An NADH Dependent Reductase for Isolated Enzyme and Whole Cell Catalysis

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By

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Abstract

Isolated enzymes and whole cell biocatalysts can both be applied for the synthesis of chiral hydroxy compounds. It is hypothesized that whole cells can easily be employed for such reactions using simple technology which is robust. This is because whole cells contain all the necessary enzymes and metabolic pathways for cofactor regeneration. This also means that the enzymes and their cofactors are well-protected within their natural cell environment. In contrast, it is hypothesized that isolated enzymes require complicated and expensive purification procedures. They also require the stoichiometric addition of cofactors (or methods employed for their regeneration), and are susceptible to inactivation since they are isolated from their natural cell environment. The aim of this thesis was to systematically compare a whole cell biocatalyst (*Trichosporon capitatum* (MY1890)) and an NADH dependent isolated reductase (tetralone reductase) in the synthesis of a chiral alcohol (6-bromo-β-tetralol). The comparison was carried out to ascertain which type biocatalyst is preferred, and also to establish whether the general hypotheses (as stated above) are true with respect to each biocatalyst.

Comparison of the isolated enzyme and whole cell biocatalyst showed that there were significant differences with respect to each of the systems. These included differences in: the biocatalytic purity, the reaction methodology, the system efficiency, and the effects on the biocatalyst from the addition of substrate and solvent. The isolated enzyme methods were much more complicated than the whole cell methods, from the preparation of the isolated enzyme through to the bioreduction. This was because a novel protein purification process needed to be set up and a cofactor regeneration system was required. However, the isolated enzyme system showed higher substrate conversions than the whole cell system. At 1g/L, a conversion of 86% after 420min was achieved, whereas the whole cell system exhibited a conversion of 79% after 450min. It was hypothesized that the whole cell system suffered from lower conversions due to the substrate and product accumulating inside the cell membrane and disrupting cell metabolism. In the same configuration, the whole cell system also suffered from lower reaction rates which were attributed to mass transfer limitations through the cell membrane. The addition of a solvent enhanced whole cell biocatalytic reaction rates, but only at low substrate concentrations. The isolated enzyme system was susceptible to inactivation, and increased solvent concentrations caused a detrimental affect on the reaction rates and conversions. This was most likely due to the solvent causing an irreversible change in the active site conformation. The similarities and differences of employing an NADH dependent reductase and a whole cell biocatalyst for the production of a chiral alcohol are discussed in this thesis.

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I would like to acknowledge the people who have led to the submission of three years of research. In particular, I would like to thank John Woodley, the BBSRC, Michel Chartrain and Merck & Co., Ltd. I would also like to thank my family, and my friends from Foster Court and UCL.

"At first he found it amusing. He coined a law intended to have the humour of a Parkinson's law that 'The number of a rational hypotheses that can explain any given phenomenon is infinite.' It pleased him never to run out of hypotheses. Even when his experimental work seemed dead-end in every conceivable way, he knew that if he just sat down and muddled about it long enough, sure enough, another hypothesis would come along. And it always did. It was only months after he had coined the law that he began to have some doubts about the humour or benefits to it."

Taken from *Zen and the Art of Motorcycle Maintenance*. Robert M. Pirsig referring to one of Phaedrus's theories. 1974, Published by Vintage.

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Abbreviations

ρ-CMB	ρ-chloromercuribenzoate
ADH	alcohol dehydrogenase
ATP	2-deoxyadenosine 5-triphosphate
BSA	bovine serum albumen
CHMO	Cyclohexanone mono-oxygenase
CLEC	cross linked enzyme crystal
DSP	downstream processing
EDTA	ethylenediaminetetraacetic acid
ee	Enantiomeric excess
FAD	flavin adenine dinucleotide
FDH	formate dehydrogenase
FMN	flavin mononucleotide
HPLC	high performance liquid chromatography
M	molar concentration
MDH	malate dehydrogenase
mM	millimolar concentration
NAD^+	β-nicotinamide adenine dinucleotide
NADH	β-nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate (reduced form)
OD	optical density
PEG	polyethyleneglycol
TN	turnover number
TTN	total turnover number
U	enzyme activity
YADH	yeast alcohol dehydrogenase

Introduction

1.1 Introduction to Biocatalysis

Isolated enzymes and whole cell microorganisms can both be applied as biocatalysts to achieve highly specific and desired conversions under controlled conditions in a bioreactor (Buchholz *et al.*, 2000). Glucose isomerase is a good example of a successfully applied biocatalyst. This immobilised enzyme isomerises glucose into fructose, and is the biggest selling immobilised enzyme in the world (Cheetham, 2000). Another example of the industrial use of biocatalysts is the manufacture of enantiopure L-amino acids through the dynamic resolution of racemic *N*-acetyl amino acids by L-acylases (Schmid *et al.*, 2002). Whole cell biocatalysts can also be employed to carry out a series of reactions where the product may be very dissimilar to the starting compound. An example of this is the use of microorganisms to produce antibiotics from glucose. Antibiotics are highly complex molecules and require a number of enzymatic steps from glucose to the final product (Dordick, 1991).

Enzymes are of particular importance as they display four types of selectivity:

1. Substrate selectivity

   The ability to distinguish and to act upon a subset of compounds within a larger group of chemically related compounds.

2. Chemoselectivity

   The ability to react on one type of functional group, leaving other groups alone.

3. Regioselectivity and diastereoselectivity

   The ability to distinguish between identical groups on the same substrate. The regioselectivity of enzymes even on complex molecules, without any need of protecting groups, is a fundamental strength of biocatalysis (Rasor *et al.*, 2001).

This reduces the number of synthetic steps and thereby cuts occupation time of chemical reactors, which is an important factor of process economy in pharmaceutical and chemical production.

4. Enantioselectivity/stereoselectivity.

   The ability to produce optically active substances from pro-chiral substrates. Enzymes have gained most attention because of their stereoselectivity, especially in the preparation of pharmaceutical compounds. The recent tendency to develop single stereoisomer pharmaceuticals instead of racemates has helped to establish enzymes as tools in organic synthesis (Rasor *et al.*, 2001)

Another major advantage of biological catalysts is that they can utilise the growing resources of biotechnology. This includes: random or site selective mutagenesis for the optimisation of enzymatic properties, transfer of the gene into high productivity organisms, and overexpression by incorporation of a promoter gene. An example of where this has occurred is in the Baeyer-Villiger reaction in which a gene expressing CHMO from *Acinetobacter calcoaceicus* NCIB 9871 was cloned into *Escherichia coli* in an L-arabinose inducible vector (Doig *et al.*, 2001). This was done because firstly, the wild type host was a class 2 pathogen, therefore unattractive for large scale fermentation. Secondly, the fermentation protocol for *Acinetobacter calcoaceicus* NCIB 9871 involved growth on a toxic carbon source which was required for induction. Finally, *Acinetobacter calcoaceicus* NCIB 9871 contained an active lactone hydrolase and therefore either purification of the CHMO or selective inhibition of the hydrogenase was required prior to the biotransformation.

Enzymes can be very efficient catalysts on their natural substrates with higher acceleration rates and lower mole ratios when compared to their chemical counterparts. Enzymes are environmentally friendly (they can be completely degraded by the environment), and they require mild reaction conditions such as neutral pHs (6-8) and low temperatures (20-40^{0}C) (Faber, 1997). These mild

reaction conditions minimise the problems associated with harsh chemical reaction conditions, such as product degradation and side-reactions, and minimise the amount of energy required in the process. Enzymes are also advantageous because they do not need to be used in their natural environment. For example, two enzymes may be isolated from two different types of cell and put together in a compatible new reaction environment. Sequential reactions can be performed in this manner by using multi-enzyme systems, thus simplifying reaction processes (Faber, 1997). Similarly, enzymes are not purely bound to their natural role and can catalyse a variety of substrates including many synthetic substrates which can be used in the synthesis of new and important pharmacological compounds. This, alongside the diverse biocatalytic systems available, can give rise to numerous reaction configurations.

1.2 The Disadvantages of Biocatalysts

There are a number of disadvantages associated with the use of biocatalysts when substituted in place of chemical catalysts, these are:

1. In general, enzymes require mild conditions; this means that the process needs to be monitored and controlled within narrow parameters as any slight change in pH, temperature, or salt concentration could denature the enzyme (Faber, 1997).

2. A large number of organic compounds which are of importance in modern biotechnology are insoluble in aqueous media (Angelova *et al.*, 1999). Solvents are required to dissolve these types of compounds, or these compounds are in fact organic solvents themselves, and biocatalysts are often liable to be denatured in the presence of organic solvents (Fukui and Tanaka, 1985). A compromise may be made with the ratio of solvent to aqueous phase and the type of solvent used, although still there may be losses in activity and stability.

3. The selectivity of enzymes means that they are specific to one type of enantiomer, and there is no way of creating mirror image enzymes (from D-amino acids). The only way to achieve conversion to the other enantiomer would be to undergo a separate screening process for another enzyme (Faber, 1997).

4. Many enzymatic reactions are prone to substrate or product inhibition; substrate inhibition can be overcome by the monitoring and controlled addition of substrate levels. Product inhibition is more complicated as *in situ* removal of the product needs to be facilitated (Faber, 1997).

1.3 Oxidoreductases

Oxidoreductases are the class of enzyme which catalyse reduction and oxidation reactions, specifically the removal or addition of hydrogen. Enzymes can be classified into six groups by the nature of their specificity:

Enzyme Classification	
EC1	Oxidoreductases which catalyse redox reactions
EC2	Transferases which catalyse the transfer of a group from one molecule to another e.g. acyl or phosphate
EC3	Hydrolases which catalyse hydrolysis reaction e.g. Esterases, lipases and amidases
EC4	Lyases catalyses the cleavage of C-C, C-O, and C-N bonds other than by hydrolysis or redox reactions. Typically these reactions involve elimination of, or addition to double bonds.
EC5	Isomerases catalyse isomerizations such as epimerization and racemization
EC6	Ligases catalyse the intermolecular bond formation coupled with the cleavage of ATP or other nucleotide triphosphates

(International Union of Biochemistry and Molecular Biology, (IUBMB); 1992)

The oxidoreductases are one of the most important classes of enzyme and this is seen through the asymmetrisation of a prochiral substrate which leads to an optically pure product with a possible 100% conversion (Anthonsen, 2000). Optical purity

(chirality) is the property which characterises a pair of enantiomers or molecules that are non-superimposable mirror images of each other. Enantiomers have different biological activities which can often influence the efficacy or toxicity of a compound. For example, the *R*-enantiomer of penicillamine is toxic whilst the *S*-enantiomer is antiarthritic (Figure 1.1). The reverse oxidative process is of limited use (for example, alcohol oxidation) as this generally leads to the destruction of a chiral centre.

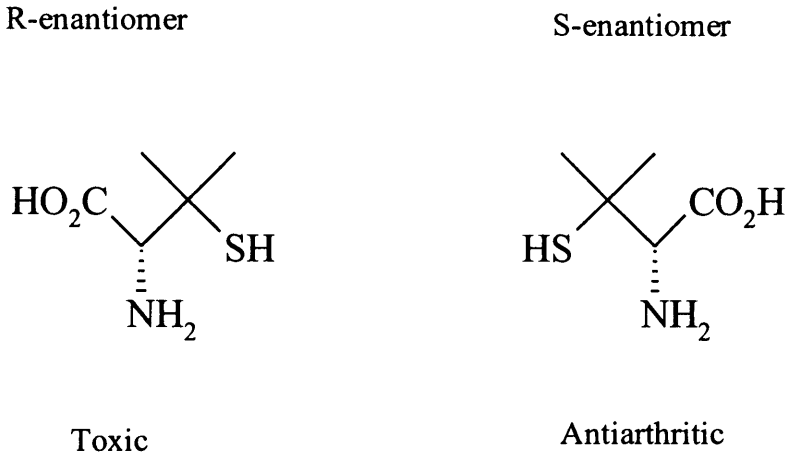


Figure 1.1 *Biological effect of penicillamine (adapted from Faber, 1997).*

Industry is finding an ever-increasing need for the production of safe products with greater purity and different chiral forms of every new drug have to be synthesised to study their individual pharmacological effects (FDA's Policy Statement on the Development of New Stereoisomeric Drugs, 1992). Chemical catalysts, in general, do not possess the specificity required to produce chiral products, oxidoreductases, in contrast, have ability to produce products of high optically purity. However, the disadvantage of using oxidoreductases for such reactions is that these enzymes require the stoichiometric use of cofactors.

1.4 Cofactors for Oxidoreductase Activity

Cofactors are a low molecular weight, (0.5 - 0.8 kD) non-protein species that are essential participants in most isolated enzyme catalysed reactions (Chenault *et al.*, 1988). Cofactors and coenzymes are distinguished from each other in that a cofactor is tightly bound to the enzyme, whereas a coenzyme can dissociate into the medium (Faber, 1997). The most common type of cofactor for the oxidoreductases are the nicotinamide derivatives (NAD(P)(H)); these cofactors are often loosely bound to the enzyme and can in many ways be considered as cosubstrates which donate or accept a functional group (i.e. a hydride, or a phosphate) (Adlercreutz, 1996). Many enzymes require coordinated metals such as iron, zinc, magnesium and manganese (Faber, 1997) as a type of coenzyme. However, this in itself does not result in a problem as many enzymes have a high affinity for these metals, and if they do not, the cost of supplying a metal rich medium is not significant.

The stability of nicotinamide cofactors is dependent on a variety of situations and conditions. Temperature and pH have the greatest impact on the degradation of both NADH and NADPH. However, the degradation of NADPH was much faster under all conditions investigated compared to the degradation of NADH (Wu *et al.*, 1986). One instability in particular is the catalysis arising from exposure to extremes of pH. Acidic conditions catalyse the hydration of the reduced cofactors (NAD(P)H) (Wong *et al.*, 1981), where basic conditions catalyse the hydrolysis of the cofactors (NAD(P))(Guilbert *et al.*, 1977). The optimal stability of NAD(H) in aqueous solutions is hard to predict because the destruction of the reduced cofactor is minimised by alkaline conditions and the destruction of the oxidised form is minimised by acidic conditions. In a two-enzyme cofactor regeneration system, the cofactor is continuously recycled between its reduced and oxidised forms (Chenault and Whitesides, 1987). Knowledge of activities of the enzymes must be acquired to allow optimisation of the process. NAD(H) is also known to be degraded in the presence of phosphates, and enzymes such as phosphodiesterases and phosphoesterases are occasionally present as impurities in enzymes (Chenault *et al.*,

1987). Reduced nicotinamide cofactors are particularly unstable in phosphate buffers and depending on the buffer or other ions present, the half life of nicotinamide cofactors in solution at pH 7, and 25^0C can range from several hours to a month. Acetate buffers also produce a similar effect (Wu *et al.*, 1986; Chenault *et al.*, 1987).

In both forms of nicotinamide cofactors, electron transfer involves only the nicotinamide moiety. Enzymatic reduction of NAD(P) directs hydride attack at the C-4 position of the pyridinium ring and forms exclusively the 1,4-dihydropyridine product, which is the only enzymatically active form of NAD(P)H (Figure 1.2). The requirement for the highly specific 1,4- reduction of the pyridinium ring severely limits the number of strategies practical for large scale regeneration of NAD(P)H (Chenault *et al.*, 1988).

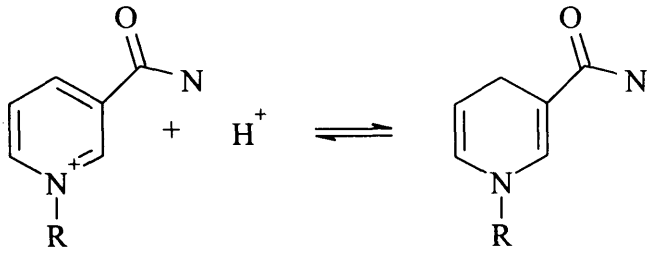


Figure 1.2 Electron Transfer in NAD(H)

Enzymatic reduction of NAD directs hydride attack at the C-4 position of the pyridinium ring and forms exclusively the 1,4-dihydropyridine product, which is the only enzymatically active form of NADH. The only difference between the nicotinamide cofactors NAD and NADP is the 2-phosphate on the adenosine ring.

1.5 Oxidoreductase Isolation and Purification

The oxidoreductase required for a specific bioreduction will be just one of a large number of components which make up a whole cell biocatalyst. To apply isolated enzyme catalysis efficiently, methods of purifying the target enzyme from the other cell contaminants are required. These contaminants may include: cell growth debris, nucleic acids, endotoxins, small molecules and other cell proteins (Bailey and Ollis, 1986). If the protein of interest is overexpressed then it will make up a large percentage of the total cell protein, if not, then the method of protein purification will need to be extremely efficient to gain a protein of high purity and concentration. Extraction of an active and intact protein from a natural source may employ the following methods: homogenisation, lysis, extraction, precipitation, centrifugation, filtration and chromatography (Harris, 1989). The purification of proteins is usually carried out by a series of chromatography columns which separate the cell components due to the different properties inherent in their structures. These include: the number of ionic charges which are accessible on the surface of the molecule; the hydrophobicity of the molecule or functional groups within the molecule; the presence of sites on the molecule which can interact with other molecules in a bio-specific binding action; the range of chemical conditions in which the molecule can stay in solution and maintain biological activity; and the molecular weight of the protein (Harris, 1989). However this mode of separation can end up in a circular motion as the properties of the protein of interest may only be ascertained after purification of the protein.

1.6 Isolated Oxidoreductase Biocatalysis

In applied isolated oxidoreductase systems cofactors are used in stoichiometric quantities and these cofactors emerge from the enzymatic reactions in an altered form. It is the high cost of these cofactors which preclude them from being used in large-scale industrial processes (Chenault and Whitesides, 1987; Hummel, 1997) and if oxidoreductases are to be employed as catalysts for large-scale enzyme catalysed synthesis of chiral products it will require an efficient procedure for the *in*

situ regeneration of the cofactor (Hummel and Kula, 1989; Hummel 1997). The regeneration of cofactors also influences the position of the equilibrium of the reaction, precludes the problem of product inhibition, eliminates stoichiometric quantities, and simplifies reaction work-up (Chenault and Whitesides, 1987).

To be practical a regeneration method must fulfill several requirements:

- An economical turnover number must be possible.
- The materials and equipment should be readily available, inexpensive, easily manipulated and stable under reaction conditions.
- The regeneration must also be kinetically and thermodynamically favourable.
- Compatible with the synthetic reaction system.

(Chenault *et al.*, 1987).

Two definitions concerning the turnover numbers for cofactors are important:

Total turnover number (TTN) is the total number of moles of product formed per mole of cofactor during the course of a complete reaction:

$$TTN = \frac{amount\ of\ product\ formed}{amount\ of\ cofactor\ present\ in\ reaction}$$

Turnover number (TN) refers to the number of moles of product formed per mole of cofactor per unit time, it is measured as a rate and has units of time (s^{-1}):

$$TN = \frac{amount\ of\ product\ formed}{amount\ of\ cofactor\ present\ in\ reaction \times time}$$

Whereas, TTN emphasises the total cost per mole of product formed, TN emphasises the cost per unit rate of product formation (Chenault *et al.*, 1988). The turnover

number required for the reaction depends on several elements, the most important of which is the cost of the process; the higher the cost of the process the higher the turnover number required. The turnover number also depends on the selling price of the product, the higher the price the lower the turnover number needed. Total turnover numbers are generally in the range of 10^2 - 10^6 (Kula *et al.*, 1989). The cofactor regeneration number is important because the cost of cofactor is inversely proportional to this number. There are a number of methods which have been researched to regenerate cofactors, these include; electrochemical methods (Somers *et al.*, 1997; Cantet *et al.*, 1996; Laval *et al.*, 1991), chemical methods (Montaine *et al.*, 1987), and light energy methods (Hatanaka et al., 1996; Karube et al., 1980; Miura *et al.*, 1980; Mandler *et al.*, 1986; Kirveliene *et al.*, 1993; Umeda *et al.*, 1993). All of these methods, however, generally suffer from lack of specificity, low specific activity, and low yields.

The most documented and successful method for regenerating cofactors *in situ* is by enzymatic regeneration. Enzymatic methods to regenerate cofactors can be divided into three classes:

1) The coupled enzyme approach where two different enzymes are used, one is the synthetic enzyme and requires a substrate, and the other enzyme is the regenerating enzyme which requires a different substrate (the cosubstrate).

2) The coupled substrate method where this method uses the same approach as the first method but the synthetic and regenerating enzymes are the same, yet the substrates are different.

3) The third method is based on the second method, but the substrate of the desired product is formed *in situ* from the precursor; both reactions depend on the same cofactor.

For systems with two different enzymes (the coupled enzyme approach) there is the advantage that the rate of reaction can be controlled by changing the recycling enzyme concentration and the substrate concentration. Ideally, the recycling enzyme reaction would not be the rate limiting step. Much of the published work on the enzymatic regeneration of NAD(H) uses formate dehydrogenase (FDH) as the regenerating enzyme.

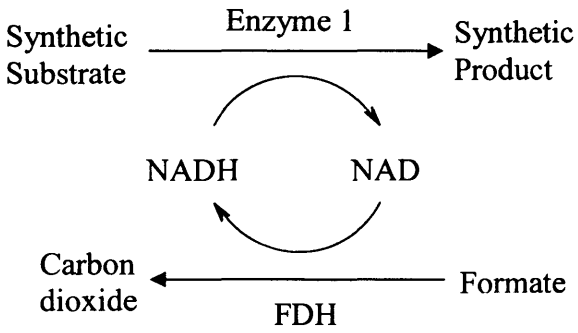


Figure 1.3 Enzymatic Cofactor Regeneration

NADH is used by enzyme 1 to reduce the synthetic substrate into the synthetic product. NADH is oxidised during the reaction into NAD and then is reduced back into NADH by the oxidation of formate to CO_2 by FDH. The recycling can performed continuously if enzyme activity is maintained.

FDH catalyses the NAD-dependent oxidation of formic acid to carbon dioxide. This method has the advantage that formate is inexpensive, stable, innocuous to enzymes and strongly reducing. The by-product, carbon dioxide, is generally innocuous to enzymes and can be easily removed from the reaction. The enzyme FDH is commercially available, readily immobilised, and stable if protected from auto-oxidation (Chenault and Whitesides, 1987). However, FDH is an expensive enzyme, but this may be justifiable if enough of the product can be formed. The

reactions have a generally favourable equilibrium strongly shifted towards carbon dioxide formation. However, due to the high K_M value a surplus of formate is required to guarantee a sufficiently high activity even at high conversions (Schutte *et al.*, 1976). It has also been suggested that there are no inhibitory effects by high formate concentrations (Kragl *et al.*, 1996). Formate dehydrogenase has also been produced in bulk quantities from *Candida boidinii* (Weuster-Botz *et al.*, 1994). The formate/FDH system has been applied in the continuous production of L-amino acids (Wichmann *et al.*, 1981) (Hummel *et al.*, 1987a, b) and can be employed for the commercial preparation of L-tert-leucine (Wandrey and Bossow, 1986). Large scale applications of NAD(P) dependent oxidoreductases, and developments have recently been reviewed (Hummel, 1999).

1.7 Continuous Isolated Oxidoreductase Biocatalysis

To enable a two-enzyme cofactor regeneration system to be continuously applied in a bioreactor both the enzymes and their respective cofactors must be retained in the bioreactor by an effective and economical method (Wichmann and Wandrey, 1981). The retention of enzymes is considered less of a problem than the retention of their respective cofactors as generally enzymes are > 40kD and are much larger than cofactors (0.5 - 0.8 kD). Methods of cofactor regeneration and retention have been developed in recent years and to be economical methods must be available to recycle the cofactors 10^2 - 10^6 times (exact numbers depend on the cost of the cofactor and the value of the product) (Chenault *et al.*, 1988).

Enzyme membrane reactors (EMRs) (Figure 1.4) are devices for performing multi-component enzymatic catalysis. In an EMR the enzymes, which are present in solution, are retained in a reaction vessel with continuous flow by means of an ultrafiltration membrane. This exploits the fact that enzymes are very much larger than the respective product or substrate molecules. This method can provide continuous catalysis with good use of the catalyst and an uncoupling of the residence periods from the reactants and the catalyst (Kragl *et al.*, 1993).

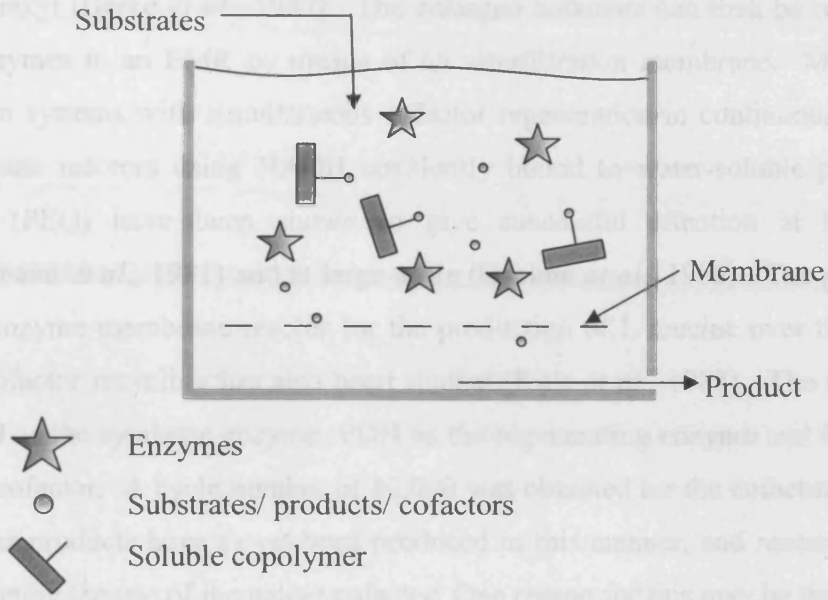


Figure 1.4 Mechanism of the Enzyme Membrane Reactor

An enzyme membrane reactor (EMR) can be employed to maintain enzymes behind a membrane whilst the product can diffuse through the membrane in a continuous operation. The cofactor can be retained behind the membrane by coupling it to a water soluble polymer, thus increasing the cofactor size. Configurations of the EMR can consist of using enforced flow, nanofiltration membranes and charged membranes.

To employ EMRs for continuous cofactor regeneration a high retention of the cofactor is required and this depends on (1) the average molecular weight of the cofactor derivative and (2) the pore distribution determining the actual cutoff behaviour of a membrane (Kula *et al.*, 1987). One method to sustain the concentration of cofactors behind a membrane is to increase the molecular weight of the cofactor and this has been achieved by synthesizing analogues by coupling cofactors to water soluble polymers. NAD(H) has been coupled to polyethylene glycol (PEG) (Buckmann *et al.*, 1981), dextran (Mosbach *et al.*, 1974) formyl PEI-

succinyl, and agarose (Wykes *et al.*, 1975). ATP has also been coupled to PEG-N6-aminohexyl (Berke *et al.*, 1987). The enlarged cofactors can then be retained with the enzymes in an EMR by means of an ultrafiltration membrane. Multi-enzyme reaction systems with simultaneous cofactor regeneration in continuously operated membrane reactors using NADH covalently bound to water-soluble polyethylene glycol (PEG) have been shown to give successful retention at bench scale (Wichmann *et al.*, 1981) and at large-scale (Oshima *at al.*, 1985). The performance of an enzyme membrane reactor for the production of L-leucine over three months with cofactor recycling has also been studied (Kula *et al.*, 1987). The system used LeuDH as the synthetic enzyme, FDH as the regenerating enzyme and PEG-NADH as the cofactor. A cycle number of 80,000 was obtained for the cofactor. However, no other products have as yet been produced in this manner, and research seems to be targeting the use of the native cofactor. One reason for this may be the likely high cost of the analogue in large scale synthesis, and there may also be a change in kinetic properties of the cofactor.

