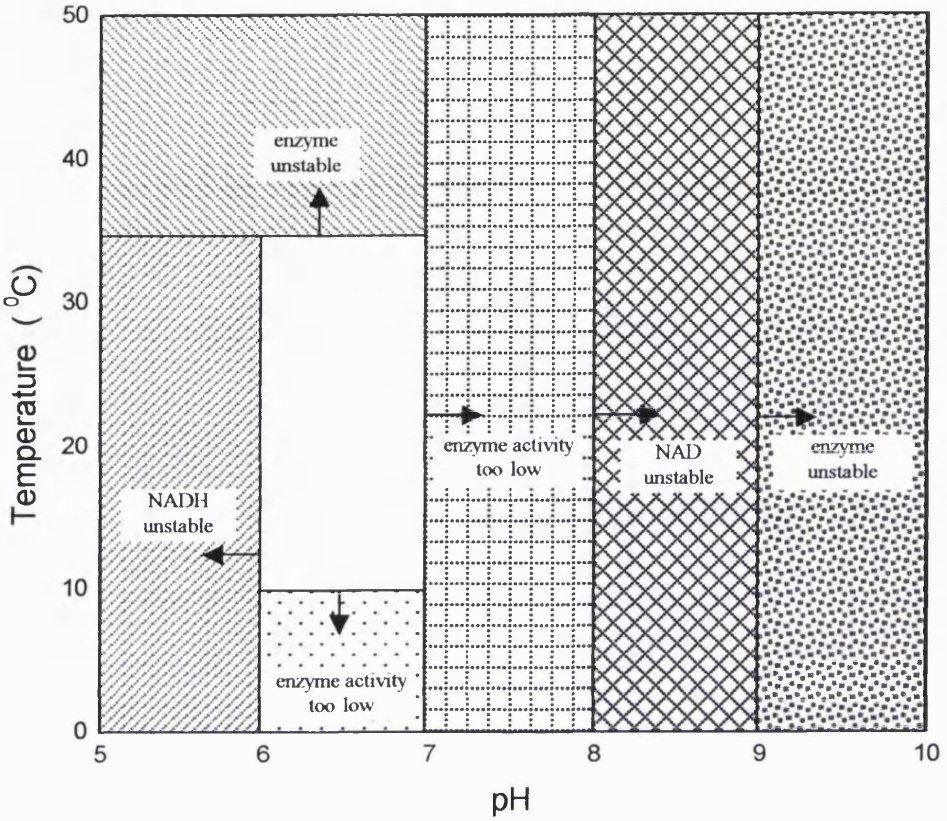


Figure 7.2:

Window of pH vs temperature for immobilised tetralone reductase enzyme. The white box indicates the area with optimum conditions.

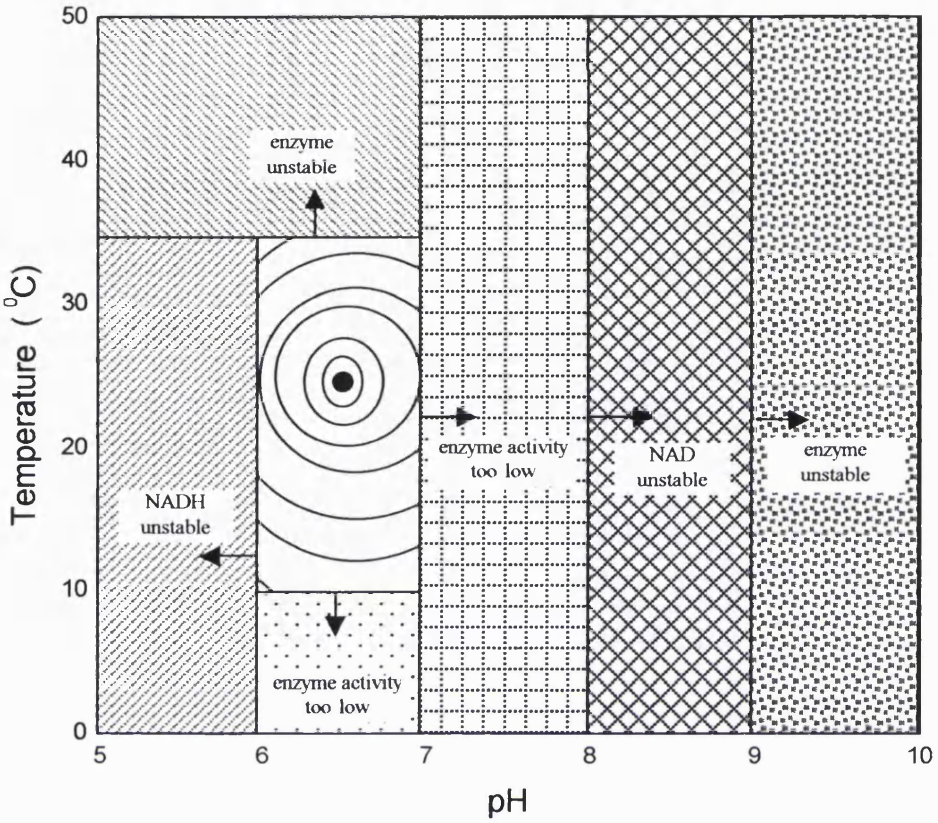


Figure 7.3:

Window of pH vs temperature for immobilised tetralone reductase enzyme, with the rate of tetralol production from the factorial experiments superimposed over the window. The black dot represents the peak optimum conditions for the two enzyme process, which decreases along the contour lines surrounding it.

to the diagram. However, a significant change in activity of the formate dehydrogenase due to a low stability or low activity in certain conditions would lead to a decrease in the overall tetralol production rate and therefore would be represented in the contour lines on the diagram.

This approach appears to be successful in funnelling the information which the researcher gathers from the general to the specific. The first information which can be added to the diagram is that about the cofactors which is established in published data. Information can then be added about the newly isolated tetralone reductase enzyme as it is collected. This information does not necessarily have to be as detailed as that described in chapter 4. The threshold values for the upper and lower limits of the chosen axes are the essential pieces of information to be found at this stage. Once something is known about the new enzyme, a second, regenerative enzyme can then be found. The combined system of both enzymes can then be tested, looking at the production of the product of interest, and more detailed data can be collected. In this case a factorial approach was found to give a great amount of information with a relatively small number of experiments. Moreover, the factorial experiments led to a model which predicts the behaviour of the system within the operational window in figure 7.3.