Nanofiltration

To maintain the cofactor in its native conformation, which should ensure full kinetic activity of the enzyme, methods of retaining the cofactor using nanofiltration membranes have been investigated. Nanofiltration is a separation process which is based on the difference in size and charge of the solutes, and depending on the membrane material used these contribute differently to the separation properties. Nanofiltration membranes, unlike conventional ultrafiltration membranes, have shown improved retention of cofactors in continuous operations (Seelbach *et al.*, 1997). One example of the recent use of nanofiltration membranes is the continuous production of L-alanine whereby glucose dehydrogenase (the regenerating enzyme) was co-immobilised with alanine dehydrogenase in a nanofiltration membrane bioreactor. The substrate, pyruvate, was supplied separately due to its instability under acidic conditions. This method for cofactor regeneration and retention proved effective, with the NAD regeneration number reaching 20000 (Lin *et al.*, 1997).

However, there were complications with respect to using a nanofiltration membrane in the reactor. Cofactors were only partially retained in the reactors so that an external supply of the cofactor was necessary in the continuous operation (Lin *et al.*, 1997). Problems associated with nanofiltration membranes (0.5-0.7 kD) include retention issues in which the nanofiltration membrane can retain not only the cofactor but also the product and substrates as well. Flux characterisations are often poor and leakage of the cofactor does occur (Nidetzky *et al.*, 1996).

Charged Membranes

To overcome the problems associated with nanofiltration membranes, charged membranes have been investigated. Charged membranes work on the principle that NAD(H) is an amphoteric molecule which carries a negative charge at pH values greater than 3. Charged membranes can be used instead of nanofiltration membranes because these membranes have well defined size exclusion. There are, however, a number of problems associated with charged membranes:

1. NAD^+ may not be as well retained as NADH, this is probably attributable to the lower net charge of NAD^+. The retention coefficients of NAD^+ are in the region of 0.65-0.85 (Nidetsky *et al.*, 1996). To prevent leakage, the ratio of NADH forming to NADH consuming activity should be high enough to guarantee a surplus of the reduced cofactor throughout the reaction (Nidetsky *et al.*, 1996).

2. Strong ionic solutions cause a decrease in the Donnan potential (in strong ionic solutions the electrostatic interaction becomes weaker and therefore the retention coefficients will decrease). Therefore buffers with a low ionic strength (e.g. triethanolamine, tris or 5-10mM phosphate) should be used to guarantee that the fixed charge density of the membrane is larger than the ionic strength of the solution. Rough optimal conditions should give the retention coefficients for NAD^+ and NADH as 0.85 and 0.99 respectively (Nidetzky *et al.*, 1996).

Selection of the most suitable reaction conditions will depend upon the activity and stability of individual enzymes, the overall thermodynamic equilibrium of the coupled reaction, and the inhibitory effects of the product. Sulfonated polysulfone has been used in an ionized (charged) membrane reactor for the retention of NADP(H) (Kitpreechavanich *et al.*, 1985). This system showed good retention of NADP(H) yet allowed the product to flow freely through the membrane, however, the cofactor was subjected to high levels of degradation. Another system has used a charged membrane for the retention of NAD(H) for the conversion of fructose to mannitol (Nidetzky *et al.*, 1996). A total turnover number between 75000 and 150000 was achieved with 45 reactor cycles, and it was predicted that with a TTN of 100,000 the total cost of the cofactor would be less than 5% of the total process cost.

Two-phase EMR Systems

Enzyme membrane reactors can also be used to achieve a high turnover number of cofactors, even when the substrates are of low water solubility. This is made feasible by the recycling of the water phase subsequent to the extraction of the hydrophobic product. Although the low solubility of the compounds could potentially be overcome by the addition of organic solvents, dehydrogenases normally show increased deactivation rates in the presence of interfaces. An EMR has been set up where a hydrophobic product of the enzyme catalysed process was extracted by means of a hydrophobic microfiltration membrane into a hexane cycle (Kragl et al., 1996). This makes the recycling of the hydrophilic cofactor in the NAD^+ cycle possible due to the decoupling of the residence times of cofactor and substrate. A TTN of 1350 was reached using FDH and ADH for the production of (S)-1-phenyl-2-propanol; this TTN was 25 times greater in comparison to standard techniques used without any cofactor retention (Kragl et al., 1996).

Dynamic Affinity Reactors

If an enzyme has a high affinity for its cofactor, it is possible to use this as a method of cofactor retention. However, a high affinity for a cofactor is a very selected

property. Enzymes, which are immobilised at high concentrations, give cause to effect high dynamic affinity. It is this property which can be used to increase the retention of the cofactors in a membrane. A theoretical study of continuous dynamic NAD recycling was investigated in a hollow fiber reactor (Miyawaki *et al.*, 1982), which was later verified experimentally (Miyawaki *et al.*, 1982). The method used a two enzyme system (ADH and Lactate DH), and the results showed an adequate turnover of NAD (6180), although the conversion was low (34.6%). Alcohol dehydrogenase (ADH) has been used as the regenerative and synthetic cofactor in an affinity chromatographic reactor (Miyawaki *et al.*, 1991). The necessary nicotinamide cofactor was regenerated through the conjugated reaction of propionaldehyde to propylalcohol and the oxidation of ethanol to acetaldehyde. Native cofactors have also been recycled and effectively used in a bioreactor with immobilised conjugated enzymes through the dynamic affinity of the immobilised enzyme and cofactor (Lin *et al.*, 1997). This method, like other methods, requires a high concentration of immobilised enzymes to obtain a high enough extent of the dynamic affinity between the immobilised enzymes and the free cofactor. The obvious disadvantage of this method for continuous regeneration of cofactors is that the methodology is only applicable for enzymes which are immobilised in high concentrations and for enzymes with an extremely high affinity for the cofactor (Nidetsky *et al.*, 1996).

1.8 Whole Cell Biocatalysis

Whole cell microorganisms can be used in instead of isolated enzymes for the bioreduction of substrates (Faber, 1997). Microorganisms, in particular yeasts, are made up of a vast number of dehydrogenase enzymes which are able to accept non-natural substrates (Levison, 2001). These microorganisms also contain all the necessary enzymes and metabolic pathways for cofactor regeneration, and can be selected to perform synthetic reactions, for example the fermentation of glucose to ethanol by exploiting the cells metabolism. In the same way, whole cells can also be

employed for the bioreduction of pharmaceutically important pro-chiral substrates (Figure 1.5).

The use of whole cells to reduce cofactors is advantageous in that the reduction reactions take place inside the cells as part of their natural metabolism. This means that the cofactors and enzymes are well-protected within their natural cell environment and cheap carbon sources can be used as auxiliary substrates (Faber, 1997). Other advantages to this method are that the processes do not introduce additional compounds into the enzymatic system, and therefore the downstream processing is not affected. This method is also cheap as the enzyme does not have to be isolated and the cofactor does not have to be added stoichiometrically.

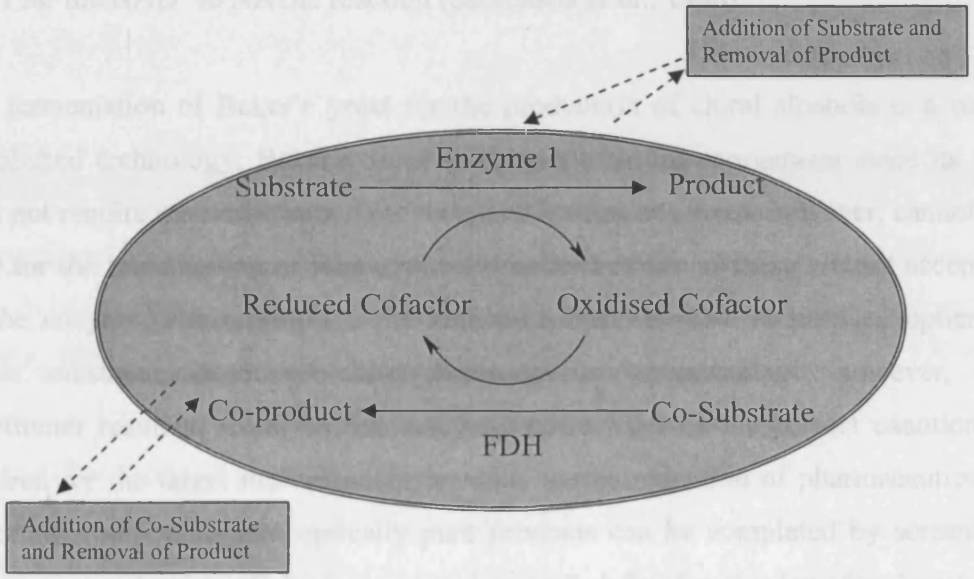


Figure 1.5 Whole Cell Cofactor Regeneration

Whole cells contain multiple enzymes and all the necessary cofactors and pathways for natural cell metabolism. They can be employed for the bioreduction of pharmaceutically important pro-chiral substrates by using these naturally occurring enzymes inside the cell membrane to complete the reaction. The converted substrate can then be isolated and purified from the cell debris.

Baker's yeast (*Saccharomyces cerevisiae*) is by far the most widely used microorganism for the asymmetric reduction of ketones (Faber, 1997). Large-scale preparations of chiral alcohols have been completed using baker's yeast (Kometani *et al.*, 1991, 1993), the method used a 19L bubble column reactor and NADH was regenerated from NAD^+ through ethanol oxidation. A scheme was also proposed for NAD(P)H regeneration coupled with the asymmetric reduction of carbonyl compounds using ethanol as the energy source (Kometani *et al.*, 1994). The regeneration of cofactors can also been achieved in immobilised yeast cells. Immobilisation of cells can promote stability, simplify reactor operation, and ease downstream processing (Stevenson *et al.*, 1993). Cells of *Saccharomyces cerevisiae* have been immobilised in calcium alginate fibres and used in a reactor as a source of ADH for the NAD^+ to NADH reaction (Stevenson *et al.*, 1996).

The fermentation of Baker's yeast for the production of chiral alcohols is a well-established technology; Baker's yeast is also an ideal microorganism since its use does not require sterile fermenters (Faber, 1997). Baker's yeast, however, cannot be used for the bioreduction of long-chain and bulky ketones as these are not accepted by the enzyme (Faber, 1997). The ability of Baker's yeast to produce optically active substances from pro-chiral substrates is an advantage; however, the enantiomer resultant from the reaction may not always be the correct enantiomer required for the target molecule. In practice, the bioreduction of pharmaceutically important compounds into optically pure products can be completed by screening numerous yeasts (from Baker's yeast to less well-defined soil microorganisms) for their reaction compatibility. The disadvantages of using whole cells include: the requirement for the use of large volumes, low productivities due to a low synthetic compound tolerance, and side reactions due to uncontrolled cell metabolism. The use whole cell biocatalysts also has a big impact on the amount of downstream processing required, and if growing cells are used process control can be difficult (Faber 1997).

1.9 Isolated Oxidoreductases and Whole Cells in Organic Media

Many pharmaceutically important compounds are hydrophobic; these can include, steroids, fats, oils, hydrocarbons and precursors of chiral synthons (Carrea, 1988). It is generally accepted, however, that enzymes require an aqueous environment to maintain the active conformations for substrate binding and catalysis (Chen *et al.*, 1989). As a result of this conflicting combination there has been interest in finding methods of applied catalysis which maintain enzyme activity yet provide adequate levels of substrate for an efficient bioconversion; such methods have been reviewed (Fukui *et al.*, 1985; Cabral *et al.*, 1997; León *et al.*, 1998; Angelova *et al.*, 1999; Loughlin, 2000). In cofactor requiring enzyme systems water-organic solvent two-phase systems (Carrea *et al.*, 1987), reverse micelles (Orlich *et al.*, 1999) and homogenous isotropic microemulsions (Larsson *et al.*, 1990) have all been employed to study the effects of organic solvents.

Increasing the solubility of a compound by adding water miscible organic solvents to the reaction medium has served to maintain enzyme stability and activity, but only at low concentrations (Klibanov *et al*, 1979). Increasing solvent concentrations progressively gives rise to enzyme inhibition, decreased specificity and denaturation (Carrea, 1984), and there are no reports of using water miscible solvents in cofactor requiring systems. One method to overcome the solvent effects on enzymes at high concentrations may be to use two-phase systems; these systems contain water and a water immiscible organic solvent (Carrea, 1984), and have been researched with regard to isolated cofactor requiring enzymes and whole cell cofactor requiring biocatalysts. The water contains the enzymes or cells and the hydrophilic cofactors, and the organic solvent contains the hydrophobic substrates. On stirring or shaking the substrates are transferred from the organic phase to the aqueous phase. There they undergo an enzyme catalysed bioconversion and the products return to the organic phase. One of the parameters which influences the reaction effectiveness is the rate of transfer of reagents and products between phases; the rate of mass transfer can be increased by increasing the mixing, however, a compromise will be made as

the enzyme will be denatured by the liquid-liquid interfacial contact (Thomas *et al.*, 1979; Carrea *et al.*, 1987). A number of dehydrogenase enzymes have been investigated with regard to the effects of water immiscible solvents these include, an $NAD(P)^+$ dependent hydroxysteroid dehydrogenase, an NAD dependent alcohol dehydrogenase and an NADPH dependent glutamate dehydrogenase (Carrea *et al.*, 1987).

Another method of trying to increase enzyme stability in organic media is to use reverse micelles. Reverse micelle systems are configured from an oil rich micro-emulsion where the reverse micelles of water are dispersed in the oil (Orlich *et al.*, 2000). These types of systems are capable of solubilising non-polar substrates so that the reaction can occur at the large internal interface between the reverse micelles and the oil (Figure 1.6).

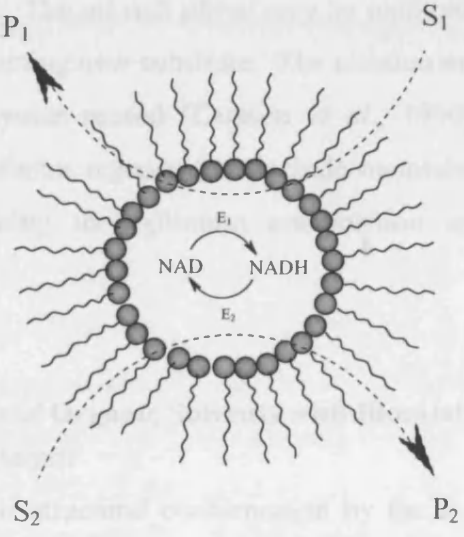


Figure 1.6 A Section of a Reverse Micelle

A section of a reverse micelle with enzyme cofactor regeneration (adapted from Orlich et al., 1999). The aqueous interior of the reverse micelles serves as a host for the water soluble enzymes and cofactors.

Reverse micelles have been employed in the enzymatic reduction of a poorly water-soluble ketone with NADH regeneration (Orlich *et al.,* 1999). A ternary mixture was developed from a surfactant (Marlipal O13/60) an organic solvent (cyclohexane) and water. It was demonstrated that in microemulsions the rate of the reduction of 2-heptanone to *S*-2-heptanol was increased up to 12 times compared to the reaction rate in water. The disadvantage of using reverse micelles for multi-enzymatic cofactor regeneration is that product recovery and reuse of the enzymes and cofactors is difficult (Orlich *et al.*, 2000).

Oxidoreductase bioreductions have also been performed in homogenous isotrophic microemulsions to try to overcome the problems associated with product recovery, and enzyme and cofactor recycling. This is possible by controlling small changes in temperature which shifts the system into the two phase regions where an oil-rich phase containing the product co-exists with a water-rich phase containing the surfactant and enzyme. The oil rich phase may be replaced in continuous operation by an oil solution containing new substrate. The reaction may then be continued and the cofactors and enzymes reused (Larsson *et al.*, 1990). Difficulties in using microemulsions for cofactor regeneration include maintaining enzyme activity and stability and determining the optimum composition and temperatures of the microemulsions

1.10 The Interactions of Organic Solvents with Biocatalysts

Isolated Enzyme Biocatalysts

Enzymes maintain their structural conformation by the forces resulting from their sequence of amino acids; in aqueous environments these forces (including hydrophobic interactions) cause enzymes to fold into compact structures with non-polar cores (Styrer, 1995). When the polarity of the surrounding environment is reduced by adding an organic solvent, the hydrophobic areas in the enzyme disperse resulting in an unfolded and inactive enzyme (Fukui, 1985).

Whole Cell Biocatalysts

Cell membranes consist of lipid bilayers with embedded proteins and are used as a barrier to the flow of polar molecules (Styrer L, 1995). When solvents are added to whole cell solutions the solvents tend to have an effect on the cell membranes, there are also interactions with the cell due to solvent toxicity on cell physiology and other related activities (Angelova, 1999).

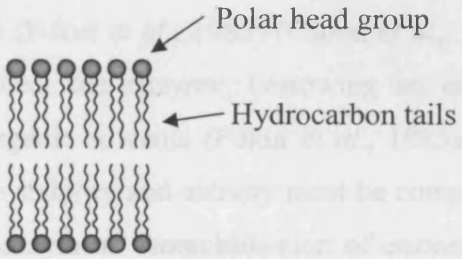


Figure 1.7 A Section of a Bilayer Membrane

A section of a bilayer membrane formed from phospholipids molecules.

Whole cells do not adequately exhibit the catalytic activities of many intracellular enzymes. This is likely to be due to the permeability barrier of the cell membrane towards the substrates and products (Gowda *et al.*, 1991). A number of procedures to permeabilise cell membranes to enhance substrate diffusion rates have been reported for microbial organisms (Gowda *et al.*, 1991; Liu *et al.*, 1999). One example is the permeabilisation of recombinant *Saccharomyces cerevisae*, these cells were permeabilised with alcohol solutions, and results demonstrated that under optimum conditions the permeabilised cells were very effective as whole cell biocatalysts (Liu *et al.*, 1999). The problems associated with permeabilised cells can include a reduction in cell viability and target enzyme leakage (Liu *et al.*, 1999).

The problems associated with employing organic solvents for delivery of the substrate to the isolated enzyme and whole cell systems have not been overcome.

Firstly, there is insufficient information on the physiological response of microbial cells to the presence of lipophilic compounds (Angelova *et al.*, 1999). Secondly, enzymes are numerous and diverse, and there are no rules concerning the inhibitory effects of various solvents on particular biocatalysts, and each solvent must be tested individually (Carrea *et al.*, 1987).

1.11 Enzyme Stability and Immobilisation

Methods such as immobilisation to retain enzyme activity in organic media have been researched (Fukui *et al.*, 1985) (Cabral *et al.*, 1997). Immobilisation has been reported to protect the enzyme, bestowing an enhanced stability against being denatured by organic solvents (Fukui *et al.*, 1985). To be industrially viable, the isolated enzyme stability and activity must be comparable to, or higher than, that of the whole cell enzymes. Immobilisation of enzymes is one method that has been developed to promote stability, but more notably immobilisation of an enzyme can be employed to separate the enzyme from the substrate and product in a continuous operation.

There are two types of immobilisation methods; adsorption and covalent linkages. Evidence has shown that the adsorption techniques are less valuable. For instance, a comparison was made for the following resins XAD-7, Sepabeads FP-DA, Eupergit C, Celite 547 and Silica Gel 60; the covalently linked enzymes could be washed with water to desorb the product with little loss in activity, however, the adsorbed enzymes could not be reused and enzyme activity was rapidly lost (Anthonsen *et al.*, 1999). Examples of the diverse type of resins which have been employed for enzyme immobilisation are: sand (Brotherton *et al.*, 1976), CN-Br-activated sepharose (Gorisch *et al.*, 1984; Schneider-Bernlohr *et al.*, 1978), polyacrylamide gel and glass balls (Julliard *et al.*, 1986), AH-Sepharose-4B, CH-sepharose 4B, DEAE-cellulose or DEAE-sephadex (Kim *et al.*, 1987) and more recently Eupergit C (Brocklebank *et al.*, 1999; Hublik *et al.*, 2000; D'Annibale *et al.*, 2000). Much of

this research has concentrated on the stabilising effect it is thought that these matrices bring about as opposed to their separation properties.

Immobilisation of isolated enzymes can affect the three-dimensional conformation specific to the enzyme; this has observed through electron paramagnetic resonance (EPR) (Clark *et al.*, 1983) using alpha-chymotrypsin immobilised on CNBr-activated sepharose 4B. EPR spectroscopy has also been used (Skerker *et al.*, 1988) to compare the active-site structural features of immobilised Horse Liver Alcohol Dehydrogenase on CNBr-Sepharose and Octyl-Sepharose Cl-Br, both of which show slightly different conformations. Contrary to these results, however, great similarity was shown in the spectroscopic properties of soluble and immobilised horse liver ADH on CNBr-activated Sepharose and it was concluded that no significant change had taken place during immobilisation (Schneider-Bernlohr *et al.*, 1978). Calorimetry studies have also confirmed this idea (Koch-Schmidt *et al.*, 1977). Changes in the conformational three-dimensional shape of some enzymes may be due to a multipoint linkage (Hernaiz *et al.*, 2000). It has also been suggested that additional multipoint attachment can be used to increase stability (Fernandez-Lafuente *et al.*, 1999; Mateo *et al.*, 2000). Changes in enzyme activity by immobilisation have been reviewed (Clark, 1994).

A variety of other methods may be applied to stabilise enzymes in addition to immobilisation. Free and immobilised Horse Liver ADH (on CNBr-activated sepharose) were both stabilized by the addition of AMP, and both types of enzyme were found to have the same stability in the presence of 5mM AMP (Gorisch *et al.*, 1983). Lactase immobilised onto controlled porosity glass has also exhibited the same stability as its free form in the presence of ethanol, acetone, methanol, and DMSO (Rogalski *et al.*, 1995). Enzyme stability may also be increased by the addition of a second protein (thus increasing protein concentration). The stability of a free enzyme was increased by adding BSA to the solution, and was made more

stable than the immobilised form and the original free enzyme form (Johnson *et al.*, 1978).

Cross linked enzyme catalysts (CLECs) is a further method by which enzymes can be immobilised. Small protein crystals are grown from aqueous solutions, or aqueous solutions containing organic solvents. These crystals are then cross-linked with a bi-functional reagent i.e. glutaraldehyde. This then enables them to function at elevated temperatures, extremes of pH, and in harsh aqueous, organic or anhydrous media. Irregular pore sizes and shapes are created between individual enzyme molecules in the underlying crystal lattice. This restricted accessibility greatly enhances the metal ion or cofactor retention characteristics of CLECs in comparison to conventionally immobilised enzymes and enzymes in solution. However this may also mean that the particle size is too small to allow for continuous process, i.e. the retention of products and substrates. Recent research has found that some CLECs will not form unless the respective cofactor is present (Lee *et al.*, 2000). This then becomes a problem in the recycling of the cofactor, and research must be carried out to see whether it is possible to form a CLEC using a two enzyme system.

1.12 Isolated Enzymes versus Whole Cells in Biocatalysis

Isolated enzymes and whole cells can both be applied for the bioreductions of prochiral substrates. The methods and controls which ensure that such reactions can take place with high efficiency and specificity in each of them are, however, considerably different.

1) Isolation and Purification

Enzymes need to be isolated and purified from their host cells and these extra steps dramatically increase the process time and costs. After the isolation and purification the enzyme can be used in predefined conditions to carry out highly controlled and specific reactions. In contrast, whole cells suffer from the presence of multiple reductases with overlapping substrate specificities but

differing stereoselectivities and, therefore, when using whole cells as catalysts instead of isolated enzymes this can often lead to mixtures of alcohol products particularly when whole yeast cells are used for ketone reductions (Stewart *et al.*, 2001). This increases the amount of downstream processing which is also made difficult due to the large quantities of biomass particularly if the product is stored inside the cell.

2) *Cofactors*

Microbial cells contain multiple dehydrogenases and all the necessary cofactors and metabolic pathways for their regeneration (Chenault and Whitesides, 1987). Consequently, cheap carbon sources can be used as auxiliary substrates for asymmetric reduction reactions. The cofactor is also protected in its natural cell environment (Faber, 1997). Isolated enzyme systems require the addition of stoichiometric quantities of cofactor and to be used efficiently these cofactors need to be regenerated *in situ*.

3) *Hydrophobic substrates*

The hydrophobicity of many pharmaceutically important substrates and products can cause problems for whole cells as there can be difficulties in the transport of these compounds in and out of the cells (Angelova, 1999) and in such systems the use of isolated enzymes seems attractive. However, hydrophobic substrates also cause problems as the substrate concentrations in aqueous reaction solutions are generally low and the addition of solvents to increase the substrate concentration can cause the isolated enzyme to be denatured.

4) *Single Step/ Multiple Steps*

One advantage of the whole cell biocatalyst has over the isolated enzyme, is that, a whole series of chemical reactions can effectively be carried out in essentially a single step using cheap carbon sources to produce specialty chemicals from amino acids to penicillins. Such syntheses are known as fermentation processes

(Faber, 1997). A large number of bioconversions often start from relatively complex organic molecules and use a single step to convert a synthetic organic compound into a desired product. Such single step bioconversions can more easily mimicked (than a multiple step reaction) by an isolated enzyme system, by isolating the enzyme from source and adding exogenous quantities of the cofactors.

5) *Substrate Inhibition*

Whole cells and isolated enzymes generally suffer similarly from substrate inhibition. This is exhibited through the enzyme catalysed reaction rate diminishing with excess substrate (Bailey and Ollis, 1986) and this enzyme behaviour can have important implications in the design of bioreactors.

The decision on whether to employ an isolated enzyme or a whole cell enzyme relies upon preliminary analysis of the constraints summarised above, the type of reaction, the scale of the reaction and also the potential costs of the process.