7.4 Use of robotics

The use of robotic technology has the potential to speed up process synthesis. Figure 7.4 shows the time span for a set of experiments which are carried out manually. The times given for the various activities are similar to those which were found by doing the factorial experiments in chapter 6 manually. Each of the experiments has to be done sequentially and the total time taken for a single experiment is given as 2.5 hours. Figure 7.5 shows the time span for the same experiments done by the semi-automatic robotic method described in chapter 6. In this case 96 experiments can be carried out simultaneously, although the time to complete one set of 96 experiments is 36 hours. Figure 7.6 shows the rate of experimentation theoretically possible with each method. This does not take into account any down time necessary for the manual operator to take breaks or for the

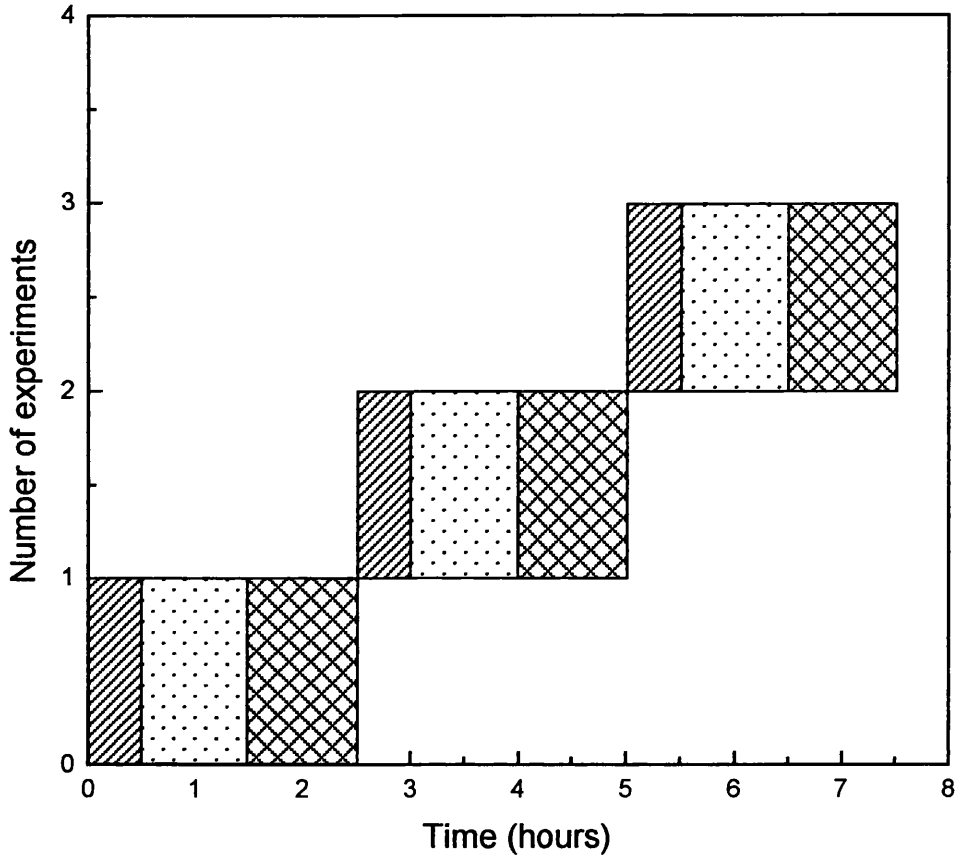


Figure 7.4:

Time span for manual experimentation. Set-up time, including making and dispensing solutions and equipment set up, taken as half and hour for the manual set-up. Assay time, set at 1 hour for this example. Analysis time, for HPLC, assumed to be 5 sets of HPLC runs at 12 minutes each, for each experiment.

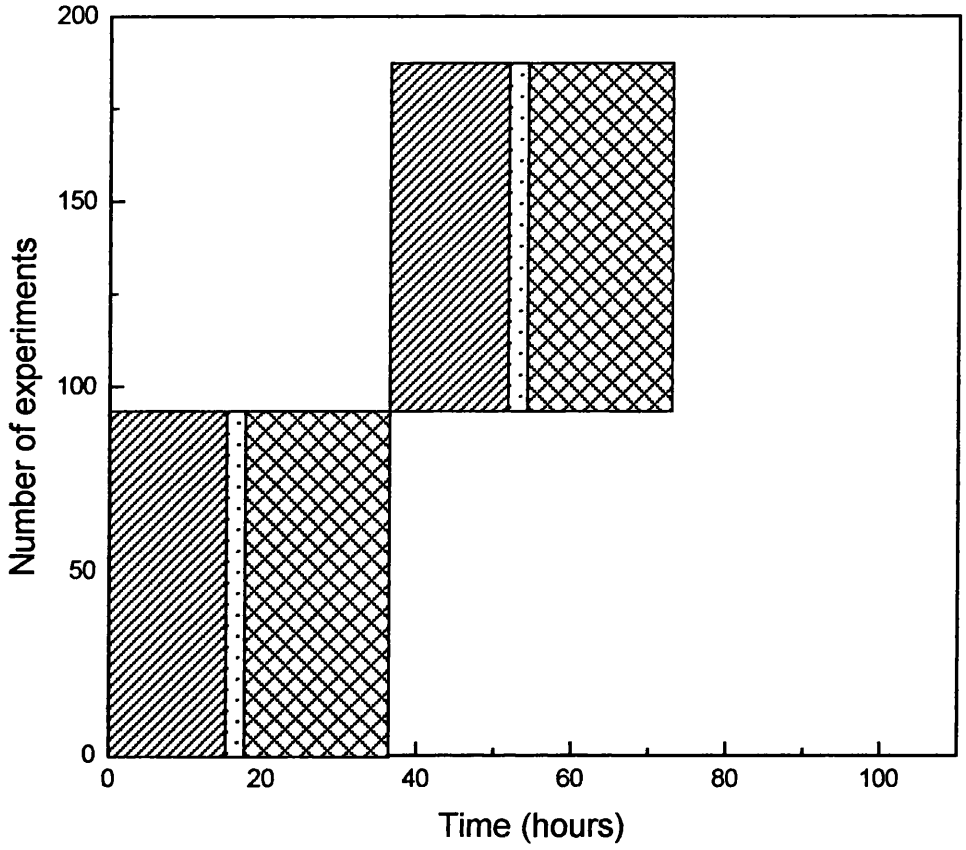


Figure 7.5:

Time span for robotic experimentation. Set-up time, including making and dispensing solutions and equipment set up, taken as 3 hours per 96-well plate. Assay time, set at 1 hour for this example also. Analysis time, for HPLC, assumed to be 5 sets of 96-well plates at 2.5 minutes per well.

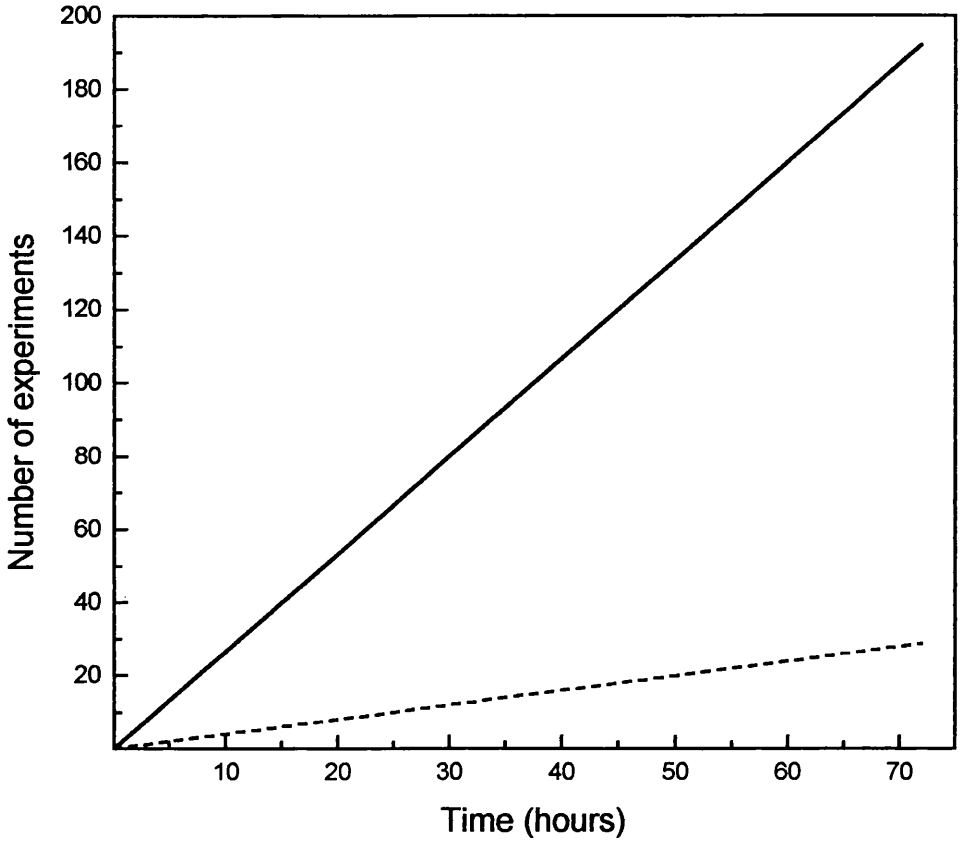


Figure 7.6:

Rate of experimentation with manual and robotic methods. (———) robotic method, (·····) manual method.

robotic liquid handler to be serviced, however, the difference in potential rates of experimentation is significant.

Figures 7.4 and 7.5 also show the proportion of the total time which is expended on each task. For manual operation the assay and analysis time are the longest durations, but all three tasks contribute significantly to the overall experimentation time. The robotic operation shows a very different pattern, with the assay time contributing an insignificant amount to the total experimentation time. Therefore to improve on the robotic process the set-up and analysis times must be reduced.

The key to the differences in duration between the assay time and set-up and analysis is that the assay can be operated in a parallel fashion, while the other tasks must still be done serially. This means that each well on a 96-well plate has to be filled separately by one pipette in the set-up sequence. This could be greatly improved by adding to the number of pipettes on the robotic station. Some robotic liquid handlers offer this facility, but it was not possible with the Gilson liquid handler used in this example. In addition, the analysis of the plates could also be accelerated by having more than one HPLC column on which to run the samples: only one was possible with this equipment. Therefore by allowing the set-up and analysis to also run parallel the total experimentation time could be reduced still further.

The significant contribution which the analysis time makes to the total time leads to the issue of redundancy in the use of robotic systems. The tendency is to fill the capacity of the system to produce a large number of experimental repeats and therefore supposedly more robust data. The discussion of accuracy in chapter 6 shows this not to be so, and after a certain point collecting more data does not lead to a better quality of result. If the issue of redundancy is not addressed while using robotic systems, the experimental time will be unnecessarily long.

7.5 Decision tool for cofactor recycle

Chapter 2 proposes a decision tool to aid the choice of cofactor regeneration method. The tool is a decision tree which aids in the rational selection of a regeneration method and offers a framework around which to investigate it's use.

Figure 7.7 shows the original decision tree, modified according to the information gained about the tetralone reductase process, with formate dehydrogenase as the regeneration method.