1.13 Aim of Thesis

The aim of this thesis is to systematically compare the isolated enzyme and whole cell biocatalyst for the bioreduction of a prochiral substrate. The comparison will be used to determine which of the systems has the most potential for carrying out a reduction reaction. The model oxidoreductase which will be used for the investigation is a NADH-dependent reductase from a soil organism which is not overexpressed and remains in its native microorganism. This in itself reflects the ever-increasing need to find new and valuable methods and compounds, and is a problem faced by industry in the search for new enzymes for biocatalysis. The substrate and product which are under investigation are novel compounds which exhibit low aqueous solubility; this low aqueous solubility is an inherent property of many pharmaceutically important compounds. This limited knowledge of the enzyme, the substrate and the product, mirrors the problems in industry of producing

new and invaluable compounds via enzyme catalysis. The comparison will investigate the isolated enzyme and the whole cell biocatalyst with respect to: the biocatalyst type, the effects on the reaction with respect to biocatalyst purity, the bioreduction methodology including methods of regenerating cofactors, the methods of substrate delivery, and the bioreduction efficiency. Lastly a comparison will determine if there are any potential improvements which could be facilitated via recombinant technology. The objectives are set out in further detail in Chapter 2.7

1.14 Discussion

Isolated enzymes and whole cells can both be employed as highly specific biocatalysts for the bioreduction of prochiral substrates. They are advantageous over chemical catalysts due to their high specificity and adaptability; however, they require strict reaction conditions which if not controlled effectively may cause the biocatalysts to become denatured. The oxidoreductases are one of the most important types of enzyme as they can reduce prochiral substrates into optically pure products with high conversions. However, isolated oxidoreductases require the stoichiometric use of cofactors which due to their high costs at large scale require regenerating. The regeneration of cofactors can be achieved through a number of methods; the most effective method seems to be the addition of a secondary enzyme and secondary substrate. There are a number of documented secondary enzyme systems, of which, the FDH/formate system appears to be the most viable; this is because the enzyme is highly active, formate is innocuous to the enzyme, and the product of the reaction is carbon dioxide which can be easily removed from the reaction (Chenault *et al.*, 1987). The problems associated with using this type of enzyme system in continuous operation are that the cofactors are often of similar size to the substrates and products, thus conventional membranes cannot be employed to retain enzymes without cofactor leakage. Enzyme membrane reactors (EMRs) have been employed using methods which have either adapted the cofactor or adapted the membrane to try to increase cofactor retention. Adaptation of the cofactor can cause a change in the kinetic properties of the cofactor and research is

currently intent on using the native configuration of the cofactor. Methods to continuously recycle cofactors have employed EMRs using nanofiltration or charged membranes, but neither type of membrane has demonstrated the required retention to ensure economical use of the cofactor. Retention of the cofactor whilst maintaining kinetic activity is a current hurdle in the use of these isolated enzymes at large scale, and therefore to use an isolated oxidoreductase with cofactor regeneration in a bioreactor means that a novel approach is potentially required. The type of bioreactor used for the bioreduction must ensure that the enzyme activity is relatively high and the cofactor is regenerated enough times to make the system economically viable. If possible the isolated enzyme bioreduction should take place in a continuous bioreactor, this would allow a greater efficiency of the enzyme and the cofactor, and would significantly reduce the associated cost of the cofactor (in comparison to batch reactors). If such an isolated enzyme bioreactor system is not viable then it may be more appropriate to use a whole cell microorganism which can potentially be employed instead of an isolated enzyme in the bioreduction of synthetic substrates. Whole cell biocatalysts recycle cofactors as part of their own cell metabolism, and therefore the high cost of cofactors will not impact the method selected for the bioreduction. Whole cells, however, contain multiple enzymes and this can cause an increase in the competition for the substrate, and therefore there will also be a concurrent increase in the number of by-products. The amount of downstream processing will also be increased (when compared to isolated enzyme systems) due to the increased number of by-products, and also because the cell debris will also need to be removed. The selection of the type of whole cell bioreactor would generally be the batch reactor since whole cell recycling can be problematic. This is because it can be difficult to transport hydrophobic compounds in and out of the cells, and thus if the product does not diffuse out of the cell it will have to be extracted by other means. This is generally through the addition of a solvent which can cause detrimental effects to the cells.

Many pharmaceutically important compounds are highly insoluble in aqueous environments, and thus methods to increase substrate concentrations without reducing biocatalyst viability have been investigated. These have included the addition of single-phase solvents, two-phase solvents, and micro-emulsions. It is generally accepted that: 1) Single phase solvents can be used at low concentrations, however, as the solvent concentration is increased enzyme activity is rapidly decreased (Klibanov *et al.*, 1979). 2) Two-phase solvents retain greater enzyme activity but by increasing the mixing to increase mass transfer causes an increase in the interfacial contact, which also rapidly decreases enzyme activity (Carrea *et al.*, 1988). 3) Microemulsions can be used for batch configurations, however, the composition of the microemulsion is difficult to define to ensure enzyme stability and activity (Larsson *et al.*, 1990). Therefore, the choice of which type of system to use will depend upon the type of biocatalyst employed (isolated enzyme or whole cell), the respective activities and stabilities, the reaction methodology including cofactor recycling requirements, and the substrate and product hydrophobicity.

Immobilisation has been investigated to promote biocatalyst stability in solvents, however there seems to be no common link between the effects of immobilisation and the stability and conformation of the enzymes. Immobilisation has a greater impact in its ability to facilitate continuously operating bioreactors by separating the isolated enzyme from the substrates and products. This separation ability is true in most enzymatic systems, however in an isolated oxidoreductase system the method of cofactor retention must be considered separately if such immobilisation techniques are to be utilized since the cofactors are often similar in size to synthetic substrates and products.

Isolated oxidoreductases have a number of advantages over whole cell biocatalysts when employed for the bioreduction of pharmaceutically substrates, equally, the contrary in also true. Isolated enzymes can be highly active and highly specific, however, they require costly isolation and purification techniques. Isolation of the

active enzyme from the cells also means that the enzyme immediately become vulnerable to extrinsic factors. Whole cells are less specific, but don't require the stoichiometric addition of cofactors, however, the other cell components are contaminants which require removal after the reaction. The enzymes and cofactors used in the whole cell bioreductions are also more protected to extrinsic factors since they remain in the cell. There currently seems to be no direct comparison between this type of biocatalyst to determine which type, isolated enzyme or whole cell, has the most potential for large scale bioreductions of low soluble pharmaceutical compounds. The aim of this research is to compare an NADH dependent reductase as an isolated enzyme and as a whole cell biocatalyst for the bioreduction of a pharmaceutically important substrate of low aqueous solubility.

1.15 Summary

Isolated enzymes and whole cell microorganisms can both be employed for the biocatalytic conversion of pharmaceutically important compounds of low solubility. The oxidoreductase class of enzyme can be employed for the asymmetric conversion of prochiral substrates to optically pure products; they also have the potential to replace chemical catalysts (which are, in general, non-specific) in the synthesis of chemical compounds. In isolated enzyme bioreductions, however, oxidoreductases require the stoichiometric addition of the appropriate cofactor, and because of the high cost of these cofactors they need to be regenerated *in situ*. Whole cell biocatalysts do not require the addition of a cofactor regeneration system as they naturally regenerate cofactors as part of their own cell metabolism. However, the use of whole cells for such bioreductions is made difficult by the multiple enzymes in the cells, and the level of downstream processing required. Many of the pre-cursors to pharmaceutical compounds exhibit low solubility in aqueous solutions. However, both isolated enzymes and whole cells suffer degradation in low aqueous environments. Therefore, a compromise may need to be made between method of substrate addition and the biocatalyst stability. To promote enzyme stability, immobilisation has been used, however, immobilisation has served a greater purpose

in separating the biocatalyst from the substrates and products in continuous operation. The difference in employing isolated oxidoreductases and whole cells can be seen through their physical appearance, the operating parameters required for each system, and the environments which are required to ensure effective catalysis. The aim of this thesis is to compare an NADH-dependent reductase for isolated enzyme and whole cell biocatalysis. In the following chapter the microorganism, enzyme, substrate, and products which will be used in the experiments are described in detail including analytical techniques; the objectives of the research are also summarised.

Background and Experimental Logic

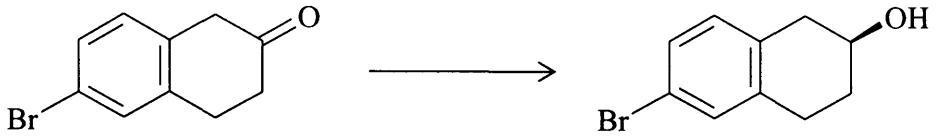
2.1 Introduction to the Model System

The model system used for this research was selected because it replicates many of the characteristics of industrially important enzymes. The active enzyme is a reductase which carries out the asymmetric synthesis of a prochiral ketone to its corresponding *(S)*-alcohol (6-bromo-β-tetralone to (*S*)-6-bromo-β-tetralol) and this reaction requires NADH as a cofactor. There is limited knowledge of the enzyme as it has not been purified, and therefore no sequencing or crystallisation has been possible. Stability studies show that the enzyme is unstable, exhibiting a half life of 7hrs at 22^0C. The model substrate 6-bromo-β-tetralone and product 6-bromo-β-tetralol are both novel compounds which exhibit low solubility in aqueous environments and 6-bromo-β-tetralone only exhibits a solubility of 10g/L in ethanol. Solvents are known to affect enzyme activity and consideration of the substrate addition will be required. If a process is to be operated with an isolated enzyme, the cofactor (NADH) which if not added stoichiometrically will require regenerating *in situ*. For this research, FDH has been selected as the regenerating enzyme for the isolated enzyme studies. This is because it has been shown to be highly effective at regenerating NADH (Chenault *et al.*, 1988) and has undergone a number of characterisation studies. Methods to maximize productivity perhaps by enzyme and whole cell regeneration/ recycling will also be analysed.

2.1.1 Inefficiency of the Chemical Catalyst

(*S*)-6-bromo-β-tetralol is a precursor to the compound MK-0499. This compound was a potentially potent potassium channel blocker, which mediated the re-polarization of cardiac tissue and was under investigation for the treatment of ventricular arrhythmias and the prevention of sudden cardiac death (Tschaen *et al.*, 1995). In the synthesis of this drug, the chemical methods for the chiral reduction of

6-bromo-β-tetralone were inefficient and microbial reduction was selected for the production of the specific enantiomer (Tschaen *et al.*, 1995) (Figure 2.1).



6-bromo-β-tetralone (*S*)- 6-bromo-β-tetralol

Figure 2.1 Model Substrate and Product

6-bromo-β-tetralone can be reduced to produce a chiral product. The figure above demonstrates the (S)-configuration, the enantiomeric excess is dependent upon the specificity of the catalyst. Chemical catalysts are generally non-specific and produce racemic mixtures; biological enzymes are highly specific and have the ability to produce substances of high enantiomeric excess.

2.1.2 Substrate and Product Stability

6-bromo-β-tetralone and 6-bromo-β-tetralol (Figure 2.1) are used as the model substrate and product and were kind gifts from Merck and Co., (Rahway, NJ, USA). 6-bromo-β-tetralone and 6-bromo-β-tetralol are stable (>99%) in both water and ethanol for over 12hrs (Thomas, 2000). 6-bromo-β-tetralone and 6-bromo-β-tetralol exhibit a solubility of 0.5g/L in water and 10g/L in ethanol (Thomas, 2000).

2.1.3 Media Component Selection

Media components used for the growth of *Trichosporon capitatum* (MY 1890) have previously been evaluated (Reddy *et al.*, 1996). Experiments demonstrated that the glucose in the growth media most likely promoted another enzyme which converted the substrate into the unwanted optical isomer giving only 70% enantiomeric excess. Further experiments replaced the glucose with glycerol, thus, causing a change in the

metabolic pathway and inhibiting the unwanted enzyme. The optical purity of the product after this change in growth media was 99%.

2.1.4 Growth of Trichosporon capitatum (MY 1890)

1ml of frozen stock stored at –80^{0}C was used to inoculate 50ml of media in a 200ml Erlenmeyer™ flask (30g/L glycerol, 25g/L hysoy peptone, 20g/L yeast extract) (Sigma, Poole, Dorset, UK). Cells were grown at 28^{0}C for up to 48hr; 10ml of the cells were used to inoculate 500ml of media in a 2L Erlenmeyer™ flask. The cells were shaken continuously at a speed of 200rpm and left to grow at 28^{0}C to a concentration of 18g/L (Figure 2.2). This protocol was adapted from (Reddy *et al.*, 1996).

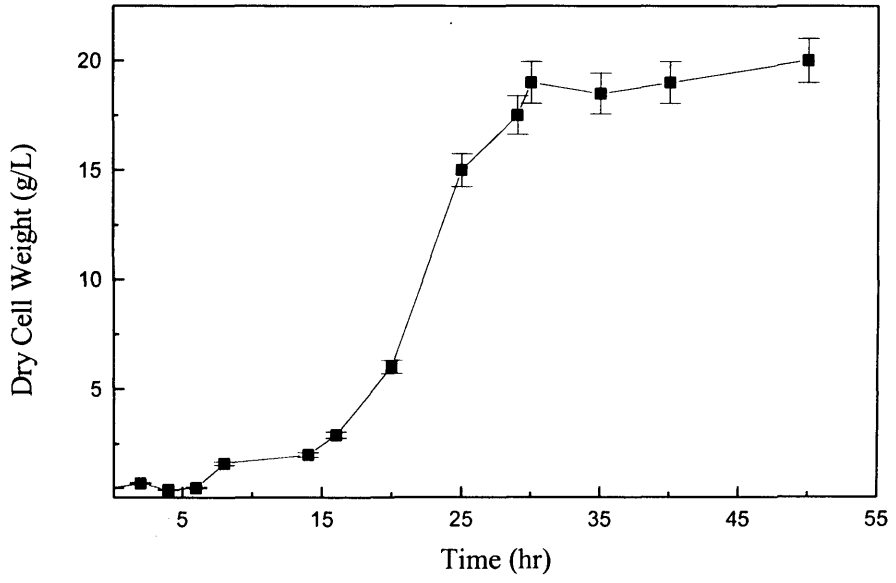
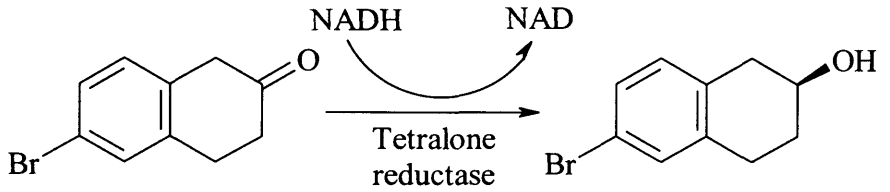


Figure 2.2 Growth curve of Trichosporon capitatum (MY 1890)
50ml of cells were grown at 28^{0}C in a 250ml Erlenmeyer™ flask inoculated with 1ml of frozen stock. 10ml of these cells were then used to inoculate 500ml of media. The cells were shaken continuously at 200rpm.

2.1.5 Whole Cell Biocatalytic Synthesis of 6-bromo-β-tetralol

The whole cell biocatalytic synthesis of 6-bromo-β-tetralol has previously been described (Reddy *et al.*, 1996). Gram quantities were produced through the asymmetric bioreduction of 6-bromo-β-tetralone (dissolved in ethanol) using growing cells (Figure 2.3).



6-bromo-β-tetralone (*S*)- 6-bromo-β-tetralol

Figure 2.3 Chiral Reduction of 6-bromo-β-tetralone

The whole cells of Trichosporon capitatum (MY1890) can be used to reduce 6-bromo-β-tetralone to (S)-6-bromo-β-tetralol (99% ee). NADH is recycled via the cells metabolic pathway.

2.1.6 Enzymatic Regeneration System

An oxidoreductase can be isolated from the cells of *Trichosporon capitatum* (MY 1890), and this enzyme requires NADH as a cofactor (Thomas, 2000). The enzyme (tetralone reductase) carries out the bioreduction of 6-bromo-β-tetralone to (*S*)-6-bromo-β-tetralol with high enantiomeric excess (99%) (Thomas 2000). However, it is unstable and has not been fully purified. The enzyme works effectively in a regenerating system using formate dehydrogenase as the regenerating enzyme and formate as the cosubstrate (Figure 2.4).

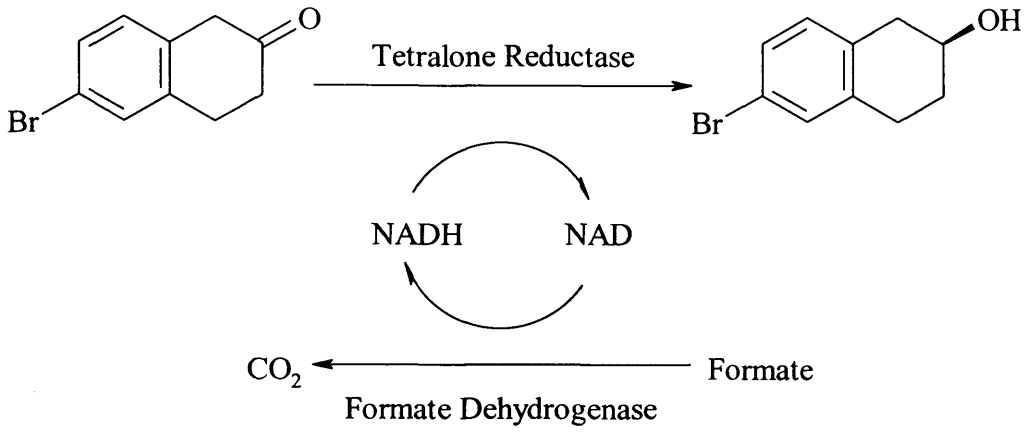


Figure 2.4 Regenerative Bioreduction

Tetralone reductase isolated from the cells of Trichosporon capitatum (MY 1890) can be used to reduction 6-bromo-β-tetralone to (S)-6-bromo-β-tetralol. This requires the addition of exogenous quantities of NADH. During the reduction reaction NADH is oxidized but can be recycled back into its reduced form by the addition of a regenerating enzyme, in this case formate dehydrogenase and the secondary substrate, formate.

2.2 Scale of Experiments

The scale of experiments is limited by a number of parameters. Firstly, the quantities available of the model substrate and product are limiting as they are novel chemicals and can not be purchased. Nicotinamide adenine dinucleotide (NAD), and its reduced form (NADH), are limiting because they are currently expensive chemicals to purchase. In addition, formate dehydrogenase is also currently an expensive enzyme. The other important limiting factor is tetralone reductase. The scale of the equipment limits the production of this novel enzyme and only small quantities of the enzyme will be produced after the purification steps. This is because the enzyme is not over-expressed and only a small percentage of the cell protein is the target enzyme. Almost all the experiments were carried out at micro-

litre scale in 96-well flat-bottomed round micro-litre plates (Nalge Nunc International, Rochester, NY, USA). Other experiments required larger volumes (ranging up to 10ml) are described in detail in later chapters.

2.3 The Use of Novel Microorganisms for Bioreductions

The bakers' yeast *Saccharomyces cerevisiae* was the first eukaryote whose genome was entirely sequenced and is one of the favoured model organisms for basic biological research (Dujon, 1996). There have been large numbers of papers describing its characteristics and biocatalytic properties under a range of conditions. Processes that use novel microorganisms suffer from the lack of process understanding which can only be accumulated through the combination of a number of experiments. Fermentation processes have been characterised to take into account temperature, pH, dissolved oxygen, substrate feeding and mixing, and therefore a process which uses whole cells as a biocatalyst can be implemented with little difficulty using established fermentation technology. This is an advantage whole cell biocatalysts have over their isolated enzymes, which require further processing for isolation and purification. There are no set rules for defining methods of enzyme purification, although previously purified enzymes with similar characteristics can be used as a guide for identifying appropriate methods. One advantage of an isolated enzyme process is that it might not require media component optimization (Chapter 2.2.3) as the competing enzyme may be removed through the enzyme purification stages. The use of a newly screened microorganism also has the complication that the active enzyme has not been isolated and purified this means that the crystal structure of the enzyme is unknown and, the enzyme cannot yet be genetically modified or over-expressed. The small quantity of enzyme in the microorganism is probably less than 0.1% of the total protein in the cell therefore the catalytic activity of the enzyme will be low in comparison to an over-expressed enzyme.

2.4 Oxidoreductases

In fermentations, cells provide all the enzymes necessary for cofactor regeneration, whereby the cofactors are synthesized and regenerated as part of cellular metabolism. In processes using isolated enzymes *ex vivo* nicotinamide cofactors must be regenerated explicitly. In addition to the system of enzymes used for the synthesis, a second reaction system, the regenerative system must be used (Chenault and Whitesides, 1986). This complicates the process engineering, as not only does the cofactor need to be added but also a secondary enzyme and substrate.

2.5 Insoluble Substrates and Products

Lipophilic compounds are of significant importance in modern biotechnology (Angelova *et al.*, 1999). Steroids, fats, oils, hydrocarbons and many chiral precursors are all poorly soluble in aqueous media (Carrea *et al.*, 1987). Substrates poorly soluble in water present difficulties as the bioconversions must be carried out using large reaction volumes and consequently large amounts of biocatalyst and cofactors are required (Carrea and Cremonesi, 1987). The bioavailability of highly insoluble compounds is currently a problem, and organic solvents or surfactants have been used mostly as mediators for increasing substrate solubility (Angelova *et al.*, 1999), although this can have a detrimental effect on cell viability. In an aqueous medium, the folding of a soluble enzyme protein is driven by the tendency of the hydrophobic amino acid residues to be excluded from water so that they are buried in the interior of the molecule, whereas, the charged and hydrophilic molecules are on the surface in contact with aqueous solvent. When water is replaced with an apolar solvent the interior hydrophobic residues tend to disperse, resulting in the reorganization of the enzyme tertiary structure (Klyosov *et al.*, 1975). Therefore, it is likely that any organic solvent will gave rise to enzyme inactivation. Methods to protect the enzyme such as immobilisation may prove to be a useful alternative. In addition, it may be useful to employ another type of medium such as an ionic liquid to preserve the enzyme conformation (Lee *et al.*, 2000).

2.6 Objectives

The aim of this thesis (detailed in Chapter 1.13) is to compare the biocatalytic systems of isolated enzyme and whole cell oxidoreductases for the bioreduction of a poorly soluble substrate. The main objectives of the thesis are as follows:

1) To investigate the isolation and purification of tetralone reductase from the cells of *Trichosporon capitatum* (MY 1890) using chromatographic methods to purify the enzyme, and to characterize the isolated enzyme to gain a greater understanding of its instability.

2) To investigate the immobilization of tetralone reductase, then to develop an enzyme regenerating system for the bioreduction of 6-bromo-β-tetralone using FDH as the regenerating enzyme and formate as the regenerating substrate. Finally, to develop a bioreactor configuration for the bioreduction with cofactor regeneration.

3) To investigate the potential of the whole cell as a biocatalyst for the bioreduction of 6-bromo-β-tetralone. The whole cell system will be characterised including an investigation of the cell substrate and solvent tolerance, the potential of cell recycling will also be examined.

4) To compare the isolated enzyme system and the whole cell system for the bioreduction of 6-bromo-β-tetralone. This will be done using comparisons of productivity and conversion, substrate and solvent tolerance, and reaction methodologies. The potential of each system will also be predicted for genetic modification and overexpression of the enzyme.

2.7 Analytical Techniques

2.7.1 Errors

To calculate the error in experimental results the standard deviation of triplicate results will be used:

$$\sigma = \sqrt{\frac{\Sigma\left(x-\bar{x}\right)^2}{n-1}}$$

Using the standard deviation should give an accurate measurement of the experimental errors which could result from equipment error, measurement of materials, timings during kinetic assays and human error.

2.7.2 HPLC

High performance liquid chromatography (HPLC) was used to determine the concentrations of 6-bromo-β-tetralone and 6-bromo-β-tetralol. Two systems were used, a Gilson Liquid Handler (Gilson, Middletown, WI, USA) and a Dionex DX500 chromatography system (Camberley, Surrey, UK). Both methods used a Zorbax reverse phase RX-C8 25×4.5 column (MacMod analytical, Chadds Ford, PA, USA) with an isocratic elution at 1.5ml/min over 10min using a mobile phase of 50% (v/v) 0.1% phosphoric acid in H_2O and 50% (v/v) acetonitrile. Tetralone and tetralol eluted at 4.7 min and 5.6 min respectively.

2.7.3 Spectrophotometric Measurement of NADH

Reduced nicotinamide adenine dinucleotide (NADH) can be detected using a UV spectrophotometer at 340nm. Enzyme activity was assessed by following the reaction at this wavelength. Tetralone reductase consumes NADH to give NAD, and so the decrease in optical density at 340nm gives an accurate measurement of the activity of tetralone reductase. Optical density is related to the concentration of NADH using the following equation:

$$A = l \times \varepsilon \times c$$

where:

A	Adsorbance
l	Optical Path length
ε	Molar Absorption coefficient (NADH 630 L/mol/mm)
c	Concentration of cofactor

and activity was measured from:

$$U = \frac{\Delta c}{t}$$

where:

U	Activity of enzyme ($\mu mol.ml^{-1}.min^{-1}$)
Δc	Change in cofactor concentration ($\mu mol.ml^{-1}$)
t	time (min)

95% of a 0.5g/L solution of NADH in buffer was added to 5% of a 10g/L tetralone in ethanol. 1ml of this reaction mixture was added to 1ml of protein to be assayed and the reaction was followed on the spectrophotometer.

Justification of the Spectrophotometric Assay

The number of moles NADH oxidised in the spectrophotometer should be equal to the number of moles of 6-bromo-β-tetralone reduced to 6-bromo-β-tetralol within the limits of error. This statement is only true if the only active NADH is the exogenous NADH added to the assay mixture. This hypothesis was proved correct as the calculated number of moles of 6-bromo-β-tetralol produced by the spectrophotometer assay was within the standard deviation of the theoretical number of moles of NADH consumed. Therefore, the spectrophotometer assay could be used as an accurate indicator of enzyme activity.

2.7.4 Protein Concentration Assay

Protein concentration was measured using an adapted Bradford assay (Bradford, 1976). 2.9ml of a 1 part Coomassie brilliant blue G-250 dye (Bio-Rad Laboratories Ltd, Hemel Hampstead, Herts, UK) diluted in 4 parts pure water was added to 100µl of protein solution in a 3ml cuvette. The solutions were left for between 5-30min, and then measured at 595nm. A calibration curve was used to determine the protein quantities (Appendix A1.3).

2.7.5 Micro-Litre NADH Assay

Round flat bottom 96-well plates (Nalge Nunc International, Rochester, NY, USA) were used for many of the screening experiments explained in subsequent chapters. The method was the same as for the larger scale experiment except smaller quantities were used. A calibration curve was used to determine the actual quantities of NADH rather than the adsorbance equation because the path length was difficult to measure (Appendix A1.4). 95% of a 0.5g/L NADH in buffer was added to 5% of a 10g/L tetralone in ethanol. 100µl of this mixture was added to 100µl of tetralone reductase solution, and the reaction was followed at 340nm.

2.7.6 Micro-Litre Protein Concentration Assay

The protein content was assayed as described for the 3ml scale (Chapter 2.7.4). All assay components were scaled down to 300µl.

2.7.7 Control Experiments

Control experiments were run alongside enzyme activity experiments. The control assay mix consisted of a 95% 10mM Tris buffer added to a 5% of a 10g/L solution of 6-bromo-β-tetralone in ethanol. These solutions were prepared immediately before use to avoid precipitation. The final concentrations in the control assay mix were 0.5g/L 6-bromo-β-tetralone and 0g/L NADH. 1ml of this solution was added to 1ml of the enzyme solution. The activity of the enzymes was followed

spectrophotometrically at 340nm (as described in section 2.7.3). For the micro-scale assay, the same method was used except 100µl of reaction mix was added to 100µl of enzyme solution.