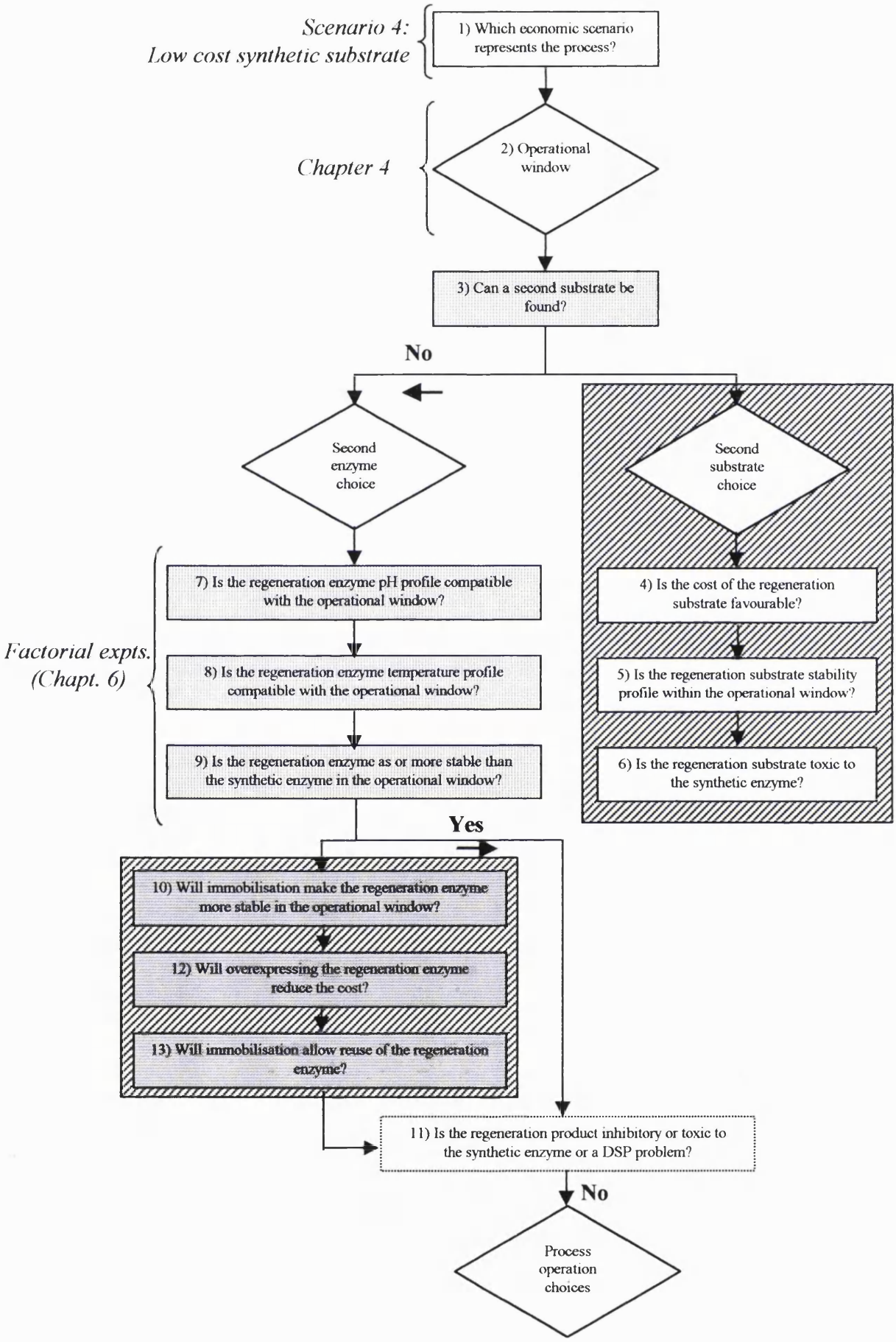
Question 1 deals with the economics of the process. As already discussed, the tetralone reductase enzyme is likely to be a relatively expensive protein to produce as extensive processing is required to produce the partially isolated enzyme from it's low concentration within the cell and the enzyme's low stability will also contribute to it's cost. Formate dehydrogenase, the enzyme chosen to be tested as a potential cofactor regeneration method, is also a relatively expensive enzyme. The formate dehydrogenase substrate, formate, is a very cheap substrate, which puts the process into the category of having a low cost synthetic substrate (scenario 4 from section 2.3). This then allows certain of the questions in the master decision tree to be ignored, in particular those concerned with the regenerative product, which do not appear in figure 7.7. The synthetic substrate can be sacrificed and used inefficiently if necessary as the use of the two expensive enzymes must be optimised.

Question 2 deals with the operational window for the synthetic reaction. This was dealt with in chapter 4, but as discussed in section 7.3 the amount of detail gained here was not entirely necessary.

Question 3 concerns the use of a second substrate rather than second enzyme method. However, as the synthetic enzyme is known to be expensive, it may be better to look for a slightly cheaper regenerative enzyme. Although formate dehydrogenase is also an expensive enzyme it is not possible to compare its cost to that of the tetralone reductase which is not commercially available. With this in mind, it was decided to follow the route of the second enzyme rather than the second substrate method.

Questions 7, 8 and 9 concern an adequate compromise between the operational windows of both enzymes. These were combined into the factorial experiments in chapter 6 which looked at the operation of both enzyme reactions together. This will certainly have streamlined the process over that which was originally proposed in the original decision tree.

Figure 7.7 (following page):
Decision tree modified for tetralone reductase catalysed process. Shaded boxes represent important questions which should be given more weight. Boxes bordered with broken lines represent less important questions which can be more flexible. Crossed out boxes represent those which are not being followed in the scheme.


Scenario 4:
Low cost synthetic substrate
1) Which economic scenario represents the process?
Chapter 4
2) Operational window
3) Can a second substrate be found?
No
Second enzyme choice
Second substrate choice
Factorial expts. (Chapt. 6)
7) Is the regeneration enzyme pH profile compatible with the operational window?
8) Is the regeneration enzyme temperature profile compatible with the operational window?
9) Is the regeneration enzyme as or more stable than the synthetic enzyme in the operational window?
4) Is the cost of the regeneration substrate favourable?
5) Is the regeneration substrate stability profile within the operational window?
6) Is the regeneration substrate toxic to the synthetic enzyme?
Yes
10) Will immobilisation make the regeneration enzyme more stable in the operational window?
12) Will overexpressing the regeneration enzyme reduce the cost?
13) Will immobilisation allow reuse of the regeneration enzyme?
11) Is the regeneration product inhibitory or toxic to the synthetic enzyme or a DSP problem?
No
Process operation choices

Each of the enzyme's operational windows are compatible and the stability of the immobilised formate dehydrogenase is not significant to the process, therefore questions 10, 12 and 13 can be missed out. Questions 10 and 13 however, deal with the immobilisation of the regenerative enzyme, which was carried out anyway. This was because both enzymes could then be retained behind a filter, while the substrate and products could be removed easily for a more straightforward downstream process. However, this decision was taken on an issue of the operation of the reactor and not due to the regeneration of the cofactor. This decision tree only proposes to offer a means to select a regeneration method and not any other area of the process. This demonstrates the limitations of a tool which only deals with one area of the whole process. However, it is proposed as an expert system and only a guide to follow while keeping a balanced view of the whole process.

Question 11 concerns the regeneration product, which in this case is carbon dioxide. This can be driven off as a gas from the reaction and therefore will not lead to any significant problems with inhibition, toxicity or downstream possessing.

Therefore the proposed decisional tree has proved to be of value in this case. Figure 7.8 shows a modified scheme to follow for developing a processes based on a reduction reaction with cofactor recycle, in light of the experience gained in this example. This is not intended to replace the decision tree proposed in chapter 2, but shows how it can be used in the context of developing a whole process. The decision tree should also be used with other synthetic and regenerative enzymes to test it's robustness. The decision tree could also be expanded farther to include other aspects of the cofactor recycle process.

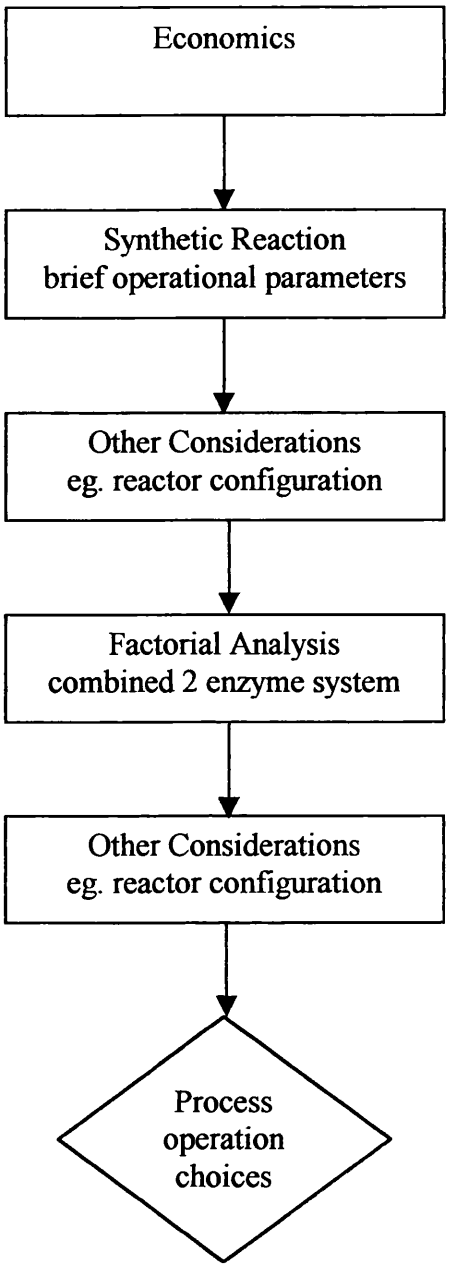


Figure 7.8:

Modified scheme to follow for developing a process based on a reduction reaction with cofactor recycle, to be used in the context of developing a whole process.

8 Conclusions

- A rational selection strategy has be drawn up for the selection of an enzymatic cofactor regeneration process.
- An NADH-specific reductase enzyme (tetralone reductase) from the yeast *Trichosporon capitatum* MY1890 has been isolated which reduced 6-bromo-β-tetralone to its corresponding (S)-alcohol, ((S)-6-bromo-β-tetralol) with an enantiomeric excess of 99.54%.
- The final purification stage of tetralone reductase resulted in a 54-fold increase in specific activity, to 430U/mg with a yield of 1.34%.
- The enzyme was found to have a preference for NADH as its cofactor over NADPH.
- Apparent Km values of tetralone reductase for NADH and tetralone were found to be 0.13mM and 0.40mM respectively. The NADH was seen to inhibit the enzyme at a concentration of 0.6mM, so such inhibition was seen for similar levels of tetralone.
- Tetralone reductase was found to immobilise onto Eupergit C in the presence of 0.5M phosphate buffer at pH7 with an optimum enzyme challenge of 0.5U/g beads.
- Apparent Km for immobilised tetralone reductase for NADH and tetralone were found to be 0.63mM and 2.7mM respectively.
- The optimum pH for activity of the free (un-immobilised) tetralone reductase was in the range 6-7 and 6-8.5 for the immobilised enzyme.
- Both free and immobilised tetralone reductase were at their most stable over the pH range 6-8.5.
- The temperature optima, a compromise between activity and stability were found to be 21°C and 31°C for the free and immobilised forms of tetralone reductase respectively.
- Optimum solvent regime was 50% hexane for the free tetralone reductase and 50% octanol for the immobilised tetralone reductase.

- The main problems with operating a robotic system for process synthesis are concerned with problems associated with immobilised bead slurry handling and accuracy of the system.
- The optimum condition of pH, temperature and solvent for a combined two enzyme system, using tetralone reductase and formate dehydrogenase, were found by factorial experiments using a robotic liquid handling system to produce a semi-automated process. The optima were found to be: pH (6.8), temperature (26°C) and solvent (30% octanol). A predictive model was also derived for these factors.
- Formate dehydrogenase was immobilised onto Eupergit C beads with an optimum enzyme challenge found to be 10U/g beads which gave a specific activity on the beads of 2.5U/g and a retained activity of 25%.
- The optimum cofactor challenge for the 2 enzyme reaction was found to be a tetralone/NAD+ molar ratio of 10/1.
- Tetralone and tetralol were found to adhere to the Eupergit C beads so that most of the tetralone and tetralol is assumed to be associated with the beads after 30 minutes. The tetralone and tetralol could be eluted from the beads with optimum conditions found to be 250µl of ethanol, which was sufficient to elute 98.9% of both reactants from 0.04g of beads.

9 Future Work

- Quantify the problems associated with the whole cell process, for example if diffusional limitation occurs.
- Sequence the isolated tetralone reductase enzyme to compare with existing enzymes. Gather more information about the type of enzyme it is, eg. if it requires a metal cation.
- Overexpress the tetralone reductase enzyme to increase its titre within the cell and make purification easier, therefore reducing the potential cost of the enzyme.
- Improve the stability of the tetralone reductase enzyme using methods such as CLEC (cross linking enzyme crystals) or protein engineering.
- Gain more information about the behaviour of formate dehydrogenase when immobilised to Eupergit C.
- Technical developments need to be made in dispensing bead slurries. Also with robotic liquid handling equipment development to provide parallel methods for liquid dispensing and HPLC analysis.
- Use the decision tree with other systems to test its robustness and make modifications as necessary.
- Expand the decision tree to take account of other methods of cofactor regeneration and also cofactor retention.
- Compare the formate dehydrogenase regenerative enzyme with other methods, both of enzymatic regeneration and chemical and electrochemical.
- Look at the economics of the system proposed here to see if it is economically feasible as it is or if the process needs to be further refined.