2.8 Summary

In this chapter, the model system was examined with respect to: the media selection and growth of the biocatalyst, previous characterisation studies, regeneration systems, and the application of novel enzymes and substrates. The analytical techniques used in the experimental chapters were also described in detail. The model reaction is the biocatalytic reduction of 6-bromo-β-tetralone to 6-bromo-β-tetralol with cofactor regeneration. The model biocatalyst is tetralone reductase which requires NADH to complete the bioreduction; NADH can be regenerated as part of whole cell metabolism or *ex vivo* using a secondary isolated enzyme. The product from the bioreduction is a pharmaceutically important precursor which cannot be prepared asymmetrically via chemical synthesis. The substrate and product are novel compounds which are both highly insoluble in aqueous environments. The isolated enzyme and whole cell biocatalytic systems both mirror a number of issues associated with the production of novel asymmetric intermediates, including low substrate and product solubility, enzyme instability, and have potential cofactor and enzyme regeneration issues. The substrate and product can be measured via HPLC analysis and the respective enzyme activity can be measured using a kinetic NADH spectrophotometer method. The analytical methods can be completed at microlitre scale or millilitre scale.

The objectives of the research are to (1) isolate, purify and stabilise the active oxidoreductase, (2) develop the isolated enzyme regenerating system and use this system in a bioreactor, (3) investigate the potential of the whole cell for the bioreduction, and (4) compare the isolated enzyme system to the whole cell system. In the following chapter, the isolation, purification and characterization of tetralone reductase will be investigated.

Isolation, Purification and Characterisation of an Oxidoreductase from *Trichosporon capitatum* (MY 1890)

3.1 Introduction

Yeasts are a rich source of proteins, particularly enzymes, and these proteins can be isolated and purified for various applications (Levison, 2001). The purification of a novel protein from the other cell proteins has a number of consequences; firstly, competing enzymes and proteolytic enzymes are removed (Harris, 1989). Secondly, more knowledge of the enzyme can be obtained by determining the crystal structure and the sequence of genes. Lastly, after sequencing, the enzyme can be cloned and overexpressed into a well-characterised microorganism. This can make it easier to obtain the enzyme at a lower cost. The sequencing of the genes also gives rise to the potential for genetic modification. To effectively purify an enzyme, the cell debris is normally removed and then the isolated enzyme is passed through a number of packed bed chromatography systems. These chromatography systems may involve ion exchange, hydrophobic interaction, affinity, size exclusion, thiophilic interaction or chiral columns; or the protein of interest may be purified through precipitation (for example ammonium sulphate) or filtration devices (Levison, 2001). Precautions must also be made to minimize proteolysis by the addition of protease inhibitors. Other considerations include minimizing the loss of activity through the adsorption of the enzyme to surfaces, and through instabilities caused by high water activities, low enzyme concentrations, oxidizing environments and metal ions (Harris, 1989). It is also important to determine the most favourable operating conditions of the reaction components in the bioreactor. This can be done at small scale or microscale using variables such as pH, temperature, alternative solvents, and other specific additives; thus, gathering essential information about the type of enzyme, stability properties, and the active site conformation. The results from the

characterisation experiments can then be applied to assess the controls required to maintain enzyme activity and stability in a bioreactor.

The main objectives of this chapter are to isolate and purify active tetralone reductase from Trichosporon capitatum (MY1890) (since previous attempts to purify tetralone reductase (Thomas, 2000) were ineffective at producing large quantities of active tetralone reductase). Tetralone reductase is maintained inside the cell membrane; therefore the isolation steps will include a homogenisation step to break open the cells and a centrifugation stage to remove the cell debris. Chromatography columns will then be employed to purify the active enzyme from the other cell proteins. The other objective of this chapter is to characterise tetralone reductase, this is essential since currently tetralone reductase has a ½ life of 7hr at 22^0C Characterisation experiments are required to define the most effective operating conditions for the active enzyme. Tetralone reductase will be characterised using parameters such as temperature, pH and additives. There will also be an investigation to try to understand the instability of the enzyme and to try to increase the stability to enable the enzyme to operate in an environment for much longer than 7hrs, thus increasing the value of the enzyme (and potentially decreasing process costs).

3.2 Materials and Methods

3.2.1 Isolation of Tetralone Reductase

Cells of *Trichosporon capitatum* (MY 1890) were washed by centrifugation (Beckman Coulter™, Fullerton, CA, USA) using a JA-10 rotor at 17000 × g and resuspended in the same volume of 10mM Tris buffer pH7 containing protease inhibitor Complete™ (Roche, Basel, Switzerland). The centrifugation and resuspension was repeated, removing most of the growth media. The washed cells were homogenised using either a microfluidizer M-100EH (Microfluidics Corporation, Newton, MA, USA) with 3 passes at 15000psi, or an APV Lab 60

homogeniser (APV Systems, Crawley, W.Sussex) with 5 passes at 500bar. The resultant broken cells were centrifuged using a JA-17 at 39000 × g at 4^0C for 30min. The active tetralone reductase was found in the supernatant (the pellet was discarded).

3.2.2 Q Sepharose Fast Flow Column Chromatography

The clarified homogenate was loaded on to an XK 50 column (Amersham Biosciences, Inc., Piscataway, NJ, USA) with Q Sepharose fast flow (900ml) at a flow rate of 15ml/min. 500ml of the protein could be applied before breakthrough. The column was washed with 10mM Tris buffer containing protease inhibitor until all unbound protein was eluted. A gradient elution from 0%-100% NaCl 10mM Tris buffer containing protease inhibitor at 15ml/min, over a volume of 1500ml, using a Biopilot FPLC system (Amersham Biosciences, Inc., Piscataway, NJ, USA) was employed. 12ml fractions were collected in a Pharmacia LKB FRAC 100 fraction collector (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The active fractions (62-78) were pooled and incubated at 4^0C. All of the purification steps were carried out at 4^0C. A second method was also used, employing a step elution whereby every 12min a change in concentration of 0M, 0.1M, 0.2M, 0.3M, and 0.4M of NaCl was applied. The column was regenerated by flushing it through with 0.5 column volumes of 1M NaCl, the column was re-equilibrated with 1 column volume of 10mM Tris buffer pH7. The isolated enzyme was stored in 20% glycerol at -20^0C.

3.2.3 Protocol Modification

The isolation (Chapter 3.2.1) and purification (Chapter 3.2.1) were repeated using EDTA free Complete™ protease inhibitor instead of Complete™ protease inhibitor, and also by using no protease inhibitor. After the Q Sepharose fast flow column, the three types of active tetralone reductase were incubated at 22^0C, 100µl samples were

removed at various time points and assayed for remaining enzyme activity using the 96-well plate assay.

3.2.4 Further Purification

A number of purification resins were screened for their affinity to tetralone reductase. C1535 Cibacron 3GA-Agarose, R2507 Reactive Blue 4-Agarose, R2632 Reactive Blue 72-Agarose, R2757 Reactive Brown 10-Agarose, R2257 Reactive Green 5-Agarose, R2882 Reactive Green 19-Agarose, R0503 reactive Red 120-Agarose, R3757 Reactive Yellow 3-Agarose, R2382 Reactive Yellow 86-Agarose; from a test kit (Sigma, St. Louis, MO, USA). Amicon Red A and Phenyl Sepharose affinity resins were also tested (Sigma, St. Louis, MO, USA). Elution buffers used were NaCl and NAD(H) (Sigma, St. Louis, MO, USA).

3.2.5 Hydroxyapatite Column Chromatography

100ml of hydroxyapatite HA Ultrogel, (Sigma, St. Louis, MO, USA) was loaded on to an XK column (Amersham Biosciences, Inc., Piscataway, NJ, USA) and equilibrated with H_2O. The protein was eluted using a step gradient of 0.1M, 0.2M, 0.3M K_3PO_4. The column was run at 3.5ml/min and 12 × 1min fractions were collected. At the final concentration, samples were collected until all the protein had eluted.

3.2.6 Toyopearl Column Chromatography

20ml of Toyopearl resin was loaded onto an XK column (Amersham Biosciences, Inc., Piscataway, NJ, USA). The column was equilibrated with 1M NH_3SO_4 (10mM Tris pH7). Protein was eluted with 6 × 1min fractions at 0.8M, 0.6M, 0.4M, 0.2M and a final 11 samples were collected at 0M of NH_3SO_4.

3.2.7 SDS-PAGE of the Protein Purification

A 10% BIS-TRIS gel (NOVEX, San Diego, CA, 92121, USA), and NuPAGE™ running buffer 20X were used to run the samples. 50ml of the running buffer was mixed with 950ml of water. Samples were prepared using 10µl of enzyme, 65µl of H_2O and 25µl of LDS 4X (Novex, CA, USA). Bio-Rad low molecular weight standards were used to determine the molecular weights. Samples were run through the gel for 40mins. The gels were stained for 20min with Coomassie Brilliant Blue R-250 (Bio-Rad laboratories, Hercules, CA).

3.2.8 pH Profile

10ml solutions of tetralone reductase were dialysed in tubing of molecular weight cut-off of 12,000-14000, 2.4nm pore size (BDH, Poole, Dorset, UK), against 1 litre of 10mM Tris buffer at pH values of 5, 6, 7, 8, 9 at 4^0C for 12 hours. The final pH of the dialysed enzymes was measured to confirm dialysis and the activity of the enzymes was determined using the spectrophotometer assay (Chapter 2.7.3).

3.2.9 Temperature Stability

10ml solutions of tetralone reductase purified using the new protocol, were incubated at 4^0C and 22^0C. 100µl samples were extracted at defined time points and used in the 96-well spectrophotometer assay to determine the remaining activity of the enzyme.

3.2.10 Activity of Tetralone Reductase with a Number of Additives

A metal cation NaCl, $MgCl_2$, $CaCl_2$, $MnCl_2$, $FeCl_3$, $CoCl_2$, $CuSO_4$, or $ZnSO_4$ was dissolved separately in tetralone reductase to a concentration of 5mM and incubated at 22^0C for 15min, a control was incubated under the same conditions with no metal cation addition. The enzyme was also tested for activity against 5mM phenylglyoxal (Sigma, Poole, Dorset, UK), 1mM ρ-chloromercuribenzoate (ρ-CMB) (Sigma, Poole, Dorset, UK) and 1mM EDTA (Sigma, Poole, Dorset, UK).

3.2.11 Inhibition of Tetralone Reductase by NADH

NADH was dissolved in the tetralone reductase solutions to the concentrations of 0.2g/L, 0.4g/L, 0.6g/L, 0.8g/L, and 1g/L. The activity was measured using the spectrophotometer assay mix (Chapter 2.7.3) with no further addition of NADH.

3.2.12 Solvent Screen for Dissolution of 6-bromo-β-tetralone

10g/L solutions of 6-bromo-β-tetralone in a number of different types of solvents (Sigma, St Louis, MO, USA) were prepared. These solutions were used as the 5% solvent required in the 96-well plate assay, and assayed for enzyme activity (Chapter 2.7.3).

3.2.13 Solubility of 6-bromo-β-tetralone

6-bromo-β-tetralone was assessed for visual solubility in ethanol and methoxyethanol. 5mg samples were added to 1ml of each solvent in a glass vial. The solutions were then vortexed to mix. Additional 5mg samples were added until a tetralone residue could be seen, this was taken to be the solubility point (+/- 5mg/ml).

3.2.14 Tetralone Reductase Activity in Ethanol and Methoxyethanol

The percentage of ethanol and methoxyethanol in the spectrophotometer assay was changed from 0%, 2%, 4.5%, to 9% (v/v). The solvent was added to the cuvette followed by 1ml of tetralone reductase and then 1ml of the spectrophotometer assay. The reaction was monitored kinetically using the spectrophotometer assay (Chapter 2.7.3).

3.3 Results

3.3.1 Isolation of Tetralone Reductase

Tetralone reductase was isolated from cells of *Trichosporon capitatum* (MY 1890) (Figure 3.1) by removing the growth media and cell debris. Centrifugation was used

to pellet the cells to allow the growth media to be decanted. Homogenisation was used to break open the cells and release the intracellular protein of interest. Centrifugation was then used to pellet the cell debris so that the protein solution could be decanted. The clarified homogenate was analysed for kinetic activity. The results show that tetralone reductase has an activity of 23U/g with a total cell protein concentration of 4.4g/L. The clarified homogenate was then applied to a chromatography column (Q Sepharose Fast Flow) for purfication of tetralone reductase from the other bulk intracellular proteins.

3.3.2 Anion Exchange Chromatography

Tetralone reductase was purified to remove the bulk impurities by using the anion exchange resin Q-Sepharose Fast Flow (Figure 3.2). Ion exchange chromatography separates molecules based on the number of positive or negative charges accessible on their surfaces. Ion exchange chromatography is widely used in the separation of proteins because the relatively mild binding conditions and elution conditions allow high protein recovery with intact biological activity. The binding mechanism is based on the ionic attraction between molecules of opposite electric charge. The functional groups of the anion exchange resin have a positive charge which separates negatively charged molecules. Ion exchange binding occurs when the salt concentration (or ionic strength) of the mobile phase is reduced to the point that the ionic groups on the sample molecules begin to serve as the counter ions for the charged groups on the stationary phase. Tetralone reductase was applied to the anion exchange column in 50mM Tris, this allowed the negatively charged surface ions to bind to the positively charged anion exchange resin. Elution takes place when the ionic strength of the mobile phase is increased; as this happens, salt molecules displace the bound sample molecules back into the mobile phase. Tetralone reductase elutes from the anion exchange resin at a salt concentration of 0.4M NaCl. This binding and elution of tetralone reductase onto an anion exchange resin demonstrates that tetralone reductase has an accessible surface which is

negatively charged. The kinetic activity of pooled tetralone reductase is 54U/g with a total cell protein concentration of 1.1g/L.

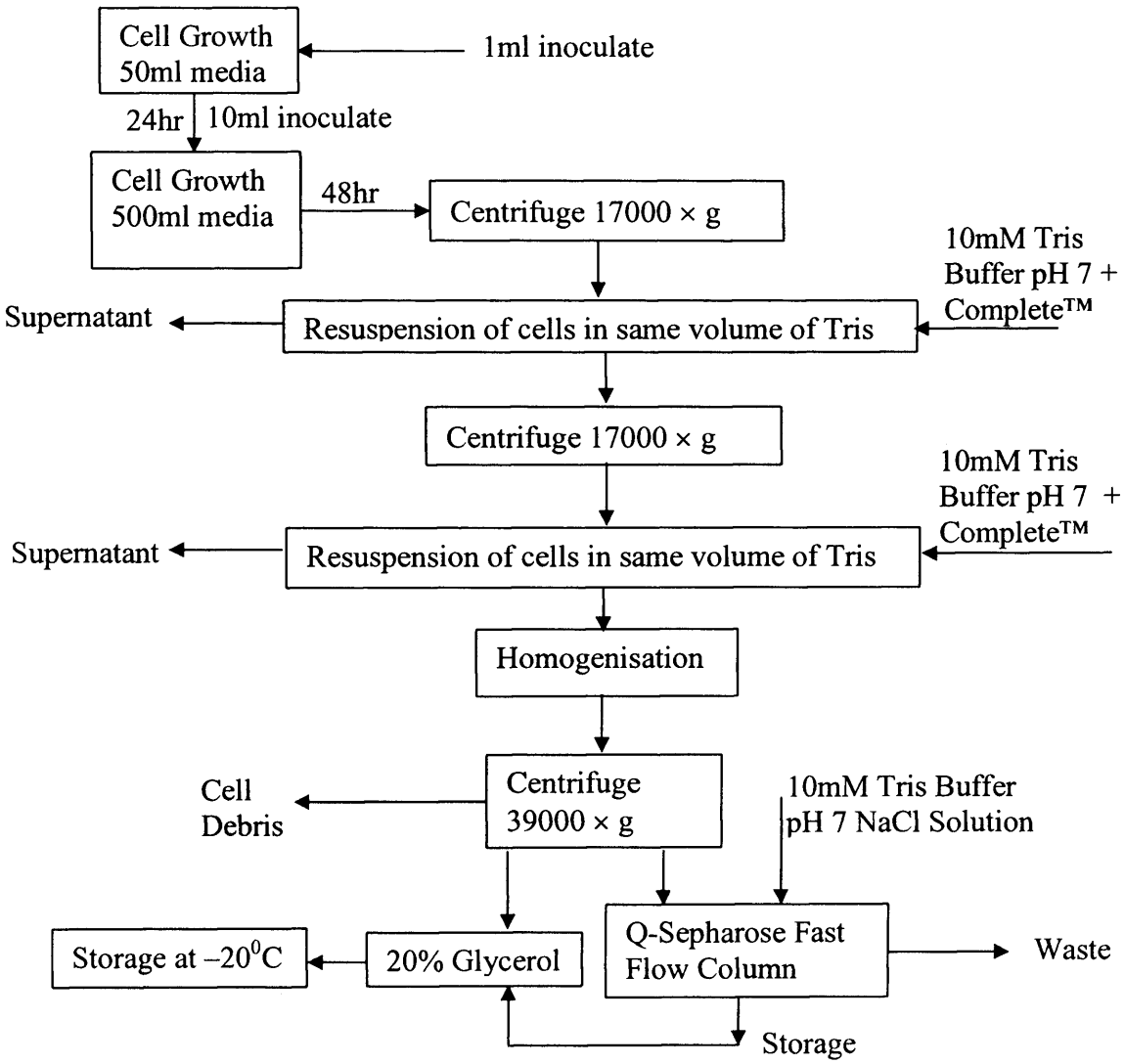


Figure 3.1 Isolation and Purification Methodology

The cells of Trichosporon capitatum (MY 1890) were centrifuged and resuspended twice in the same volume of buffer to remove the cell growth media. The resuspended cells were then homogenised to break open the cells and then centrifuged to pellet out the cell debris from the protein of interest. The supernatant from this was either stored in 20% glycerol at –20^{0}C or passed through a Sepharose Fast Flow anion exchange column then stored in 20% (v/v) glycerol at –20^{0}C.

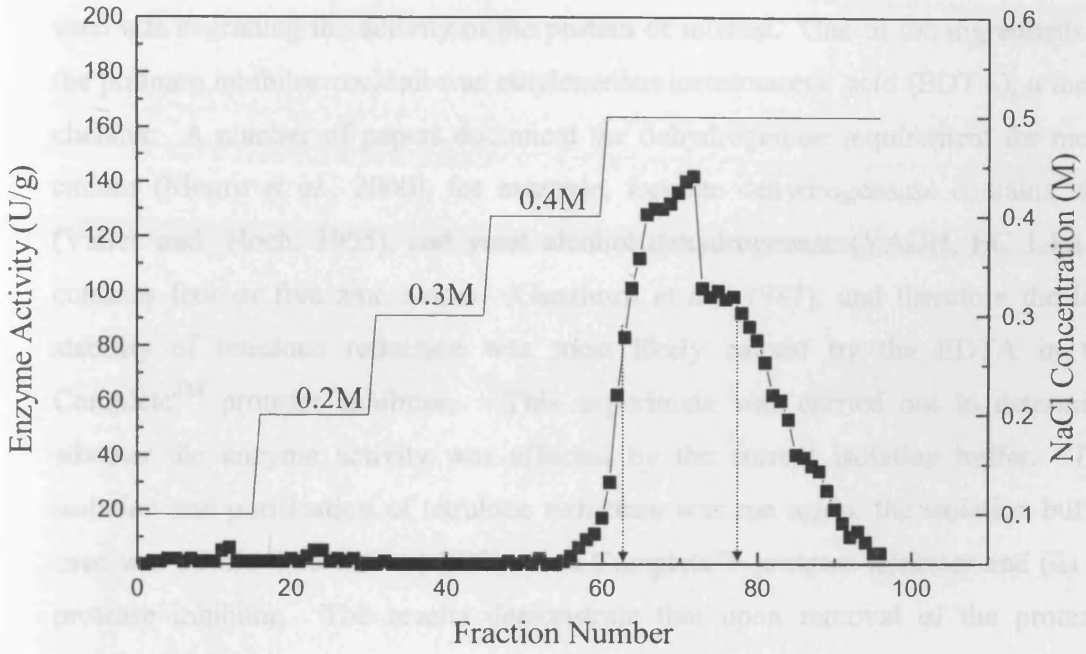


Figure 3.2 Purification of Tetralone Reductase using Q Sepharose Fast Flow Resin

The elution of the enzyme occurs due to the step change of NaCl over time (___) at 15ml/min. 12ml fractions were collected and the active protein (■) eluted between the fractions 62 and 78. The concentration of NaCl is measured going into the column. The activity is measured coming out of the column by assaying each of the fractions for kinetic activity.

3.3.3 Protocol Modification

The half-life of tetralone reductase after the isolation and purification from the Fast Flow Q Sepharose anion exchange column was 7hr at 22^0C (Figure 3.3). The initial procedure for the isolation used a 50mM Tris buffer containing a cocktail of protease inhibitors (Thomas, 2000), this buffer was used in the resuspension of the cell pellet after centrifugation, the buffer was also used as a mobile phase in the anion exchange chromatography. The cocktail of protease inhibitors was used to

prevent unnecessary degradation of the protein of interest via proteolysis. However, upon further investigation it was hypothesized that the cocktail of protease inhibitors used was degrading the activity of the protein of interest. One of the ingredients of the protease inhibitor cocktail was ethylenediaminetetraacetic acid (EDTA), a metal chelator. A number of papers document the dehydrogenase requirement for metal cations (Munro *et al.*, 2000), for example, formate dehydrogensase contains zinc (Vallee and Hoch, 1955), and yeast alcohol dehydrogenase (YADH, EC 1.1.1.1) contains four or five zinc atoms (Ganzhorn *et al.*, 1987); and therefore the low stability of tetralone reductase was most likely caused by the EDTA in the Complete™ protease inhibitor. This experiment was carried out to determine whether the enzyme activity was affected by the current isolation buffer. The isolation and purification of tetralone reductase was run again; the isolation buffer used was 50mM Tris with (i) EDTA free Complete™ protease inhibitor and (ii) no protease inhibitor. The results demonstrate that upon removal of the protease inhibitor from the purification buffer the half-life of tetralone reductase increases from 7hr to 92hr (Figure 3.4) at 22^{0}C. The addition of EDTA-free Complete™ protease inhibitor instead of the Complete™ protease inhibitor also decreases the initial activity of the enzyme, but the decrease is not as apparent as when Complete™ inhibitor is used. There is also an initial sharp drop in tetralone reductase activity in the buffer containing EDTA-free protease inhibitor, however, over the time course tetralone reductase activity begins to parallel the activity of tetralone reductase in just 50mM Tris buffer. It is unknown why this is the case, it may have been due to another ingredient of the protease inhibitor which may have caused a degradation to both tetralone reductase and the proteolytic enzymes, thus, although there was an initial sharp drop in tetralone reductase activity, the activity was maintained over time as the inactivated proteolytic enzymes could not cause further degradation of tetralone reductase. Tetralone reductase is most stable when the Complete™ and EDTA-free Complete™ protease inhibitors are removed from the buffers. The half-life of tetralone reductase when employing Complete™ protease inhibitor in the buffers is 7hr at 22^{0}C and 85hr when EDTA-free

Complete^{TM} is employed. The half-life of tetralone reductase in a 50mM Tris buffer without protease inhibitors is 92hr. The increase in tetralone reductase stability from just under 7hr to 92hr means that tetralone reductase can be used in further reactions including immobilisation and applied biocatalysis.

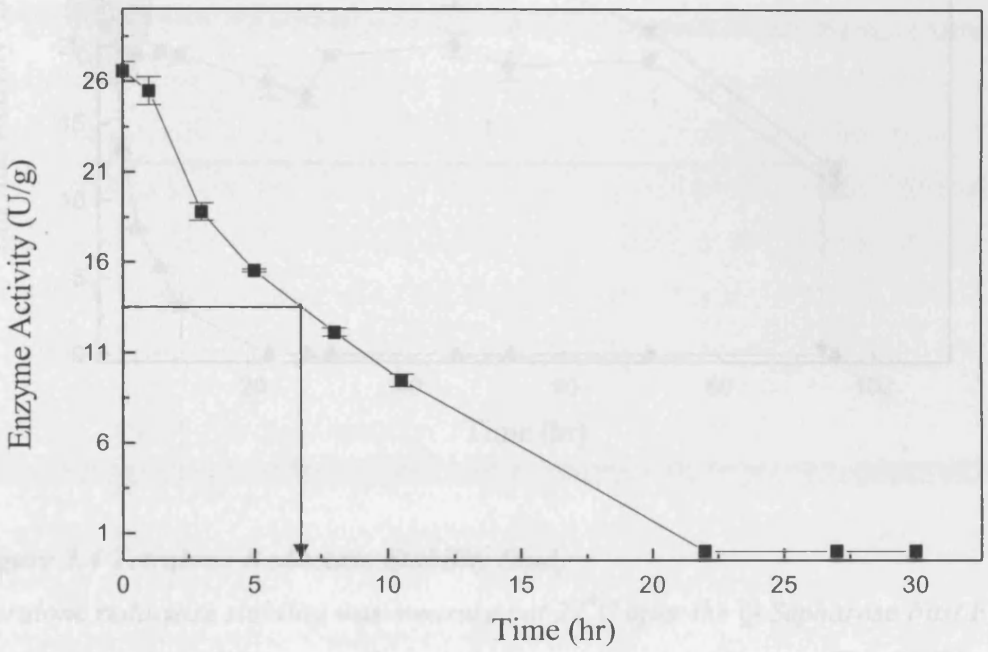


Figure 3.3 Enzyme Stability

This graph illustrates the stability of tetralone reductase stored at 22^{0}C after elution from the Q Sepharose Fast Flow anion exchange chromatography column. The stability of tetralone reductase was measured by assaying for kinetic activity at regular time points. The half-life of tetralone reductase is calculated using the graph and drawing a vertical line where the activity has decreased by half. The half-life of tetralone reductase using the graph is 7hr at 22^{0}C. Error bars were calculated as the standard deviations of three separate samples.