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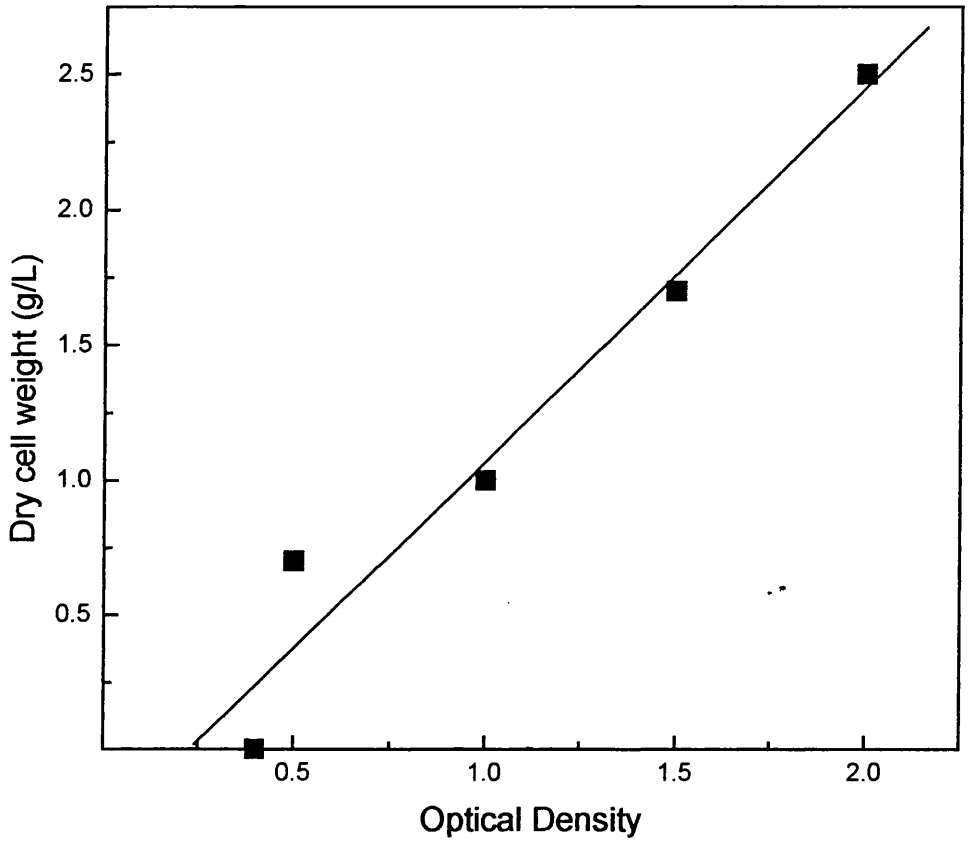
Appendix I : List of suppliers

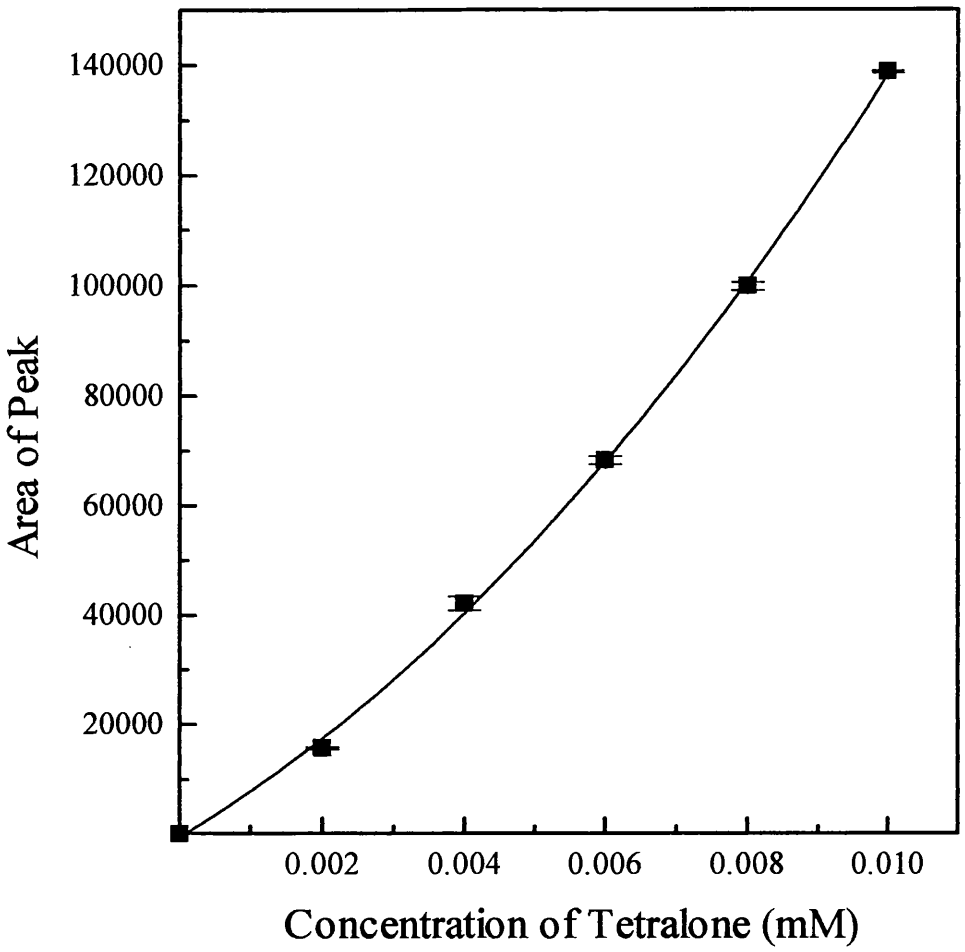
APV Eura	23 Gatwick Road Crawley W Sussex, RH10 2JB UK
Beckman Instruments	2500 Harbour Boulevard Fullerton CA 92634-3100 USA
Boehringer-Mannheim	F. Hoffmann – La Roche Ltd. CH – 4070 Basel Switzerland
BioRad	1000 Alfred Nobel Drive Hercules CA 94547 USA
Chiral Technologies	730 Springdale Drive Exton PA 19341 USA
Eppendorf	10 Signet Court Swanns Road Cambridgeshire, CB5 8LA USA
Fisher Scientific	2000 Park Land Pittsburg PA 15275 USA
Gilson, Inc., USA	3000 West Beltline Highway PO Box 620027 Middleton WI 53562 USA
Grant Instruments (Cambridge) Ltd	Shepreth Cambridgeshire, SG8 6GB UK
Mac-Mod Analytical, Inc.	127 Commons Court Chadds Ford PA 19317 USA

Merck & Co., Inc.	PO Box 2000 Rahway NJ 07065 USA
Microfluidics Corp.	30 Ossipee Road Newton MA 02164 USA
Millipore (UK) Ltd	The Boulevard Blackmore Land Watford Hertfordshire, WD1 8YW UK
Pharmacia	100 Route 206 North Peapack NJ 07977 USA
Phenomenex	2320 North 205th Street Torrance CA 90501-1456 USA
Röhm GmbH	Chemische Fabrik Kirschenalle 64293 Darmstadt Germany
SAS Institute Inc.	SAS Campus Drive Cary NC 27513-2414 USA
Sigma-Aldrich-Fluka	The Old Brickyard New Road Gillingham Poole Dorset, SP8 4BR UK
USA Scientific Inc.	PO Box 3565 Ocala FL 34478-3565 USA

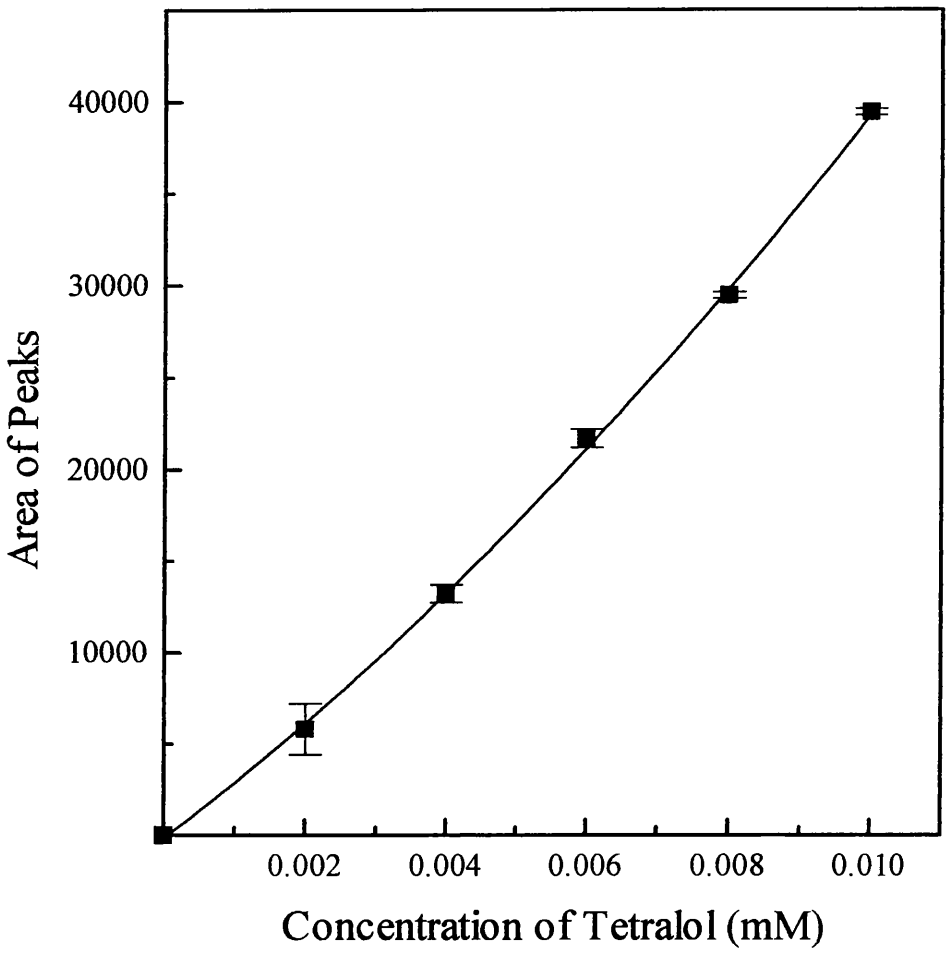
Appendix II: Standard Curves



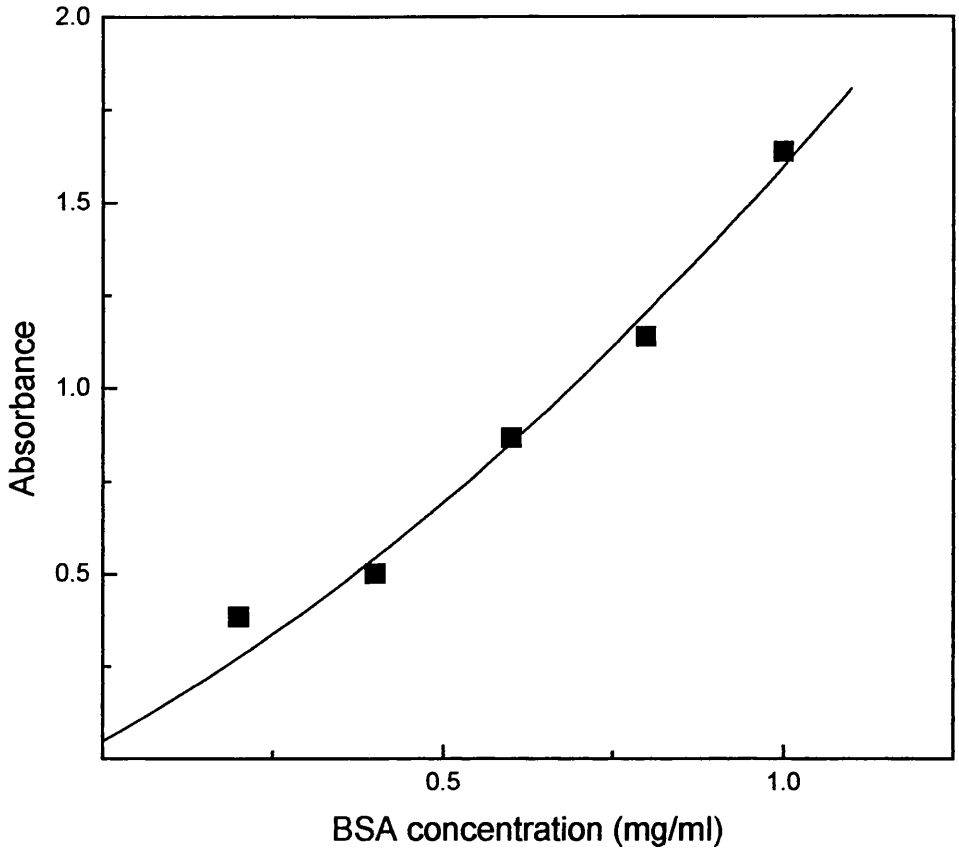
Representative standard curve for *Trichosporon capitatum* grown on production medium. Optical density measured at 650nm.