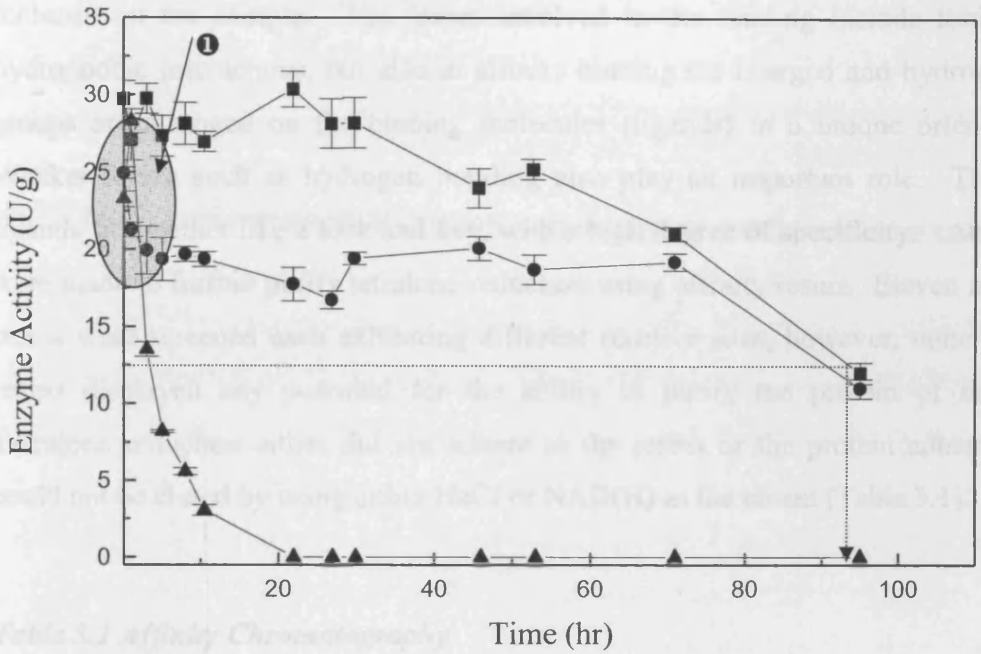


Figure 3.4 Tetralone Reductase Stability Study

Tetralone reductase stability was measured at 22^{0}C after the Q Sepharose Fast Flow chromatography column, purified with no protease inhibitor (■), EDTA free Complete™ protease inhibitor (●), and Complete™ protease inhibitor (▲). Error bars were calculated as standard deviations of three separate samples. The graph illustrates the increase in stability from a 7hr half-life to a 92hr half-life by removal of the protease inhibitor from the elution buffer. The graph also shows the rapid initial decrease in tetralone reductase activity (❶), in the first few hours, after the addition of EDTA-free Complete™ protease inhibitor.

3.3.4 Affinity Chromatography

The anion exchange chromatography step was used as a capture step to remove the bulk contaminants from the tetralone reductase solution. To further purify tetralone reductase a number of affinity resins were screened for tetralone reductase binding

and elution. Affinity chromatography is based on bio-specific binding interactions between a ligand chemically bound to the chromatography packing and a target molecule in the sample. The forces involved in the binding include ionic and hydrophobic interactions, but also in affinity binding the charged and hydrophobic groups are arranged on the binding molecules (ligands) in a unique orientation. Weaker forces such as hydrogen bonding also play an important role. The two ligands fit together like a lock and key, with a high degree of specificity. Attempts were made to further purify tetralone reductase using affinity resins. Eleven affinity resins were screened each exhibiting different reactive sites, however, none of the resins displayed any potential for the ability to purify the protein of interest. Tetralone reductase either did not adhere to the resins or the protein adhered but could not be eluted by using either NaCl or NAD(H) as the eluant (Table 3.1).

Table 3.1 Affinity Chromatography

Table 3.1 shows the affinity resins which were screened for the purification of tetralone reductase. Tetralone reductase either bound to the affinity resin (✓), but could not be eluted with either NaCl or NADH in the elution buffer; or flowed through the affinity column without binding (×).

	Binding	Comments
C1535 Cibacron 3GA-Agarose	×	
R2507 Reactive Blue 4-Agarose	×	
R2632 Reactive Blue 72-Agarose	×	
R2757 Reactive Brown 10-Agarose	×	
R2257 Reactive Green 5-Agarose	✓	No elution
R2882 Reactive Green 19-Agarose	✓	No elution
R0503 Reactive Red 120-Agarose	✓	No elution
R3757 Reactive Yellow 3-Agarose	×	
R2382 Reactive Yellow 86-Agarose	×	
Amicon Red A	✓	No elution
Phenyl Sepharose	✓	No elution

3.3.5 Pseudo Affinity Chromatography

A further protein purification stage was carried out to increase the purity of the enzyme solution. Hydroxyapatite is a pseudo affinity resin and works similarly to other affinity resins. The binding properties of tetralone reductase to this resin are unknown, however, hydroxyapatite has been previously used to purify enzymes and could be used for the purification of tetralone reductase (Figure 3.5). The enzyme was applied to the column in the same buffer as it had left the Fast Flow Q Sepharose column (50mM Tris, 0.4M NaCl). This ability to put the protein of interest from one column straight onto the next column is extremely useful as there is no requirement to add another stage between the columns such as diafiltration to ensure the protein is in the correct loading buffer. This reduced time in processing, by putting the enzyme from one column straight onto another chromatography column, means that there is less time for degradation of the enzyme. The enzyme was eluted using a step change of K_3PO_4, the results show that tetralone reductase elutes between the 0.2M-0.3M step change. A number of peaks were observed during the elution of tetralone reductase, these peaks may have been other reductases isolated from *Trichosporon capitatum* (MY 1890). It would not be uncharacteristic of a yeast cell to have a number of reductases (Levison, 2001) which could potentially also perform the bioreduction. The main peak was pooled giving a specific activity of 0.145U/mg and a concentration of 0.63g/L.

3.3.6 Anion Exchange Chromatography

Tetralone reductase after the hydroxyapatite column was still not yet fully purified (reference SDS-PAGE Figure 3.7), therefore, another purification stage was required. Toyopearl is an anion exchange resin where the recommended elution buffer is a decreasing ion buffer. Tetralone reductase was washed off the previous column (hydroxyapatite) in 0.2-0.3M K_3PO_4 and this buffer was used to bind tetralone reductase. The column was washed in purified water and tetralone reductase was eluted using a decreasing step gradient starting from 1M NH_3SO_4. Tetralone reductase elutes from the column at a 0.4M concentration of NH_3SO_4

(Figure 3.6), the pooled enzyme has a specific activity of 0.189U/mg and a concentration of 0.34g/L.

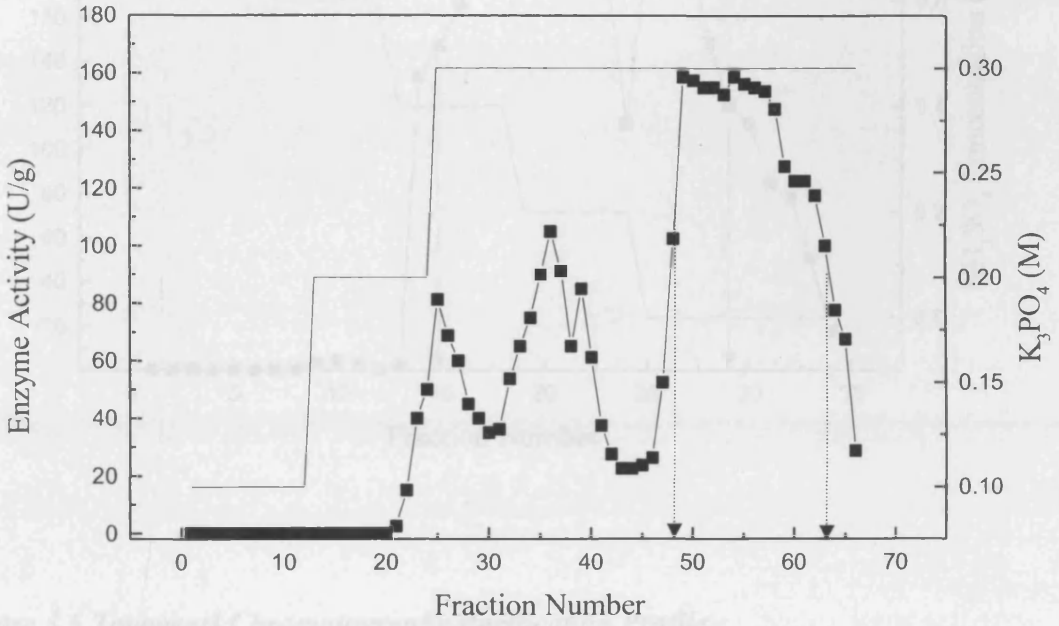


Figure 3.5 Hydroxyapatite Chromatography Purification Profile

Purification of tetralone reductase using a hydroxyapatite pseudo-affinity chromatography column. The elution of the enzyme occurs due to the step change of K_3PO_4 over time (___), elution of the active protein (■) occurs between the fractions 48 and 63. The concentration of K_3PO_4 is measured going into the column. The amount of tetralone reductase eluted is measured coming out of the column by assaying each of the fractions for kinetic activity.

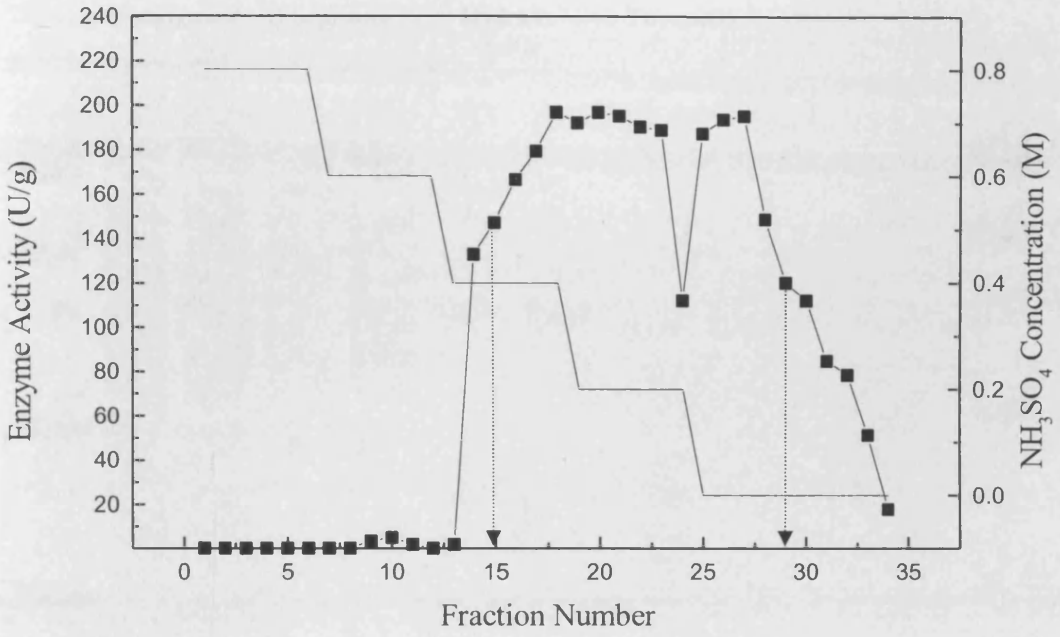


Figure 3.6 Toyopearl Chromatography Purification Profile

Purification of tetralone reductase with toyopearl 650M resin. The elution of the enzyme occurs due to the step change of NH_3SO_4 over time (____), elution of the active protein (■) occurs between the fractions 15 and 28. The concentration of NH_3SO_4 is measured going into the column. The amount of tetralone reductase eluted is measured coming out of the column by assaying each of the fractions for kinetic activity.

3.3.7 SDS-PAGE

An SDS-PAGE gel was run to determine the purity of the protein of interest (Figure 3.7). Samples of the protein solution were run on the gel to show the purification of tetralone reductase after each step. Molecular markers were run each side of the gel

to indicate the size of the protein molecule. A band is visible at about 43kDa in the protein solution after the Toyopearl resin purification stage, this is consistent with a number of dehydrogenases (Munro, 2000).

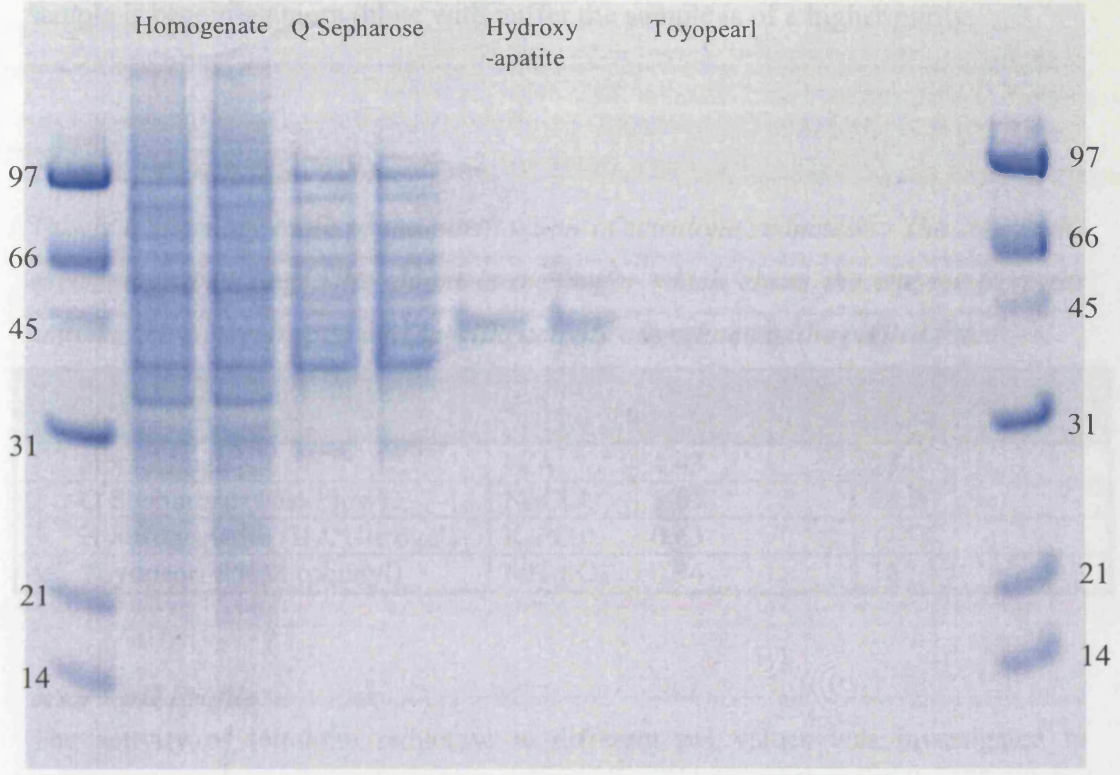


Figure 3.7 SDS-PAGE of Tetralone Reductase and Impurities during Purification
This SDS-PAGE gel illustrates the purity of the protein solution during the purification of tetralone reductase. The samples are: crude homogenate from Trichosporon capitatum (MY1890) after homogenisation and centrifugation, the protein solution after purification through the Q Sepharose fast flow anion exchange chromatography column, the protein solution after purification through the hydroxyapatite pseudo-affinity chromatography column, and the protein solution after purification via the toyopearl anion exchange chromatography column.

3.3.8 Purification Table

This table (Table 3.2) displays the results for the three purification steps. The table illustrates that as the amount of purification increases the concentration of the

protein decreases. This is an expected result because as more buffer is applied to the system less enzyme is pooled from the fractions. The specific activity of the enzyme increases with each purification step, likewise, this is expected because although the sample is becoming more dilute with buffer the sample is of a higher purity.

Table 3.2 Purification Table

This is a summary table of the purification of tetralone reductase. The step is the chromatography stage, the eluant is the buffer which elutes the enzyme from the column, the concentration and specific activity are related to the pooled fractions.

	Step	Eluant	Concentration (g/L)	Specific Activity (U/g)
1	Homogenate	N/A	4.43	23.0
2	Q Sepharose (Fast Flow)	NaCl	1.05	54.0
3	Hydroxyapatite (HA Ultrogel)	K_3PO_4	0.63	145.0
4	Toyopearl 650M (phenyl)	NH_3SO_4	0.34	189.0

3.3.9 pH Profile

The activity of tetralone reductase at different pH values was investigated to determine the optimal pH conditions for the enzyme. Tetralone reductase exhibits highest activity at the slightly acidic conditions of between pH 6.5 and 7 after a 12hr dialysis at 4^0C (Figure 3.8). A sharp drop-off in tetralone reductase activity can be observed at acidic pH values of less than 6 and at alkali conditions of pH values of greater than 7.5. This gives a relatively small operating range for any biocatalytic reactions.

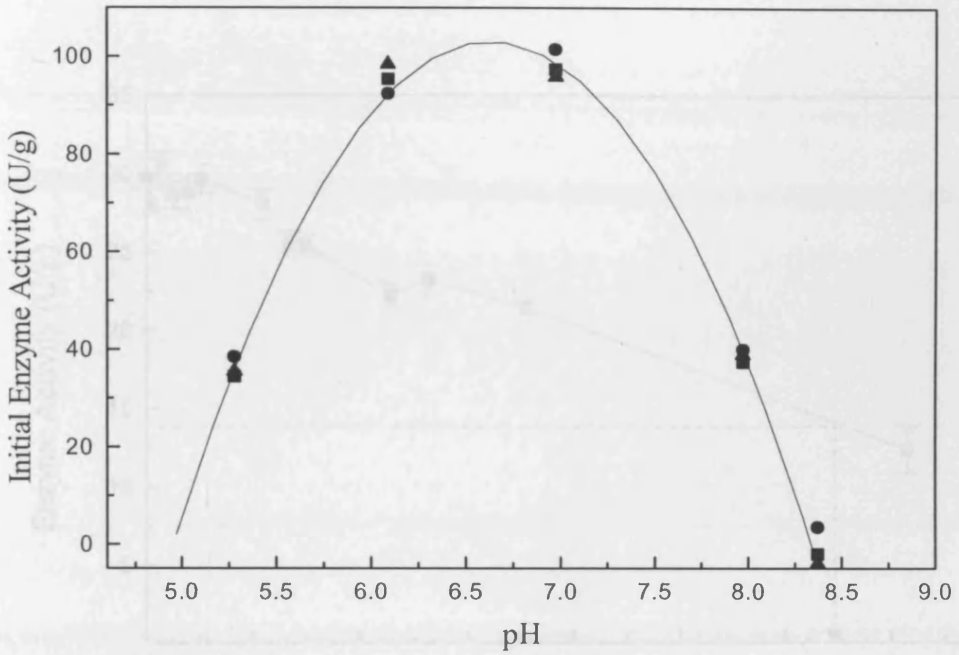


Figure 3.8 Tetralone Reductase pH Profile

The graph shows the pH profile for tetralone reductase activity after a 12hr dialysis at 4^0C. It can be observed that the highest enzyme activity is between pH 6.5 and pH 7.0. The enzyme rapidly loses activity at extremes of pH; at pH values of greater than 8.5 and lower than 5.0 all tetralone reductase activity is lost.

3.3.10 Temperature Stability

The stability of tetralone reductase purified from the Q Sepharose Fast Flow anion exchange column was investigated at temperatures of 22^0C and 4^0C to determine potential bioreactor operating conditions. The protein solution was incubated at the defined temperatures and assayed kinetically for activity. Tetralone reductase exhibits good stability at both the temperatures investigated, possessing a half-life of 130hr at 4^0C and a half-life of 92hr at 22^0C (Figure 3.9, 3.10). This means that there is a lot of potential to use tetralone reductase in a bioreactor.

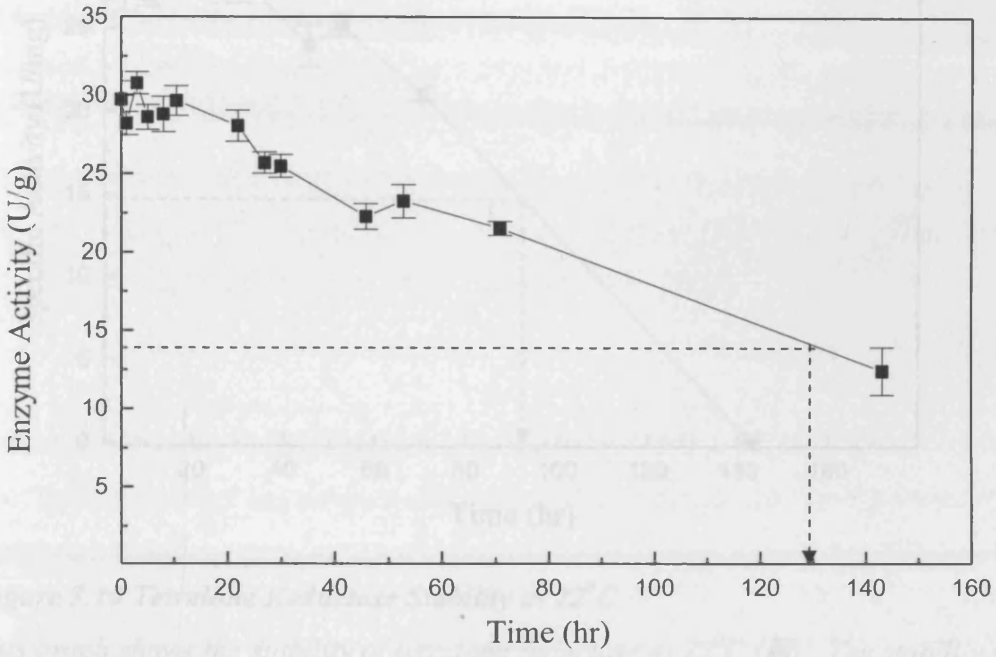


Figure 3.9 Tetralone Reductase Stability at 4^0C

This graph shows the stability of tetralone reductase stability at 4^0C (■). The stability was measured by assaying for the kinetic activity of tetralone reductase at regular time points. Error bars were calculated as standard deviations of three sample reactions. This graph illustrates the high stability of tetralone reductase at 4^0C in a 50mM Tris buffer preparation with no other additives. There is a linear decrease and a half-life of 130hr.

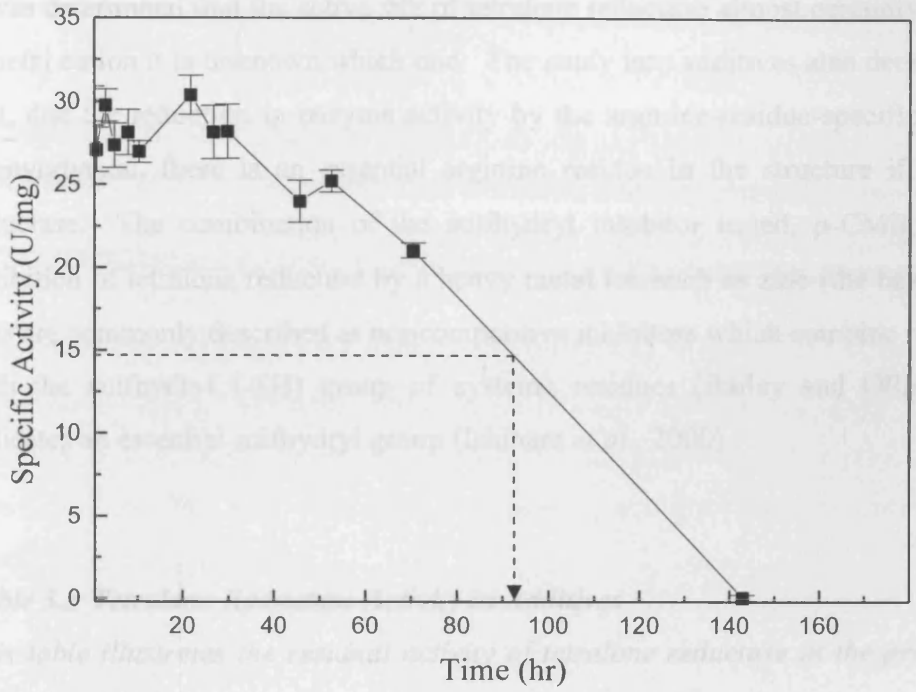


Figure 3.10 Tetralone Reductase Stability at 22^{0}C

This graph shows the stability of tetralone reductase at 22^{0}C (■). The stability was measured by assaying for the kinetic activity of tetralone reductase at regular time points. Error bars were calculated as standard deviations of three sample reactions. This graph illustrates the high stability of tetralone reductase at 22^{0}C in 50mM Tris with no other additives. The half-life is 92hr.

3.3.11 Activity of Tetralone Reductase with a Number of Additives

Tetralone reductase activity was measured against a number of additives (Table 3.3) to increase the understanding of the enzyme behaviour. The metal chelating reagent EDTA severely decreases tetralone reductase activity; similarly, the heavier cations also severely decrease enzyme activity. However, lighter cations such as sodium and calcium slightly increase tetralone reductase activity. It is hypothesized from this information that the enzyme has a metal cation in its active site, however it is very likely that the enzyme has a high enough affinity for the metal cation so

additional quantities of the cation in the reaction buffers are not required. Although it was determined that the active site of tetralone reductase almost certainly requires a metal cation it is unknown which one. The study into additives also demonstrates that, due the reduction in enzyme activity by the arginine-residue-specific reagent, phenylglyoxal, there is an essential arginine residue in the structure if tetralone reductase. The combination of the sulfhydryl inhibitor tested, ρ-CMB, and the inhibition of tetralone reductase by a heavy metal ion such as zinc (the heavy metal ions are commonly described as noncompetitive inhibitors which combine reversibly with the sulfhydryl (-SH) group of cysteine residues (Bailey and Ollis, 1986)) indicates an essential sulfhydryl group (Ishihara *et al.*, 2000).

Table 3.3 Tetralone Reductase Activity in Additives

This table illustrates the residual activity of tetralone reductase in the presence of additives at 5mM.

Additive	Molarity	Relative Activity (%)	Comment
None		100[a]	
NaCl	5mM	104	Alkali
$MgCl_2$	5mM	108	Alkaline earth metal
$CaCl_2$	5mM	106	Alkaline earth metal
$MnCl_2$	5mM	103	
$FeCl_3$	5mM	5.3	Transition metal cation
$CoCl_2$	5mM	34.4	Transition metal cation
$CuSO_4$	5mM	47.1	Transition metal cation
$ZnSO_4$	5mM	13.9	Transition metal cation
Phenylglyoxal	5mM	84	Essential Arginine residue
ρ-CMB	1mM	36	Essential Sulphydryl group
EDTA	1mM	56	Metal Chelator

[a] *The activity in absence of additive was determined by arbitrarily setting to 100. Activity measurements were taken immediately after the additive was added.*

3.3.12 Inhibition of Tetralone Reductase by NADH

Tetralone reductase requires NADH as a cofactor (Thomas, 2000), an experiment was executed to determine at which concentrations NADH becomes inhibitory and also to find out at which concentration of NADH tetralone reductase has the highest activity (Figure 3.11). Results show that tetralone reductase has the highest activity at 0.4g/L of NADH but loses all activity by 1g/L. These levels of NADH give a good indication of the levels which can be used in a bioreactor before enzyme inactivation occurs. At the low levels of NADH the low enzyme activity is likely to be because there is not enough cofactor to fill the available active sites, at higher levels of NADH, the reduction in enzyme activity may be attributed to product/substrate inhibition.

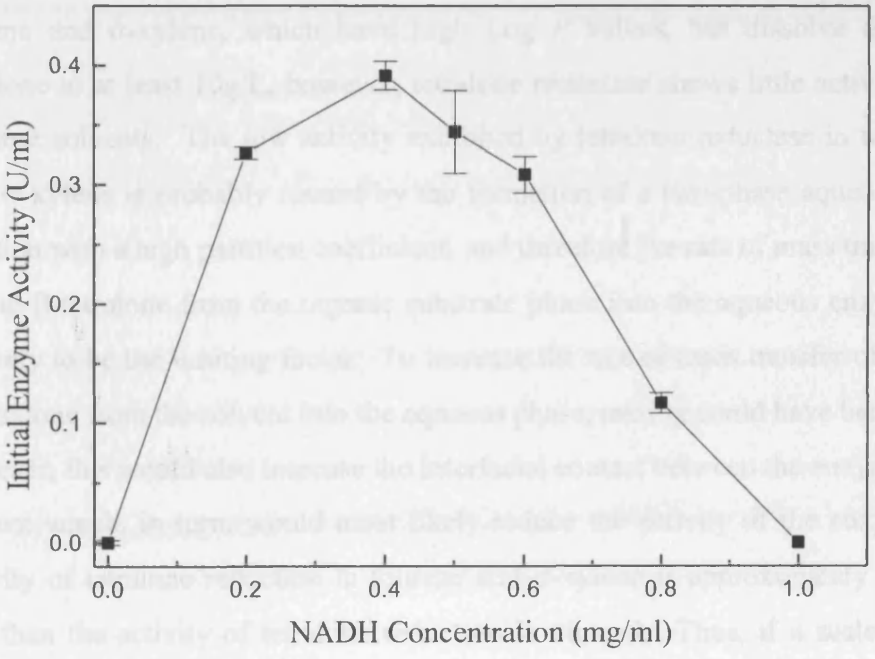


Figure 3.11 The Effect of NADH on Tetralone Reductase Activity

Tetralone reductase activity in NADH (■). The error bars were calculated as standard deviations of three reactions. This graph illustrates how tetralone

reductase is affected by the addition of NADH, there is a small operating ranging of between 0.2 and 0.6mg/ml within which tetralone reductase can operate.

3.3.13 Solvent Screen for the Dissolution of 6-bromo-β-tetralone

6-bromo-β-tetralone is insoluble in water above 0.5g/L. It cannot be added as a solid to the reaction mixture as the rate of dissolution is too slow for an effective bioreduction. Solvents were screened using Log *P* as a basis for selection to find a solvent which could improve the current assay by increasing the substrate solubility whilst maintaining tetralone reductase activity. The solvent selection was determined by assessing the application, the solubility and the enzyme activity. This was completed by dissolving 6-bromo-β-tetralone in the solvents to a concentration of 10g/L and performing the kinetic assay to determine enzyme activity. The results show that (Table 3.4), in general, solvents with higher Log *P* values have a low solubility of 6-bromo-β-tetralone (less than 10g/L). The exceptions to this rule are toluene and σ-xylene, which have high Log *P* values, but dissolve 6-bromo-β-tetralone to at least 10g/L, however, tetralone reductase shows little activity in both of these solvents. The low activity exhibited by tetralone reductase in toluene and also σ-xylene is probably caused by the formation of a two-phase aqueous-organic solution with a high partition coefficient, and therefore the rate of mass transfer of 6-bromo-β-tetralone from the organic substrate phase into the aqueous enzyme phase is likely to be the limiting factor. To increase the rate of mass transfer of 6-bromo-β-tetralone from the solvent into the aqueous phase, mixing could have been applied, however, this would also increase the interfacial contact between the enzyme and the solvent which, in turn, would most likely reduce the activity of the enzyme. The activity of tetralone reductase in toluene and σ-xylene is approximately five times less than the activity of tetralone reductase in ethanol. Thus, if a scaled reaction took 5hr to complete using ethanol as the solvent to solubilise 6-bromo-β-tetralone, it is likely that it would take 25hr to complete using either toluene or σ-xylene as the solvent (using the assumption that mass transfer in not increased, for example, via

mixing). In general, solvents with low Log *P* values dissolve 6-bromo-β-tetralone to concentrations of 10g/L or higher. Tetralone reductase activity tends to increase with solvents of decreasing Log *P* values (Figure 3.12). The two ketones which were analysed, 4-methyl-2-pentanone and 3-pentanone both display high activity on the spectrophotometer assay. However, on analysis of the HPLC chromatograms it is possible to see that that 4-methyl-2-pentanone and 3-pentanone are themselves reduced not 6-bromo-β-tetralone, demonstrating that tetralone reductase has a higher affinity for the straight chain ketones than 6-bromo-β-tetralone. It is not unusual for dehydrogenases to have multiple substrate specificity (Faber 1997) and open chain methyl- and ethyl ketones are readily reduced by dehydrogenases such as ADH from *Thermoanaerobium brockii* (TBADH) (Faber 1997), however, more complicated structures are not. The ketones screened were derivatives of pentanone, a straight chain ketone and, although this reduction of the ketones was not useful for the application of a solvent to dissolve 6-bromo-β-tetralone, it does demonstrate the catalytic power of tetralone reductase. Amines cannot be used as they react into an unknown black solution. Ethanol and methoxyethanol were found to be the most compatible solvents as they retain a higher level of tetralone reductase activity than the other compatible solvents.

3.3.14 Solubility of 6-bromo-β-tetralone

Ethanol has previously been used to solubilise 6-bromo-β-tetralone for the kinetic assay to determine enzyme activity, however, ethanol only solubilises 6-bromo-β-tetralone to a concentration of 10g/L. In the previous screening experiment, tetralone reductase had shown comparable activity in methanol, acetonitrile and methoxyethanol, and therefore 6-bromo-β-tetralone was examined for its solubility in these solvents. The results show that at 22^0C 6-bromo-β-tetralone has a solubility of 0.5g/L in H_2O, 10g/L in ethanol, 30g/L in methanol, 120g/L in acetonitrile and 250g/L in methoxyethanol (Table 3.5). A comparison of the solubilities against the Log *P* values shows that the solubility of 6-bromo-β-tetralone is not completely

dependant on the Log *P* values. Methanol has the lowest Log *P* value of –0.76 and 6-bromo-β-tetralone is soluble in methanol to a concentration of 30g/L, yet methoxyethanol has a Log *P* value of –0.50 and a solubility of 6-bromo-β-tetralone of 250g/L. This may mean that the solubility of 6-bromo-β-tetralone in a solvent may be dependent on other criteria such as the structure of the solvent. Methoxyethanol was selected as the solvent to be used in further experiments because it dissolves 6-bromo-β-tetralone to a solubility of 250g/L, a factor a 25 times more than ethanol.

Table 3.4 The Effects of Solvents on Tetralone Reductase Activity

Solvent	Initial Activity (U/mg)	Comments
4-methyl-2-pentanone	0.23	The high activity displayed was not concurrent with the HPLC assay
3-Pentanone	0.18	

Solvent	Initial Activity (U/mg)	Comments	Log *P* values
2,2,4 – trimethylpentane	0.08	Partially Soluble	No data
1,2-Propandiol	0.09	Partially Soluble	No data
n-Dodecane	0.06	Partially Soluble	No data
n-Tetradecane	0.06	Partially Soluble	No data
Ethylene Glycol	0.17	Partially Soluble	No data
1-octanol	0.04	Partially Soluble	2.72
Cyclohexane	0.09	Partially Soluble	3.4
Hexanes	0.09	Partially Soluble	3.9
n-heptane	0.08	Partially Soluble	4.66

Solvent	Initial Activity (U/mg)	Comments	Log *P* values
Ethanolamine	-0.04	Reacted to give a black solution	No data

Solvent	Initial Activity (U/mg)	Comments	Log *P* values
N,N-Diethyl Formamide	0.04	None	No data
Pyridine	0.03	None	0.65
Toluene	0.05	None	2.69
σ-xylene	0.05	None	3.12
2-propanol	0.10	None	0.05
Ethyl Acetate	0.11	None	0.73
N,N – Dimethyl Formamide	0.11	None	-0.87

Solvent	Initial Activity (U/mg)	Comments	Log *P* values
Dichloromethane	0.15	None	1.25
DMSO	0.18	None	-1.35
Methanol	0.19	None	-0.76
Acetonitrile	0.19	None	-0.3
2-Methoxyethanol	0.20	None	-0.50
Ethanol	0.21	None	-0.32

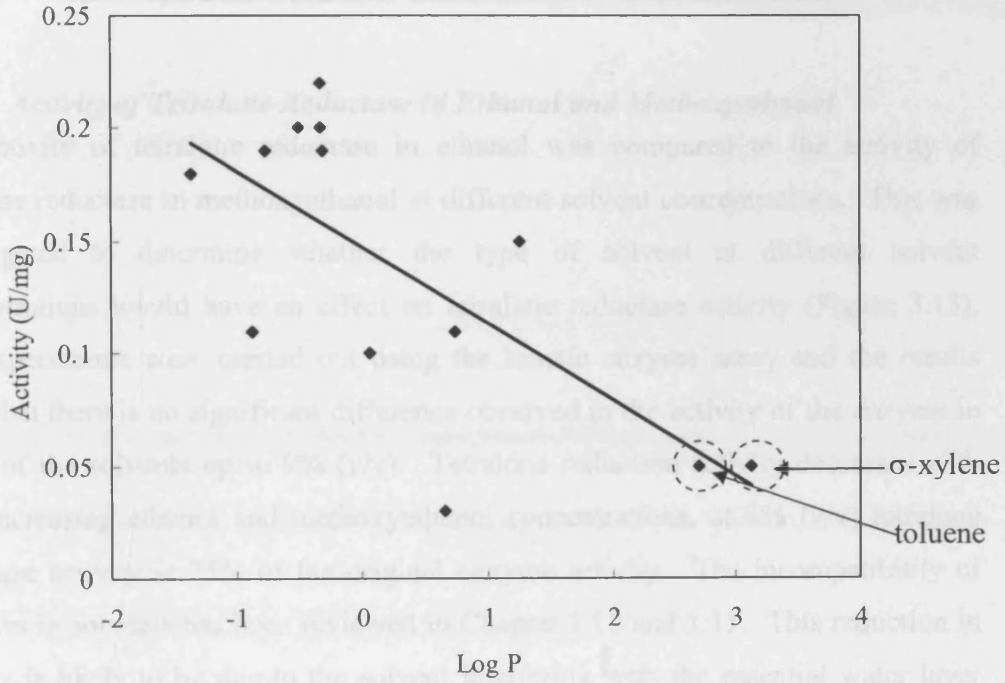


Figure 3.12 The Effect of Log P on Tetralone Reductase Activity

This graph illustrates the decrease in enzyme activity with increasing Log P values (The logarithm of the partition coefficient of a given compound in the standard n-octanol/water two-phase system). (The solvents which did not dissolve 6-bromo-β-tetralone fully to 10g/L are not illustrated on this graph).

Table 3.5 6-bromo-β-tetralone Solubility

Solubility of 6-bromo-β-tetralone in 100% solvent at 22^{0}C.

Solvent	Solubility (g/L)	Log *P*
Water	0.5	
Ethanol	10	-0.32
Methanol	30	-0.76
Acetonitrile	120	-0.30
Methoxyethanol	250	-0.50

3.3.15 Activity of Tetralone Reductase in Ethanol and Methoxyethanol

The activity of tetralone reductase in ethanol was compared to the activity of tetralone reductase in methoxyethanol at different solvent concentrations. This was investigated to determine whether the type of solvent at different solvent concentrations would have an effect on tetralone reductase activity (Figure 3.13). The experiments were carried out using the kinetic enzyme assay and the results show that there is no significant difference observed in the activity of the enzyme in either of the solvents up to 9% (v/v). Tetralone reductase activity decreases with both increasing ethanol and methoxyethanol concentrations, at 9% (v/v) tetralone reductase activity is 75% of the original enzyme activity. The incompatibility of enzymes in solvents has been reviewed in Chapter 1.10 and 1.11. This reduction in activity is likely to be due to the solvent interfering with the essential water layer surrounding the enzyme (Leön *et al.*, 1998). Ethanol and methoxyethanol both have the same detrimental effect on the enzyme activity, it also seems that the availability of the substrate to the enzyme is sufficient in both ethanol and methoxyethanol for the reactions to be similar. This, however, may be due to the low concentration of enzyme in the reaction, a higher concentration of enzyme in the reaction may change the reaction rates. Methoxyethanol will be used in further experiments, this is because although tetralone reductase shows comparable activity in ethanol the solubility of 6-bromo-β-tetralone in methoxyethanol is 25 times greater. Thus, inactivation of tetralone reductase can be minimized by reducing the amount of solvent required for the addition of the substrate.

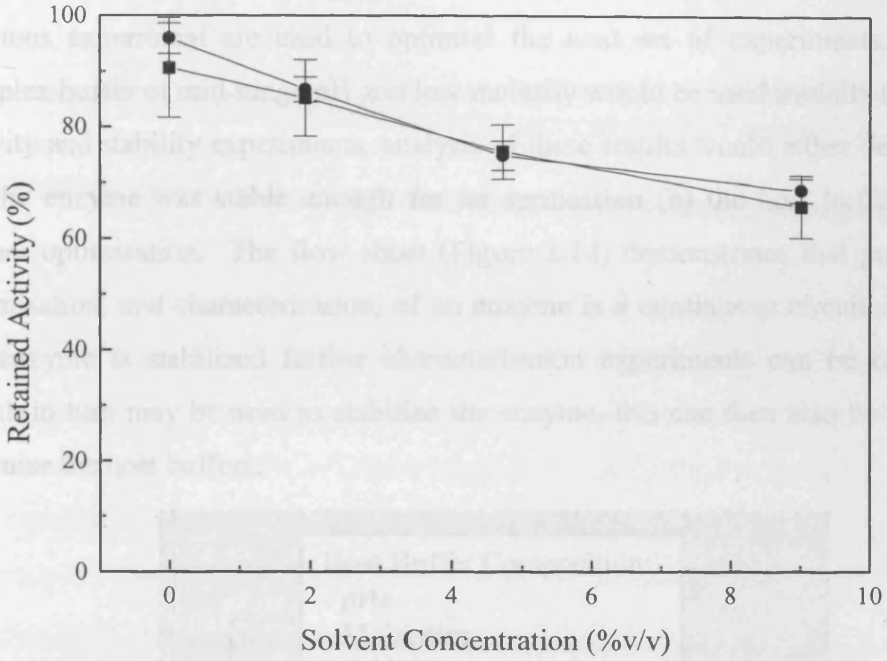


Figure 3.13 Effect of Ethanol and Methoxyethanol on Tetralone Reductase Activity

Tetralone reductase activity retained in ethanol (■) and methoxyethanol (●). Error bars were calculated as standard deviations of three samples.

3.4 Discussion

Enzymes isolated from their native cell environments immediately become subject to many inactivating conditions. The active site of an enzyme is very reactive and is therefore susceptible to inactivation by a number of denaturants. Extremes of pH, temperature, and organic solvents are examples of denaturants, and careful consideration of the host buffer is essential to minimize loss of activity and stability of the required enzyme (Harris, 1986). In the purification of tetralone reductase, a pre-defined method was employed in which the host buffer had not been optimized, in this instance, the stability of tetralone reductase was measured as 7hr at 22^0C.

The stability of tetralone reductase was increased to 92hr at 22^0C by optimising the host buffer. One method to find the optimal host buffer conditions for enzyme activity and stability is to employ an iterative procedure, where the results of the previous experiment are used to optimise the next set of experiments. A non-complex buffer of mid-range pH and low molarity would be used initially to perform activity and stability experiments, analysis of these results would either demonstrate (a) the enzyme was stable enough for its application (b) the host buffer requires further optimisation. The flow sheet (Figure 3.14) demonstrates that purification, optimisation, and characterisation, of an enzyme is a continuous circuit, in that, as the enzyme is stabilized further characterisation experiments can be carried out which in turn may be used to stabilize the enzyme, this can then also be applied to optimise the host buffers.

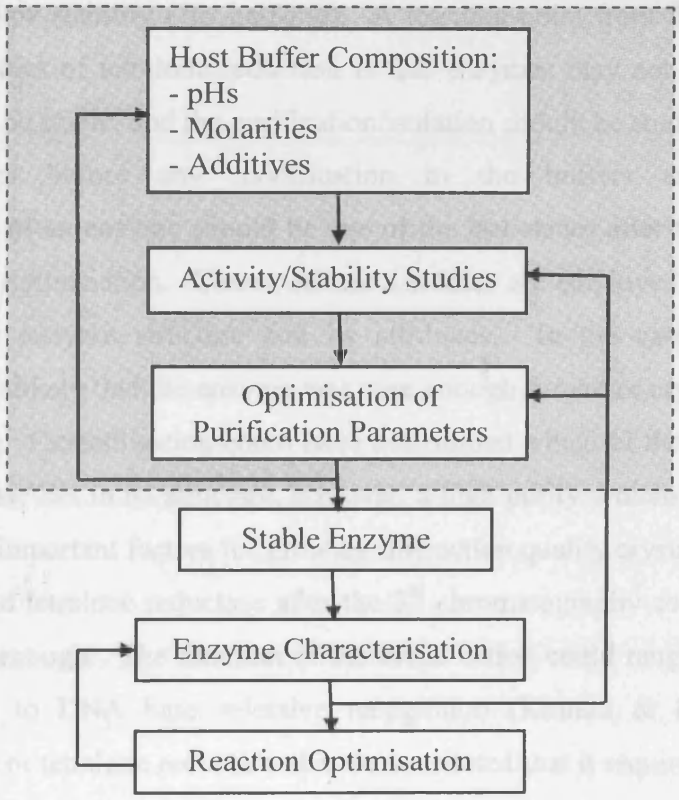


Figure 3.14 Proposed Procedure for Enzyme Purification

A simple buffer of low molarity would be employed for the purification. Activity and stability studies can then be employed (e.g pH range, additives, and temperature) to

optimise the host buffer. A number of iterations of the first three stages can be performed until a stable enzyme is established. The more iterations of the first part (shaded in grey) the more stable the enzyme should be for the purification and ultimately should remain stable for further use. The stable enzyme can then be characterised which may lead to further optimisation, or the characterisation results could be used to optimise the reaction.

In the purification of tetralone reductase it became evident through characterization studies that the host buffer had not been optimised. If the host buffer had been optimised initially using simple non-complex buffers (low molarity, mid-range pH, no additives) and then appropriate additives added after an iterative cycle of optimisation (instead of before) this would most likely have removed the problem encountered of low stability (7hr half-life). A learning point from the purification and stability studies of tetralone reductase is that enzymes may not need complex environments to be stable, and the purification/isolation should be studied using non-complex buffers before any modification to the buffers are employed. Characterisation of an enzyme should be one of the last stages after the host buffer and purification optimisation. Characterisation studies are employed to understand more about the enzyme structure and its attributes. In the case of tetralone reductase, it is unlikely that the enzyme was pure enough for either crystallization or gene sequencing. Crystallisation could have determined which of the metal cations tetralone reductase has in its structure, however, a high purity protein preparation is one of the most important factors for growing diffraction quality crystals (Wood and Coker, 2001) and tetralone reductase after the 3rd chromatography column may not have been pure enough. The function of the metal cation could range from simple biological roles to DNA base selective recognition (Kimura & Kituta, 2000). Characterisation of tetralone reductase also demonstrated that it requires NADH as a cofactor; however, tetralone reductase is also inhibited at high concentrations of NADH. Initially, it was presumed that the reason cofactors need to be recycled was due to their high cost (Chenault *et al.*, 1986); however, the inhibition of tetralone

reductase at low levels of NADH suggests that it is more important to recycle cofactors to prevent enzyme inactivation than it is to maintain a low cost process. If the cofactor is not recycled then it would need to be controlled and monitored in the reaction. However, as the cofactor is not used up in the reaction and just altered into its oxidised form, this would cause a build up of the oxidised form and may cause enzyme inhibition via the oxidised cofactor. A cofactor regeneration system, therefore, has a number of positive attributes over the stoichiometric use of cofactors:

- Removes the problems of inhibition by the cofactor by ensuring that only quantities of cofactor which maintain enzyme activity are employed.
- Reduces cost through regeneration of the cofactor (Chenault *et al.*, 1987, 1988).
- More easily controlled reactors, as cofactor levels will not need monitoring throughout the reaction. The reaction can be monitored via analysis of the substrate and/ or product.

The delivery of cofactors to the reaction mix is not seen to be a problem due to their high solubility in aqueous solutions. In contrast, the delivery of the substrate, particularly for substrates which exhibit a low solubility in aqueous environments, is currently a limiting factor in the efficient use of isolated oxidoreductases. In the bioreduction of 6-bromo-β-tetralone, the substrate is added to the reaction dissolved in a solvent; this method of substrate delivery is effective, in that, if the substrate was added as a solid to the aqueous enzyme phase the rate of dissolution would be very slow and the biocatalytic reaction times would be extensive. Selection of an effective method of substrate delivery to the reaction solution is critical in the maintenance of the enzyme activity and stability. Log *P* values have previously been described as the main criterion for selecting solvents for biocatalysis (Laane *et al.*, 1986). However, subsequent to these findings, there have been papers which suggest that Log *P* values alone are not an appropriate guide for selecting solvents, and there are a number of other parameters which need to be considered for the choice of solvent particularly for NAD(P)(H) requiring enzymes (Filho *et al.*, 2003),

this suggestion is also consistent with the results from this chapter. It might be more appropriate to consider the functionality of the solvent alongside the Log *P* values. The solvents tested for tetralone reductase compatibility were screened on a variety of Log *P* values, this gave an initial method for selection, the results show that, in general, the more negative the Log *P* value the higher the enzyme activity. This is likely to be associated with the amount of substrate available to the enzyme, this is because as the Log *P* value is increased the partition of the solvent from the aqueous solution is also increased, thus, decreasing the availability of the substrate to the enzyme. Tetralone reductase exhibits high activity in solvents which have negative Log *P* values, thus, confirming that a higher log *P* value decreases the availability of the substrate to the enzyme. Retention of enzyme activity is of paramount importance in obtaining a functional enzyme for biocatalytic reactions. It has been reported that activity retention increases with increasing Log *P* values in a sigmoidal-shaped correlation (Laane *et al.*, 1986) (Figure 3.15). This theory contrasts the method by which the solvent selection can be made, for example, for good substrate-enzyme interaction, a solvent with a low Log *P* value is required; however, for high activity retention, a solvent with a high Log *P* value, is essential (Figure 3.15).

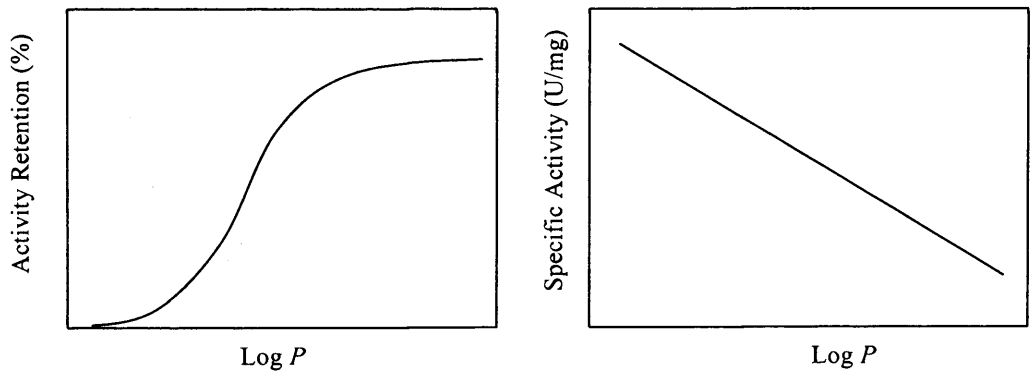


Figure 3.15 Activity Retention and Specific Activity versus Log *P*
Enzyme activity retention increases with increasing Log P values and it is possible to plot retention of enzyme activity against Log P using a sigmoidal-shaped graph. This graph is adapted from Laane et al., 1986, where a number of enzymatic systems

were found to have similar correlations. Tetralone reductase specific activity decreases with increasing Log P values, figure adapted from Figure 3.12.

Results also demonstrate that the solubility of 6-bromo-β-tetralone is not completely dependent on Log *P* values, on analysis of 6-bromo-β-tetralone solubility it was found to have a solubility of 250g/L in methoxyethanol (Log *P* = -0.50) compared to 10g/L in ethanol (Log *P* = -0.32). However, in methanol, which has a Log *P* value of -0.76, the solubility of 6-bromo-β-tetralone is only 30g/L. This result is consistent with the theory that the choice of solvent for cofactor requiring enzymes should be made on the combination of a number of parameters:

- Log *P*.
- Chemical name (e.g. alcohol, amine) - The chemical name is useful when selecting a system for substrate delivery as the choice may be incompatible with the substrate, product, or even the enzyme. For example, in the bioreduction of 6-bromo-tetralone, the amines reacted into an unknown compound (detected via a colour change), and the ketones reacted with the enzyme and were reduced to their respective alcohols.
- Molecular properties (e.g. presence of heteroatoms and residues, molecular geometry, electronic density distribution and frontier orbitals, surface tension and solvation energy (Filho *et al.*, 2003)
- Solubility of the substrate as it is necessary to ensure that there is an adequate amount of substrate available for a reaction.

The selected method of substrate delivery for a biocatalytic reaction must ensure a high enzyme activity and significant retention of that activity over the course of the reaction(s); it must also ensure a good turnover of substrate to product.

3.5 Summary

Tetralone reductase can be isolated from the cells of *Trichosporon capitatum* (MY 1890) by centrifugation and homogenisation, and purified through Q Sepharose, Hydroxyapatite, and Toyopearl chromatography columns respectively. Affinity resins were examined for further purification of tetralone reductase but were found to be ineffective due to tetralone reductase either binding to the chromatography resin but not eluting, or not binding at all. The purification protocol was refined and the enzyme stability was increased from just under 7hr to over 92hr at 22^0C. An SDS-PAGE gel of the purification stages illustrates a protein band at approximately 43kDa. Characterisation of tetralone reductase revealed that the enzyme's active site requires a metal cation, however it was concluded that the enzyme has a high enough affinity for the metal cation that additional quantities are not required in the process buffers. Inhibition of tetralone reductase by phenylglyoxal indicates an essential arginine residue; and the combination of the inhibition of tetralone reductase by the sulfhydryl inhibitor tested, ρ-CMB, and a heavy metal ion (such as zinc) indicates an essential sulfhydryl group (Ishihara *et al.*, 2000). Tetralone reductase activity is also decreased by NADH, and therefore it may be easier to maintain the enzyme activity by using NADH in a regeneration system. Tetralone activity was also screened against a number of solvents with varying Log *P* values, the results demonstrated that tetralone reductase exhibits its highest activity in ethanol and methoxyethanol, however, 6-bromo-β-tetralone has a higher solubility in methoxyethanol than ethanol and was selected as the solvent to improve the current assay. In the following chapter, methods to immobilise tetralone reductase will be analysed to enable the separation of the substrates and products from the enzymes in a continuous bioreactor. Immobilisation could also promote tetralone reductase stability in a solvent. Bioreactor conformations and reaction methodologies for the regeneration and retention of the cofactor will also be examined.

An Alternative Bioreactor Configuration for an Isolated Oxidoreductase

4.1 Introduction

Isolated oxidoreductases have found difficulty in being applied industrially; one reason for this is because these enzymes require efficient steps for the regeneration and retention of their expensive cofactors (Chenault *et al.*, 1987, 1988) and the process problems related to the regeneration of these cofactors at industrial scale have not yet been overcome, although there have been a number of recent developments (Huisman *et al.*, 2002) (van der Donk *et al.*, 2003) (Kroutil *et al.*, 2004). The application of isolated oxidoreductases requires consideration of the regeneration of the cofactor and reuse of the enzyme, the retention of the enzyme and cofactor, and the separation of the substrate, by-products and products (which often have similar molecular size to the cofactor and cannot be separated by conventional filtration processes) (Hummel *et al.*, 1989). There are also problems which are generic for many enzymes; for example, maintenance of enzyme activity and stability, substrate and product inhibition, and methods of delivering the substrate into the reaction solution.