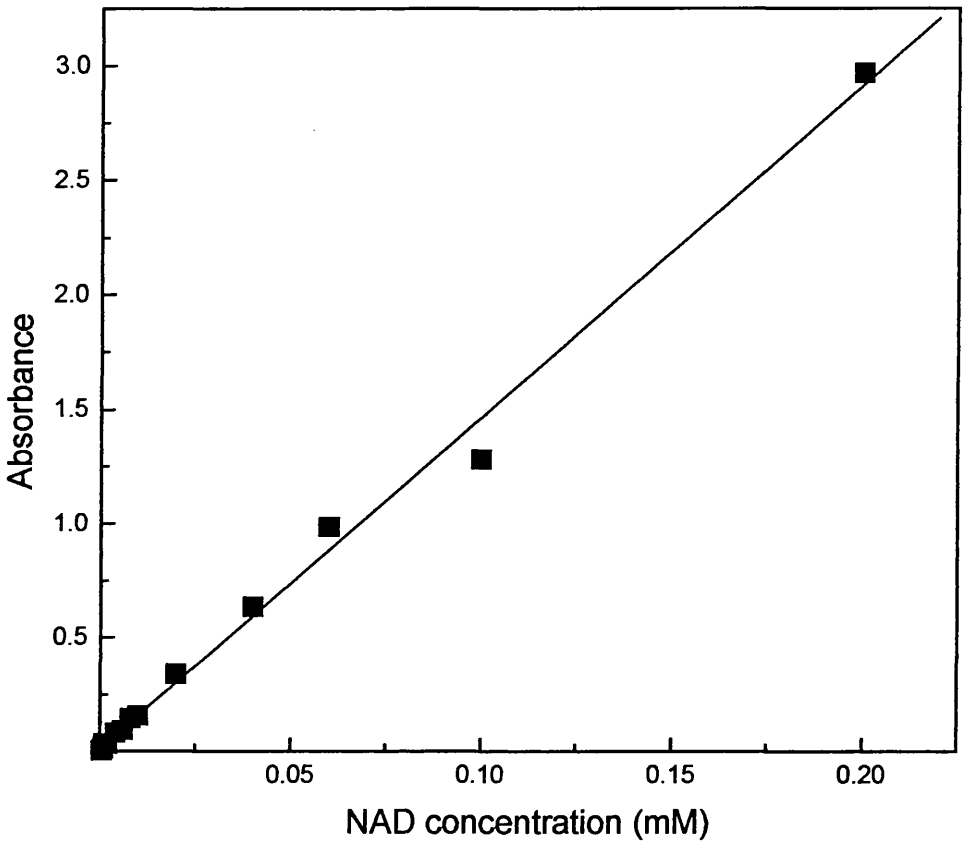
Representative calibration curve for reverse phase HPLC of tetralone standard with Zorbax RX-C8 column.



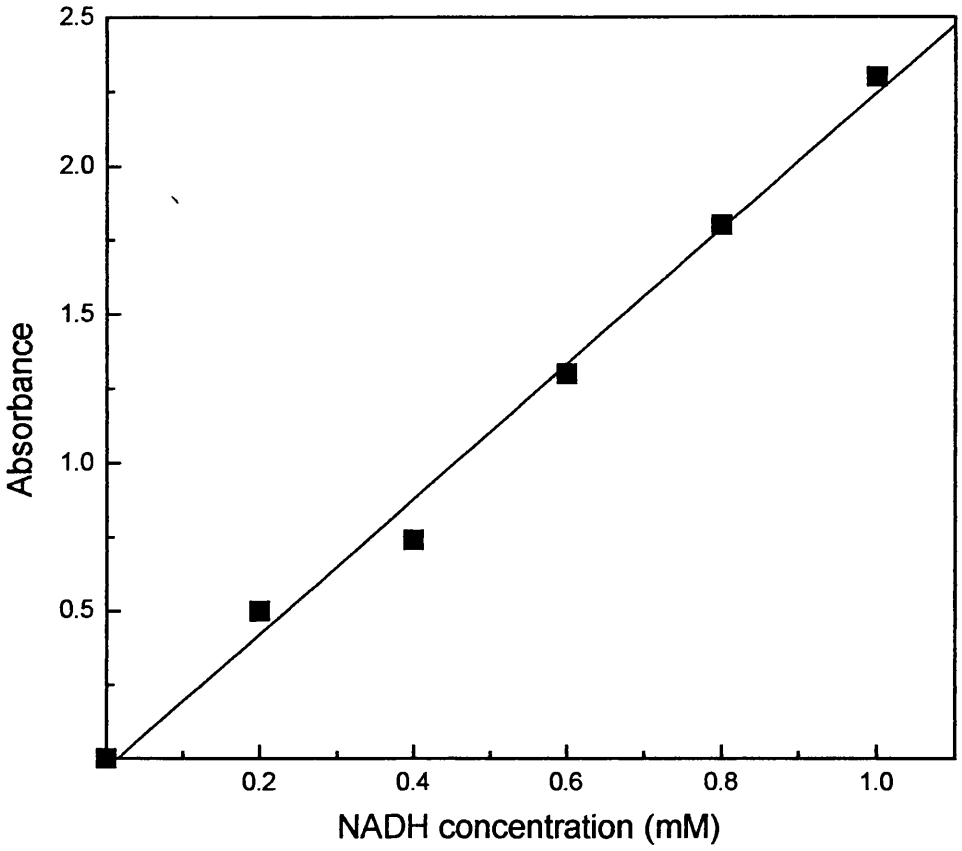
Representative calibration curve for reverse phase HPLC of tetralol standard with Zorbax RX-C8 column.



Representative calibration curve for protein concentration by Bradford method, using BSA as a standard. Absorbance measured at 595nm.



Representative standard curve for NAD^+ concentration vs absorbance at 260nm.



Representative standard curve for NADH concentration vs absorbance at 340nm.