A great deal of the research that has been carried out with respect to continuously operating bioreactors for the application of oxidoreductases has concentrated on the use of the enzyme membrane reactor (EMR). By employing a reactor containing a membrane the enzymes can be retained behind the membrane whilst the product can diffuse out (Kragl *et al.*, 1993). Cofactors can be retained alongside the enzymes through a number of methods (these were reviewed in Chapter 1). However, there are no enzyme membrane reactors which have, as yet, been applied at industrial scale. The main problem associated with the EMR is the membrane which can cause problems due to protein fouling (Huisman *et al.*, 2000). There may also be potential retention and separation difficulties (Buckmann *et al.*, 1981). Therefore,

much of the current research in cofactor regeneration has employed the batch reactor. A number of batch reactor systems have been developed for the reduction of poorly soluble ketones with cofactor regeneration via a secondary enzyme system. Reverse micelles have been employed for the reduction of 2-heptanone to *S*-2-heptanol where cofactor regeneration was performed by FDH and formate. It was found that the reaction rate was increased up to 12 times compared to in water (Orlich *et al.*, 1999). It was also suggested that in order to separate and reuse the enzyme and the cofactor, an ultrafiltration system might be employed (Schomacker *et al.*, 1997). Two-phase systems have also been employed in cofactor regeneration reactions (Carrea *et al.*, 1988; Loughlin, 2000). A recent example of a two-phase cofactor recycling system with a ketone as the substrate is the preparative asymmetric reduction of *p*-chloroacetophenone (Gröger *et al.*, 2004). In this work (Gröger *et al.*, 2004), aqueous 5mM *p*-chloroacetophenone was reduced by (*S*)-ADH from *Rhodococcus erythropolis* over-expressed in *E.coli* to its respective product with 97% conversion after a reaction time of 21hours. The cofactor recycling was carried out using FDH in a batch reactor at 30^0C. The main problem with the *p*-chloroacetophenone bioreduction was the low ketone concentration leading to non-satisfactory productivities (Gröger *et al.*, 2004). The addition of solvent in this system (Gröger *et al.*, 2004) permitted an increase of the substrate concentration in the reaction. This increase in solvent concentration was concurrent with a decrease in conversion. A 10mM substrate conversion using a water/*n*-heptane 4:1 (biphasic) reaction system gave a conversion of 69%. This work using *p*-chloroacetophenone with cofactor recycling is similar to the model system in which 6-bromo-β-tetralone also exhibits low aqueous solubility (0.5g/L, 2mM) and tetralone reductase exhibits decreased activity when exposed to a solvent. One difference of the *p*-chloroacetophenone system is that the synthetic enzyme was over-expressed and cloned in to *E.coli*; whereas, in the model system the synthetic enzyme is not over-expressed and is native to *Trichosporon capitatum* (MY 1890).

For many industrial purposes there may be a requirement to immobilise the enzyme; not necessarily to increase stability, but to optimise productivity by using this as a method to reuse/regenerate the enzyme. Immobilisation has the potential to result in an industrially efficient process with a stable, recoverable enzyme. Immobilising the enzyme to a solid phase also means that the downstream purification of the enzyme from the product will not be required. The choice of which bioreactor to employ, whether it is continuous or batch, biphasic, single phase or reverse micelles, and whether immobilised or free enzymes are used will ultimately depend upon the requirements for the reaction and the constraints of the process.

The main objective of this chapter is to develop a reactor system for the enzymatic bioreduction of 6-bromo-β-tetralone. The enzyme system will require the addition of a secondary enzyme and cofactor to allow cofactor regeneration. If a continuous system is employed, it will also require a method of separating the cofactor and the enzyme from the product. Tetralone reductase was previously isolated, purified and characterised (Chapter 3). The results from Chapter 3 show that tetralone reductase is a stable enzyme and can potentially be employed for the bioreduction with high activity. To examine the potential for tetralone reductase to be applied in a reactor the following factors will be investigated: enzyme immobilisation to enable separation of the enzyme from the products, characterization of the enzyme with respect to substrate and solvent tolerance, and characterisation of the reaction components. Lastly, a potential bioreactor configuration will be recommended for the regeneration and recycling of the synthetic and regenerative enzymes and cofactors.

4.2 Materials and Methods

4.2.1 Production of Tetralone Reductase

Tetralone reductase was isolated from *Trichosporon capitatum* (MY 1890) as previously explained (Chapter 3).

4.2.2 Immobilisation of Tetralone Reductase

1g of dry Eupergit C® (Röhm GmbH, Darmstadt, Germany) beads were mixed with 5ml of enzyme solution in 1M potassium phosphate buffer pH 7.5 and left at room temperature for 72hr (as described in the manufacturer's literature).

4.2.3 Resin Screen for NADH Adsorbance

3ml of a 0.5g/L NAD(H) solution dissolved in water was added to 0.2g of resin (XAD 4, XAD 7HP, Dowex Optipore 285, XAD 1180, XAD 2000, L323, XAD 2010, or L493) (Supelco, Poole, Dorset, UK). The solutions were shaken continuously for 30min at 22^0C and then filtered using Puradisc™ syringe filters (Whatman plc, Maidstone, Kent, UK). The remaining NADH was assayed by spectrophotometer at 340nm (Chapter 2.7.3).

4.2.4 Resin Screen for Protein Adsorbance

1g of the XAD resins (XAD 4, XAD 7HP, Dowex Optipore 285, XAD 1180, XAD 2000, L323, XAD 2010, or L493) (Supelco, Poole, Dorset, UK) was added to 3ml of a 0.6g/L tetralone reductase solution. After 30min of continuous shaking at 22^0C, the samples were filtered using Puradisc™ syringe filters to remove the XAD. The filtered samples were then assayed for total protein using the Bradford spectrophotometer assay (Chapter 2.7.5).

4.2.5 Adsorbance of 6-bromo-β-tetralol on XAD L-323

5 × 10ml solutions of H_2O containing 100µl of a 100g/L 6-bromo-β-tetralone solution in methoxyethanol (final concentration 1g/L), were added to, 0.05g, 0.1g, 0.2g, 0.4g, 0.6g of XAD L-323. The samples were left continuously shaking for 30mins. Samples were filtered using Puradisc™ syringe filters and assayed by HPLC (Chapter 2.7.2) for 6-bromo-β-tetralol.

4.2.6 Adsorbance of 6-bromo-β-tetralone on XAD L-323

5 × 10ml solutions of H_2O containing 100µl of a 100g/L 6-bromo-β-tetralol in methoxyethanol (final concentration 1g/L), were added to, 0.05g, 0.1g, 0.2g, 0.4g, 0.6g of L-323. The samples were left continuously shaking for 30mins. Samples were filtered using Puradisc™ syringe filters and assayed by HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone.

4.2.7 Effect of Solvent Type on Rate of Enzyme Reaction

2 × 9ml solutions of tetralone reductase solution were added to 4.5mg formate, 1.43mg NAD, and 10mg FDH. The reaction was started by the addition of either 1ml of a 10g/L 6-bromo-β-tetralone in ethanol solution, or 100µl of a 100g/L 6-bromo-β-tetralone in methoxyethanol solution. The reaction was run for just under 4hr and samples were taken and assayed by HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone and 6-bromo-β-tetralol at various time points.

4.2.8 Effect of the Substrate Concentration on the Enzyme Activity

6 × 10ml solutions of tetralone reductase solution were added to 1.43mg NAD, 4.5mg formate and 10mg FDH. The reactions were initiated by the addition of tetralone dissolved in methoxyethanol. The starting concentrations of 6-bromo-β-tetralone in the reaction mixtures were, 0.5g/L, 1g/L, 1.5g/L, 2g/L, 2.5g/L, 3g/L. (The volume of methoxyethanol was kept constant and the substrate concentration was changed). The reaction was run for 7hr and samples were taken and assayed by HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone and 6-bromo-β-tetralol at various time points.

4.2.9 Effect of Solvent Volume on the Enzyme Activity

5 × 10ml tetralone reductase solutions were added to 1.43mg NAD, 4.5mg formate and 10mg FDH. The reactions were initiated by the addition of 6-bromo-β-tetralone dissolved in methoxyethanol. The volumes of solvent used were 1%, 2%, 3.9%,

7.4%, 10.7% (v/v). (The 6-bromo-β-tetralone concentration was kept constant and the volume of the solvent was changed). The reaction was run for 7hr and samples were taken and assayed by HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone and 6-bromo-β-tetralol at defined time points.

4.2.10 Effect of Formate on the Enzyme Activity

Formate was added to the spectrophotometric assay to give the final concentrations of 0g/L, 0.5g/L, 1g/L, 2g/L, 4g/L, 5g/L in the final reaction mixture. The reaction was initiated by adding 1ml of the assay mix to 1ml of the enzyme and monitored over time using the spectrophotometer assay (Chapter 2.7.3).

4.2.11 Effect of FDH on the Rate of Enzyme Reaction

A reaction mixture of 0.45g/L formate, 2.7ml tetralone reductase, 0.14g/L NAD, and 300µl of 10g/L 6-bromo-β-tetralone in ethanol was used with varying concentrations of FDH (6g/L, 12g/L and 18g/L). Samples were mixed using aspiration and dispersion through a pipette tip. Samples of 200µl were taken and diluted with 800µl ethanol and assayed via HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone and 6-bromo-β-tetralol.

4.2.12 Effect of NAD on the Rate of Enzyme Reaction

A reaction mixture of 0.45g/L of formate, 2.6g/L FDH, 2.7ml tetralone reductase, and 300µl of a 10g/L 6-bromo-β-tetralone in ethanol was used with varying concentrations of NAD. NAD concentrations were 0.14g/L, 0.28g/L and 0.56g/L which gave TTNs of 20, 10 and 5 respectively. Solutions were mixed using aspiration and dispersion through a pipette tip. Samples of 200µl were taken and diluted with 800µl ethanol and these were assayed via HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone and 6-bromo-β-tetralol.

4.2.13 Effect of Tetralone Reductase Concentration on Reaction Rate

Samples were set up as before, but keeping the NAD concentration constant at 0.14g/L. Tetralone reductase was diluted to 75% and 50% of its original concentration (0.780g/L). Solutions were mixed through aspiration and dispersion through a pipette tip. Samples of 200µl were taken and diluted with 800µl ethanol and these were assayed via HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone and 6-bromo-β-tetralol.

4.2.14 Regenerative Bioconversion

8mg formate, 0.43mg NAD, 8mg FDH were dissolved into the tetralone reductase solution (2970µl, 2850µl and 2700µl). The reactions were initiated through the addition of a 100g/L 6-bromo-β-tetralone solution in 30µl, 150µl and 300µl, final concentrations 1g/L 5g/L and 10g/L respectively. (Formate was added in excess). The reactions were monitored over time by HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone and 6-bromo-β-tetralol.

4.2.15 Scaled Bioreactor (10ml) Pass 1

10ml of tetralone reductase was added to 1.43mg NAD, 20mg FDH, 4.5mg of formate. The reaction was initiated with 100µl of a 100g/L 6-bromo-β-tetralone in methoxyethanol (final reaction concentration 1g/L) solution. To assay the reaction mix, 200µl samples were taken (a total of 1ml of the reaction mixture was removed for assaying). 0.5g of XAD L-323 was added to the reaction components facilitating *in situ* product removal. The reaction components were then filtered using a syringe filter, into a new sterile Sterilin™ reaction vessel (Bibby Sterilin Ltd, Stone, Staffs, UK). A sample of the filtered mix was assayed to check that the product and remaining substrates had been removed. The reaction was run for 4hr and the samples were taken and assayed by HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone and 6-bromo-β-tetralol at defined time points.

4.2.16 Scaled Bioreactor (10ml) Pass 2

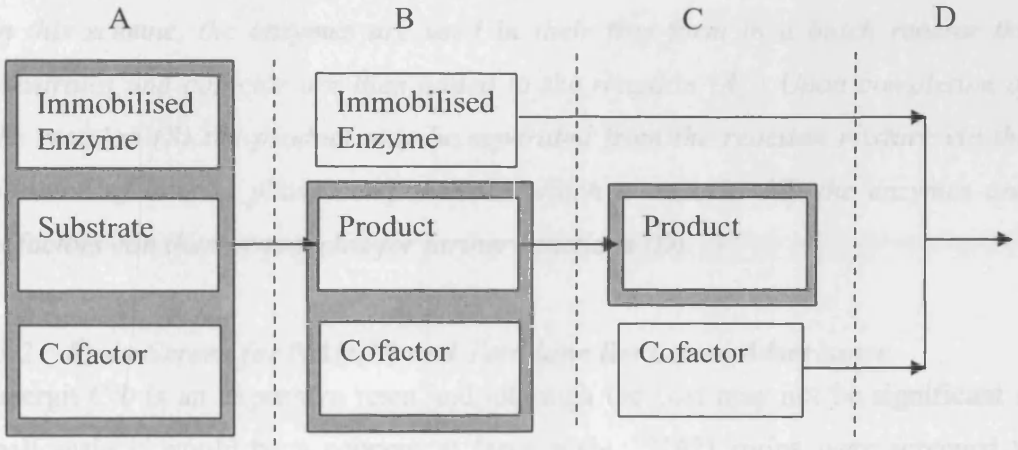
1ml (in total) of tetralone reductase was removed in the assaying of the first reaction. An extra 1ml of tetralone reductase was added to the filtered reaction components with 4mg formate, 2mg FDH and 0.143mg NAD. The reaction was initiated again with 100µl of a 100g/L 6-bromo-β-tetralone in methoxyethanol. The reaction was run for 7hr and samples were taken and assayed by HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone and 6-bromo-β-tetralol at defined time points.

4.3 Results

4.3.1 Immobilisation of Tetralone Reductase

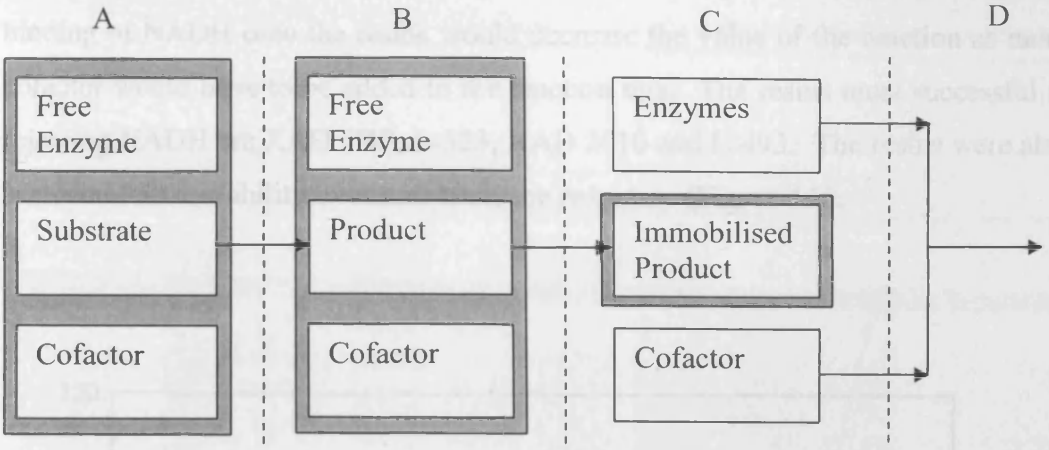
The immobilisation of tetralone reductase on to Eupergit C® was proposed as a method to separate the substrates and products from the enzymes in continuous operation. However, on assaying for enzyme activity it was found that 6-bromo-β-tetralone and 6-bromo-β-tetralol had adsorbed on to Eupergit C®, and therefore the requirement to separate the substrate and product from the immobilized enzyme (for a continuously operating bioreactor) could not be met. Another issue regarding the use of Eupergit C® was that the manufacturer's protocol required an ionic solution for the immobilisation procedure. The recommended buffer was phosphate, however, NADH (Wu *et al.*, 1986) and tetralone reductase (Thomas, 2000) are both deactivated in high molarity phosphate buffers. The adsorbance property exhibited by the substrate and product, and the high ionic buffer required for immobilisation precluded the use of Eupergit C® as an immobilisation resin. It was therefore decided that the most effective way of applying tetralone reductase in a biocatalytic process would be to use the enzyme in its free state and in batch operation, however, an alternative methodology for enzyme and cofactor regeneration and retention would be required. The unexpected binding of 6-bromo-β-tetralone and 6-bromo-β-tetralol to Eupergit C® was proposed as a method which could be used for cofactor recycling; by employing Eupergit C® to adsorb the product instead of the enzyme via *in situ* product adsorption the enzymes and cofactors could be separated and reused in a multiple batch configuration. A cofactor-requiring enzyme reactor

system which employs a solid phase component to capture the product (and remaining substrate) from the reaction system negates the requirement to ensure the expensive cofactors are maintained inside the reactor system via other methods such as cofactor immobilisation (Wichmann *et al.*, 1981) or via the employment of less conventional membranes; for example, charged membranes (Nidetsky *et al.*, 1996). The theoretical movement of the reaction components through a reactor if: (i) the enzymes are immobilized, and if (ii) the product is immobilized, is shown in Schemes 4.1 and 4.2 respectively.



Scheme 4.1

In this scheme, the enzymes are immobilised on a support and the substrates and cofactors are reacted in either continuous or batch operation (A). The output from the reaction would be a mix of the product and cofactor (B). The cofactor would need to be separated from the product (C) before it could be recycled in a further reaction with the immobilised enzyme (D).



Scheme 4.2

In this scheme, the enzymes are used in their free form in a batch reactor the substrates and cofactor are then added to the reaction (A). Upon completion of the reaction (B) the product may be separated from the reaction mixture via the addition of a solid phase component to which it adsorbs (C) the enzymes and cofactors can then be recycled for further reactions (D).

4.3.2 Resin Screen for NAD(H) and Tetralone Reductase Adsorbance

Eupergit C® is an expensive resin and although the cost may not be significant at small scale it would be a concern at large scale. XAD resins were screened to determine if an inexpensive polymeric resin could be used as a replacement for Eupergit C® for this *in situ* product adsorption and removal. The parameters analysed were: (1) the ability of the resin to reject the cofactors and hydrophilic enzymes; and (2) the ability of the resin to retain the hydrophobic substrate and product. The resins screened were XAD-4, XAD 7HP, Dowex Optipore 285, XAD 1180, XAD 2000, L323, XAD 2010 and L-493 (Figure 4.1). NADH is a component of the enzyme reaction which requires recycling and should not bind to the resin. The results demonstrate that Dowex Optipore 285 shows significant adsorbance of NADH, the other resins which adsorbed NADH were XAD 1180 which adsorbed about 40%, XAD 200 which adsorbed about 30% and XAD 4 which adsorbed approximately 15%. The difference in the binding capacities of the cofactor to the

resin is likely due to the different properties exhibited by the resins; any amount of binding of NADH onto the resins would decrease the value of the reaction as more cofactor would have to be added to the reaction mix. The resins most successful at rejecting NADH are XAD 7HP, L-323, XAD 2010 and L-493. The resins were also screened for their ability to adsorb tetralone reductase (Figure 4.2).

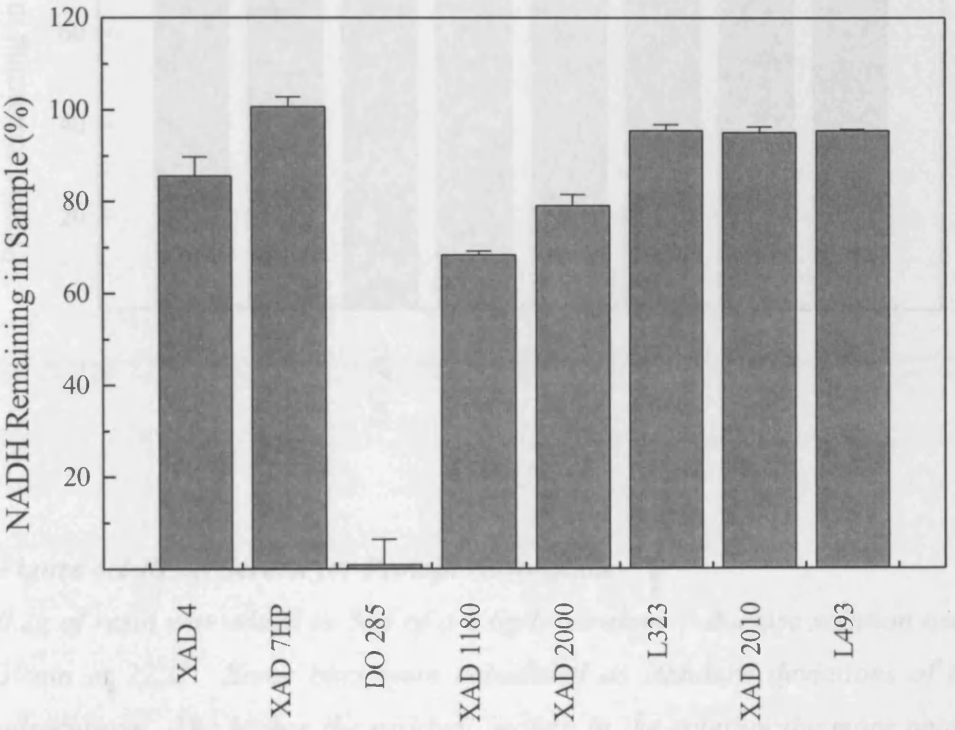


Figure 4.1 Resin Screen for NADH Adsorption

0.2g of resin was added to 3ml of a 0.5mg/ml NADH solution in water and left for 30min at 22^0C. Error bars were calculated as standard deviations of three adsorptions. The higher the residual NADH in the solution, the more potential the resin has to be used in a bioreactor as a method of in situ product removal. XAD 7HP, L 323, XAD 2010 and L493 all show potential. Dowex Optipore (DO) 285 shows least potential (Reference Section 4.2.3 for methodology).

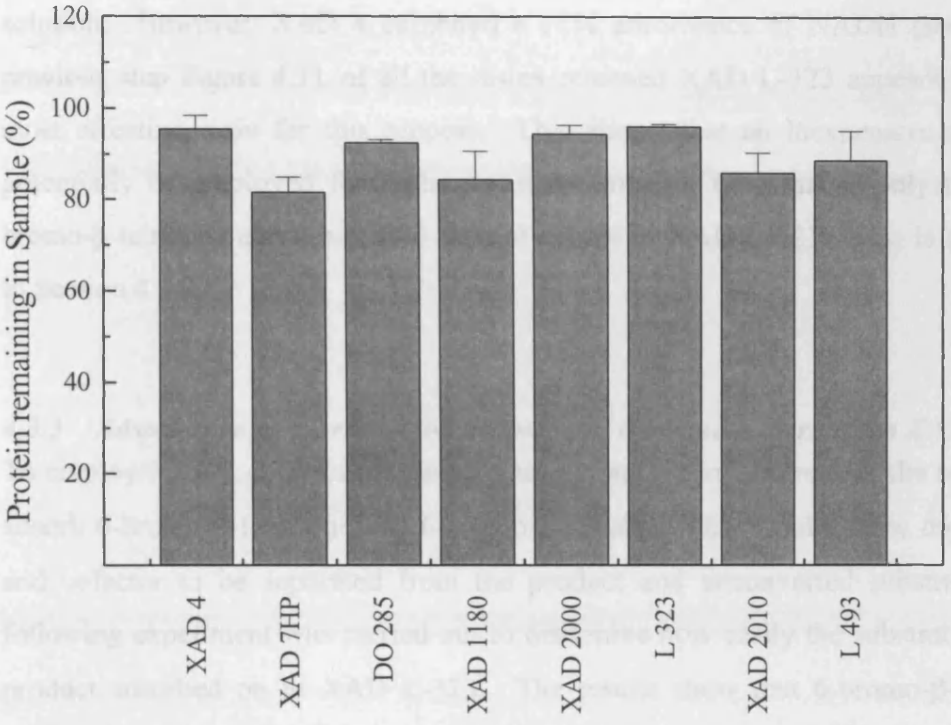


Figure 4.2 Resin Screen for Protein Adsorption

0.2g of resin was added to 3ml of a 0.6g/L tetralone reductase solution and left for 30min at 22^{0}C. Error bars were calculated as standard deviations of the three adsorptions. The higher the residual protein in the solution the more potential the resin has to be used in a bioreactor as a method of in situ product removal. None of the resins demonstrate high enzyme binding (Reference Section 4.2.4 for methodology).

To reuse tetralone reductase in a bioreactor, the enzyme must not adhere to the selected resin. Adsorbance of the enzyme to a resin will decrease the separation and may also reduce the efficacy of the enzyme. The results from the experiments

demonstrate that minimal adsorbance of the enzyme is observed on all of the resins tested. The two most effective resins for the least adsorption of tetralone reductase are XAD 4 and L-323. These two resins maintained nearly 100% of the enzyme in solution. However, XAD 4 exhibited a 15% adsorbance of NADH (seen in the previous step Figure 4.1), of all the resins screened XAD L-323 appears to be the most effective resin for this process. This shows that an inexpensive resin can potentially be employed for replacement of Eupergit C®; this is only true if 6-bromo-β-tetralone and 6-bromo-β-tetralol adhere to XAD L-323. This is examined in Section 4.3.3.

4.3.3 Adsorbance of 6-bromo-β-tetralone and 6-bromo-β-tetralol on XAD L-323

To employ XAD L-323 for *in situ* product adsorption in a bioreactor the resin must adsorb 6-bromo-β-tetralone and 6-bromo-β-tetralol. This would allow the enzyme and cofactor to be separated from the product and unconverted substrate. The following experiment was carried out to determine how easily the substrate and the product adsorbed on to XAD L-323. The results show that 6-bromo-β-tetralone easily adsorbs onto the XAD resin L-323 (Figure 4.3). A mass of 0.1g of the resin adsorbs the 10mg of 6-bromo-β-tetralone in the solution. 6-bromo-β-tetralol exhibits a lower binding capacity on to the XAD resin L-323 (Figure 4.4) than 6-bromo-β-tetralone. This may be explained as it is less hydrophobic that 6-bromo-β-tetralone, and thus, has a tendency to remain in the aqueous phase. A mass of 0.2g of the resin adsorbs the 10mg of 6-bromo-β-tetralol in the solution. This shows that XAD L-323 can potentially be used in the adsorption of the substrate and product. This mass of resin will be used comparatively in subsequent experiments as this will ensure that all of the product and unconverted substrate have the potential to adhere to the resin. It will also ensure that the resin is used in the most efficient quantities.

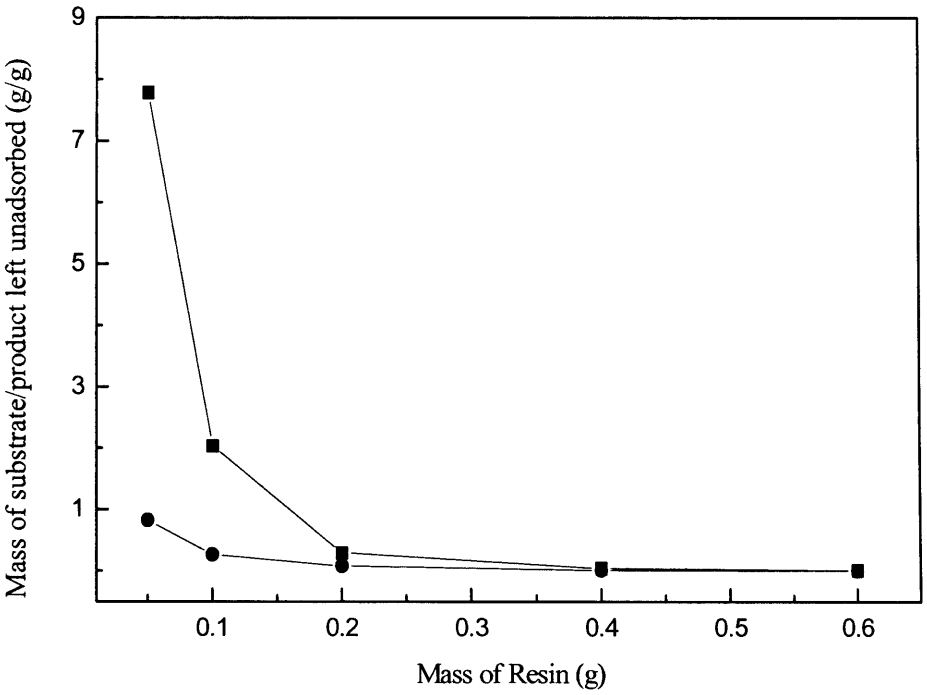


Figure 4.3 The Adsorbance of Substrate and Product on XAD L323

This graph illustrates the adsorbance of 6-bromo-β-tetralol (■) and 6-bromo-β-tetralone (●) on to XAD L-323. The components were firstly solubilised in methoxyethanol before diluted using water to 1g/L. The resin was then incubated in 10ml of the substrate and product solutions at 22^{0}C for 30min. The graph demonstrates that both 6-bromo-β-tetralone and 6-bromo-β-tetralol adsorb onto the selected resin, however, the amount adsorbed per gram of resin is higher for the substrate than for the product. (Reference Sections 4.2.5 and 4.2.6 for methodologies).

4.3.4 Effect of Type of Solvent on Rate of Reaction

A major constraint for developing a bioreactor configuration for the reduction of 6-bromo-β-tetralone is because 6-bromo-β-tetralone is a solid (with a low aqueous solubility) it needs to be solubilised in a secondary medium. To facilitate *in situ*

product adsorption via a polymeric resin it was deemed necessary to maintain a single-phase solution. This was because 6-bromo-β-tetralol needs to have a higher affinity for the polymeric resin than for a secondary phase hydrophobic solvent or the resin will become redundant as a two-phase system would likely elute the product from the resin into the secondary phase. A water miscible solvent will affect the enzyme conformation (Adlercreutz, 1988), and therefore the enzyme activity. However, by decreasing the volume of the solvent required by selecting a solvent which has a high solubility of the substrate will most likely ensure that the enzyme activity is maintained at the highest levels feasible. This experiment was carried out to determine whether a miscible solvent can be used to solubilise 6-bromo-β-tetralone, which in turn, can be applied in the bioreduction of 6-bromo-β-tetralone. The experiment was also carried out to determine if a solvent which has a low solubility of the substrate can be substituted with a solvent which has a higher solubility of the substrate. Solvents were previously screened in Chapter 3.3.13 and the results demonstrated that ethanol and methoxyethanol were the solvents which maintained most of the activity of tetralone reductase in a single phase system. Comparatively, both these solvents also retained the same amount of enzyme activity (Figure 3.13). However, ethanol dissolves 6-bromo-β-tetralone to a concentration of 10g/L yet methoxyethanol dissolves 6-bromo-β-tetralone to a much higher concentration of 250g/L. In this experiment, a bioreduction was run where ethanol was employed at higher volumes than methyoxyethanol but with similar substrate concentrations. The results (Figure 4.4) show that the rate of reaction significantly increases when methoxyethanol is used. Not only is the initial rate of reaction increased by decreasing the volume of solvent, the reaction is also completed two hours faster in a time of approximately 3hrs. The yields, however, after 4hr are relatively similar. This reduction in initial reaction rates is most likely caused by an increase in the solution polarity surrounding the enzyme; this most likely disrupts the enzyme's tertiary structure and active site conformation. Analytical techniques such as circular dichroism (CD) could be used to analyze the affects of the enzyme conformation in different solvent solutions. In Figure 4.4 a

reduction in solvent shows an increase in the enzyme reaction rates, however there may also be other parameters which affect the reaction rates. For example, the amount of substrate may affect the enzyme kinetics, this is investigated in Chapter 4.3.5.

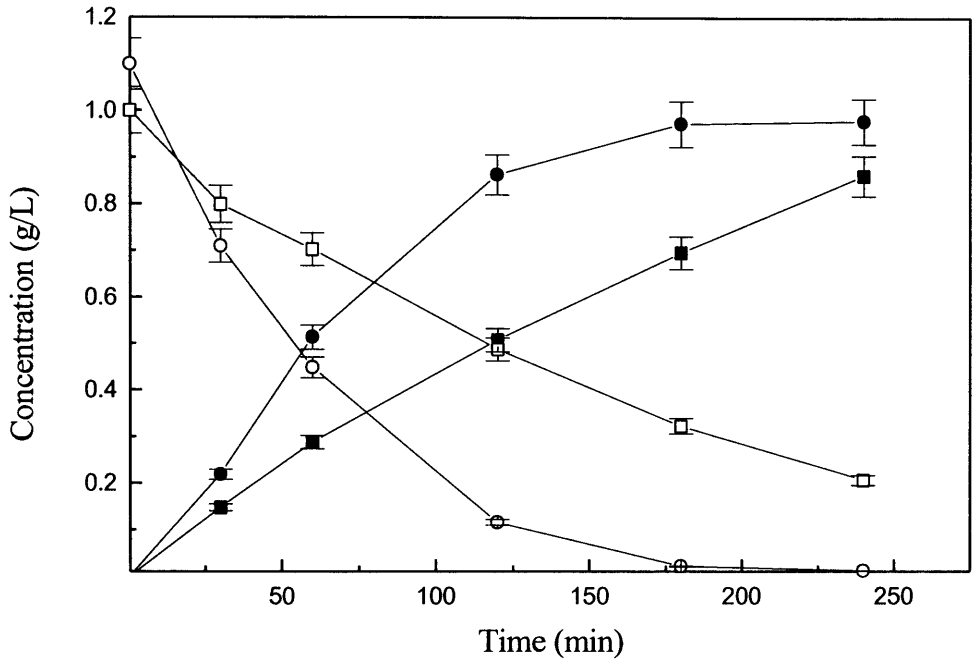


Figure 4.4 The Effects of the Type of Solvent on the Bioreduction

Bioconversion profiles for reactions using, 1ml of a 10mg/ml 6-bromo-β-tetralone in ethanol as the solvent (□) with respective product (■); and methoxyethanol, 100μl of a 100mg/ml 6-bromo-β-tetralone in methoxyethanol as the solvent (○) with respective product (●). Reactions were carried out in a volume of 10ml. The graph demonstrates that the bioconversion depends on the volume of solvent added to the reaction mixture. The addition of a 10-fold decrease in solvent whilst maintaining substrate concentration can significantly increase the reaction rates. (Reference Section 4.2.7 for methodology).

These results indicate that a miscible solvent can be used in the isolated enzyme bioreduction of 6-bromo-β-tetralone. It also demonstrates that a solvent with a higher solubility of the substrate should be employed for the bioreduction since there is less of an effect on tetralone reductase. Because of these findings, it is methoxyethanol (and not ethanol) will be used in subsequent enzyme regeneration reactions.

4.3.5 Effect of Substrate Concentration on Tetralone Reductase Activity

The change in initial substrate concentration was investigated to determine the effects on the activity of the isolated enzyme. This experiment was carried out using the single enzyme cofactor requiring system, where NADH was added stoichiometrically (not recycled). The results (Figure 4.5) show that the initial rate of reaction increases rapidly up to an initial substrate concentration of approximately 1g/L; this is most likely the point at which the enzyme becomes saturated with substrate. It was expected that the initial enzyme activity would only increase up to 0.5g/L, since this is the solubility of 6-bromo-β-tetralone in water. However, 6-bromo-β-tetralone was solubilised in methoxyethanol first and this could have increased the solubility of the substrate in the whole solution. The solubility of the substrate in different ratios of methoxyethanol and water should be investigated to confirm this hypothesis. The point at which the reaction rates level out (1g/L) is most likely representative of the enzyme becoming the rate limiting factor. At this point it is hypothesized that the transfer of the product from the active site and the transfer of further substrate to the active site become the rate-limiting steps. This hypothesis could be tested by adding more enzyme to the reaction; an increase in the amount of enzyme would increase the ratio of enzyme to substrate, thus most likely increasing the point at which the initial reaction rate levels. If the point at which the reaction levels out did not increase then it would likely mean that there is another component which limits the reaction. One potential component could the required cofactor (NADH), where there could potentially be less units of cofactor in the reaction solution than required by the enzyme; this could be tested by adding more

cofactor to the reaction. It is unlikely that the amount of cofactor added is causing the leveling out of reaction rates since this amount was calculated with respect to the amount of substrate in the reaction. It is more probable that there is not enough enzyme (since only small quantities are isolated and purified from the cell). Hypotheses for the rate limiting factors are discussed, including the effects of the amount of tetralone reductase on the bioreduction, in further detail in Section 4.3.7.

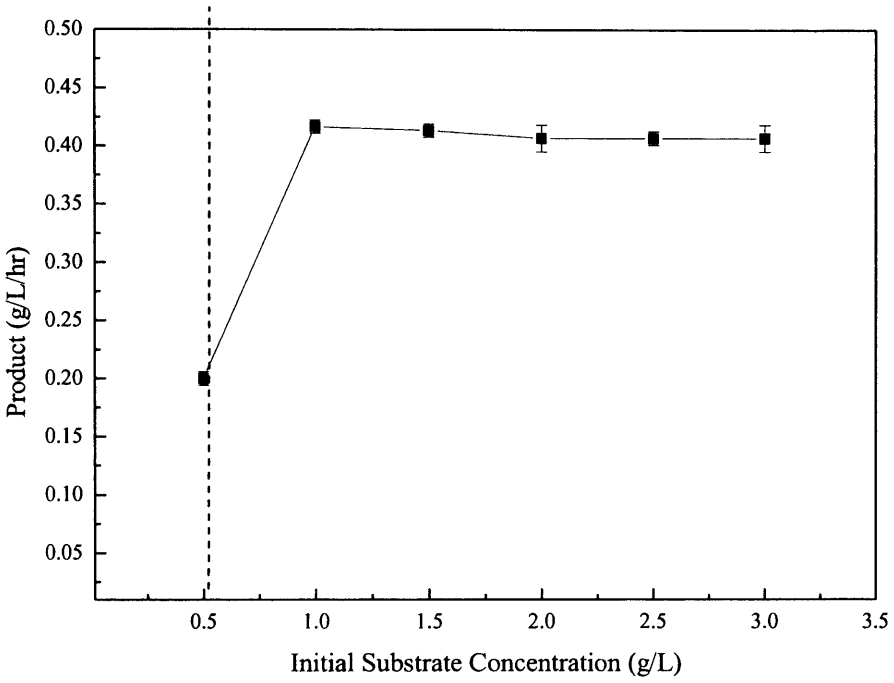


Figure 4.5 The Effects of Initial Substrate Concentration on Biocatalytic Activity

100μl of methoxyethanol was used to dissolve 6-bromo-β-tetralone in 10ml of reaction mixture. The vertical dotted line (---) illustrates the point at which water would become saturated with substrate. The graph shows an initial increase in the rate of reaction before the rate of reaction levels out. This is most likely the point at which tetralone reductase becomes saturated with 6-bromo-β-tetralone. This is at approximately 1g/L (where the initial activity no longer increases). (Reference Section 4.2.8 for methodology) (See Appendix B1.1 for raw data).

4.3.6 Effect of Solvent Volume on Tetralone Reductase Activity

The amount of solvent in the reaction mix in this system is related to the amount of product required. This is because as more substrate is added more solvent is required to solubilise the substrate. In this experiment, the effects of solvent volume on tetralone reductase activity were investigated. This could determine the amount of solvent which could be added to the reaction before tetralone reductase becomes denatured. The results from the experiment show that the initial reaction rates significantly decrease when methoxyethanol is added to the reaction mix (Figure 4.6). A solvent concentration of 6% (v/v) decreases the initial enzyme activity by approximately 50% when compared to the enzyme activity at a solvent concentration of 1% (v/v). The final conversions are less affected by the solvent volume at the substrate concentration used (Figure 4.7), there is however a small decrease and it is likely that if more solvent was added then this would have a more adverse effect. The high substrate conversions which were maintained even at high solvent concentrations may be attributed to the fact that low substrate concentrations were employed. Solvent concentrations of up to 11% show no significant decrease in final conversion, however, at the same solvent concentrations the activities are reduced to very low levels. This observed reduction in activity is likely to be due to the solvent interfering with the essential water layer surrounding the enzyme (Leön *et al.*, 1998). The disturbance in the water layer most likely causes the enzyme's conformation to become perturbed and therefore it is difficult for the substrate to bind into the 'lock and key' complex in the active site of the enzyme. The change in the solution polarity may also cause the weak attractive forces which can help create the complex to become unstable. If there is a level of disturbance of the 'lock and key' mechanism at higher solvent concentrations, this will likely cause a decrease in the reaction rates, since the reaction may take longer to complete. This may, however, not cause a decrease in the conversion (just longer for the reaction to complete). If an even higher solvent concentration was employed, this would likely cause an even greater change in the enzyme's active site and it would be even more likely that the substrate would not bind, consequently lowering the conversions

further. These results indicate that miscible solvents can be used in the bioreduction of 6-bromo-β-tetralone, however to maximise enzyme activity the solvent concentration should not be used above 10% (v/v) in bioreactor experiments.

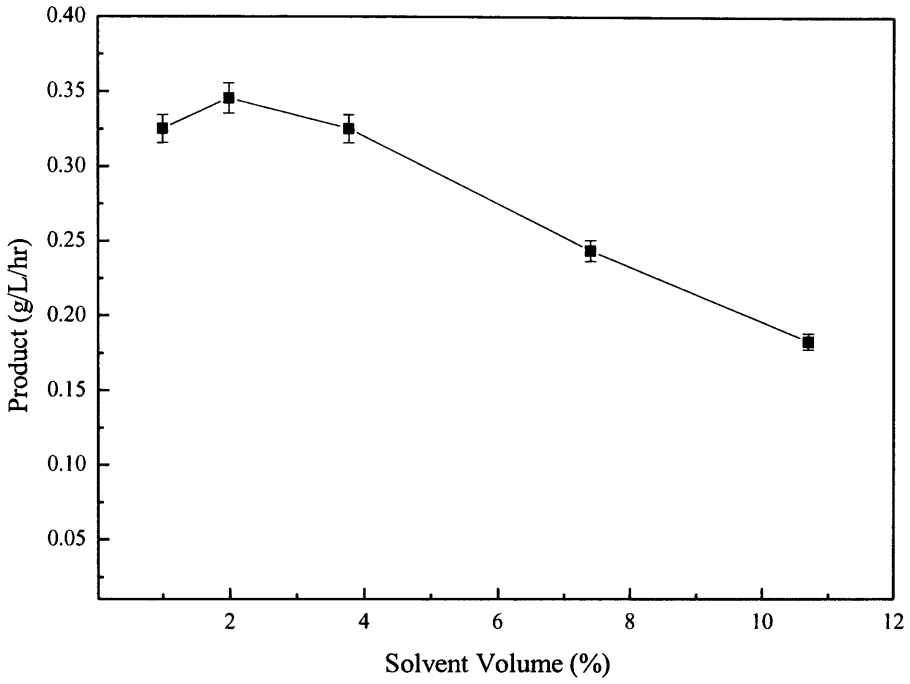


Figure 4.6 The Effects of Initial Solvent Concentration on Biocatalytic Activity

Initial substrate concentration was 0.5mg/ml. This graph illustrates how the initial enzyme activity is affected by the volume of solvent in the reaction solution. There is an initial increase in the activity up to approximately 2% (v/v) before a linear decrease can be observed. The initial enzyme activity is minor at about 11% solvent (v/v). 0% solvent (v/v) was not measured as 6-bromo-β-tetralone is highly insoluble in aqueous solutions. ((Reference Section 4.2.9 for methodology). (See Appendix B1.2 for raw data).

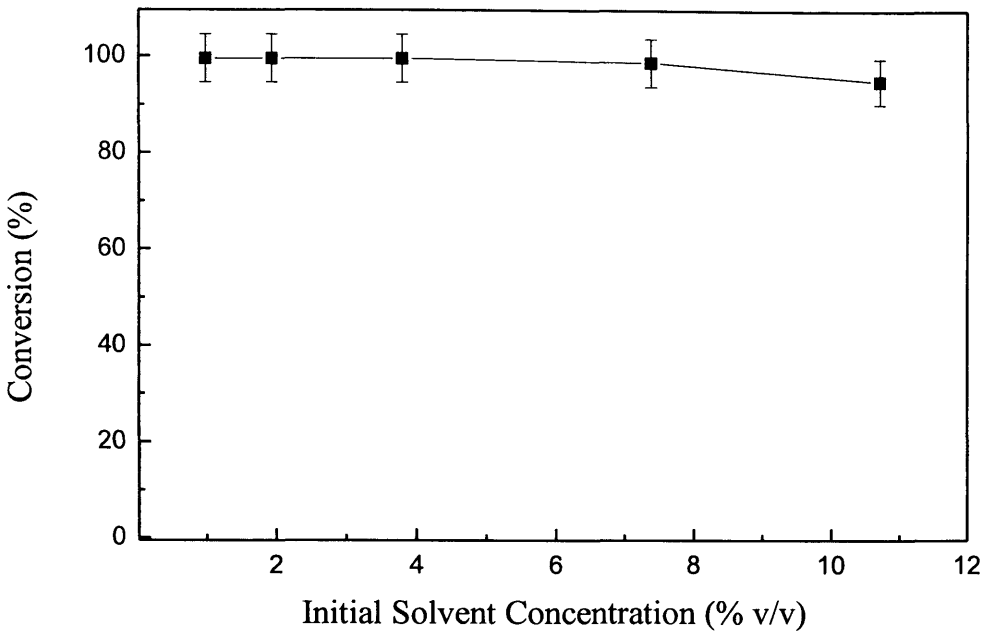


Figure 4.7 The Effects of Methoxyethanol Concentration on the Conversion

These results come from the methodology used in Figure 4.6. The results demonstrate that although the initial enzyme activity was highly affected by an increase in solvent volume (Figure 4.6), the conversions of the substrate were much less affected. There is a small decrease in the final product concentration and this becomes more pronounced as the amount of solvent is increased. (Reference Section 4.2.9 for methodology). (See Appendix B1.2 for raw data).

4.3.7 Determining the Rate-Limiting Component

In the two enzyme cofactor regenerating system there are a number of components which could affect the rate of the synthetic reaction these are: formate, formate dehydrogenase, NAD(H) and tetralone reductase. The following experiments were carried out to determine which of the components used in the bioreduction was the rate-limiting factor. Firstly, tetralone reductase was examined for activity at concentrations of formate which would be available in the bioreactor. The results show that at the formate concentrations investigated the enzyme activity is not

affected (Figure 4.8). This means that formate can be employed effectively as the regenerating substrate without affecting tetralone reductase activity, it also means that it is not the rate-limiting factor in the reaction. This result, in which formate was determined to be innocuous to tetralone reductase, is consistent with the results from a review paper (Chenault and Whitesides, 1987) (see Chapter 1.7). Secondly, FDH which is a highly stable, purified enzyme purchased in lyophilized form was examined to show whether it was the rate-limiting component. The results show that increasing the concentration of FDH does not affect the rate of the reaction (Figure 4.9). This was expected as the enzyme is well characterised and highly stable in comparison to tetralone reductase. Thirdly, the initial NAD concentration in the reaction mixture was increased to examine whether NAD was the limiting component. NAD was added to the reaction mix instead of NADH for two reasons. Firstly, NAD is more stable in a number of reported environments than NADH (Wu *et al.*, 1986; Chenault *et al.*, 1987). Secondly, the addition of NAD demonstrates that cofactor regeneration occurred in the reaction, and is not just used stoichiometrically. An increase in initial NAD concentration would ultimately decrease the TTN, and the lower the TTN the higher the process costs (Chenault and Whitesides, 1987). Results show that the enzyme activity does not change with varying NAD concentrations and NAD is therefore not the rate-limiting component (Figure 4.10). Lastly, tetralone reductase was employed at three different concentrations of 0.4g/L, 0.6g/L and 0.8g/L to determine if it was the rate limiting component. The results demonstrate that as tetralone reductase concentration is increased the rate of reaction also increases (Figure 4.11). The results show that under the current operating parameters, tetralone reductase is the rate-limiting component in the regenerative bioconversion. It is also very probable that the rate-limiting step is the conversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol. The most logical reason that tetralone reductase is limiting, is that it is highly diluted and not fully purified, unlike FDH which is an over-expressed enzyme and is in a highly pure form. This means, however, that the TTN of NAD to NADH is not accurate as tetralone reductase is probably over saturated by the cofactor (there are more units of

NAD than units of enzyme). The addition of higher concentrations of NAD does not increase the initial rate of reaction and indicates that FDH and tetralone reductase were already saturated with NAD(H). Reducing the quantity of NAD to the minimal amount would significantly increase the TTN whilst keeping the reaction rate the same. Calculating the most efficient TTN could also give an indication of how much active enzyme is in the sample. As the enzyme activity was not affected by the addition of excess NAD over-saturation would only be of concern at large scale where its cost would be more prominent. If tetralone reductase was over-expressed the reaction could be significantly enhanced with faster reaction rates and increased TTNs. The reaction would also be more likely to be limited by the transfer of the cofactor from one enzyme to the other, rather than the low concentration of tetralone reductase in the reaction solution.

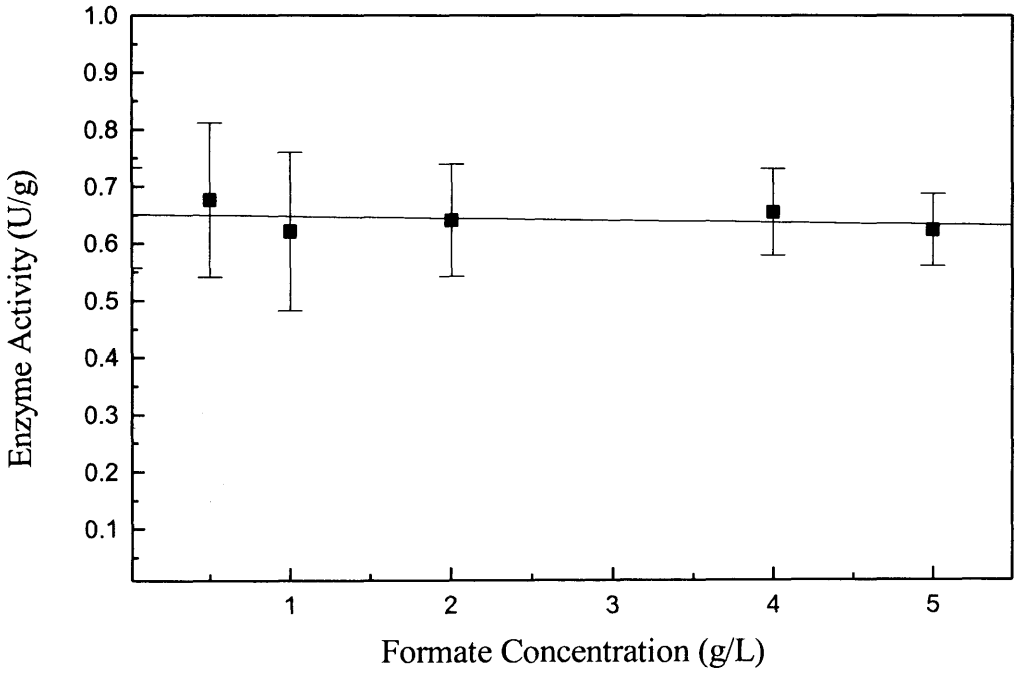


Figure 4.8 The Effects of Formate on Enzyme Activity

This graph illustrates how the amount of formate (the regenerative substrate) affects the activity of tetralone reductase (the synthetic enzyme). The experiment was

completed at formate levels consistent with, and higher than, the regenerative bioconversions levels. The results demonstrate that formate does not affect the enzyme activity at the levels tested. The error bars on the graph are calculated from the standard deviations of three separate experiments. (Reference Section 4.2.10 for methodology).

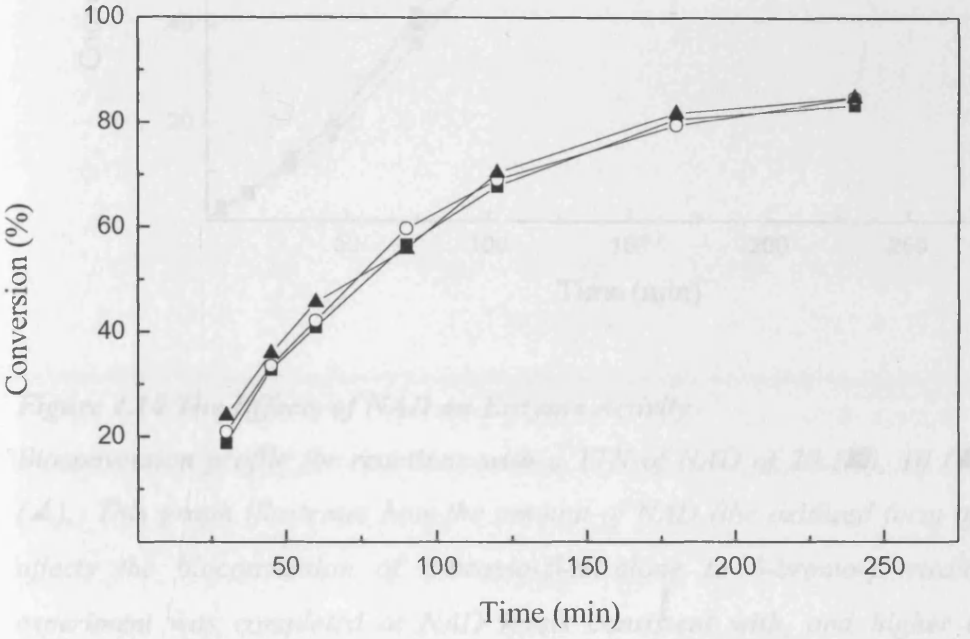


Figure 4.9 The Effects of FDH on Enzyme Activity

Bioconversion profile for reactions with 6mg FDH (■), 12mg FDH (○),and 18mg FDH (▲). This graph illustrates how the amount of FDH (the regenerative enzyme) affects the bioconversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol. The experiment was completed at FDH levels consistent with, and higher than, the regenerative bioconversions levels. The results demonstrate that FDH does not affect the bioconversion at the levels which will be employed in the bioreactor. (Reference Section 4.2.11 for methodology).

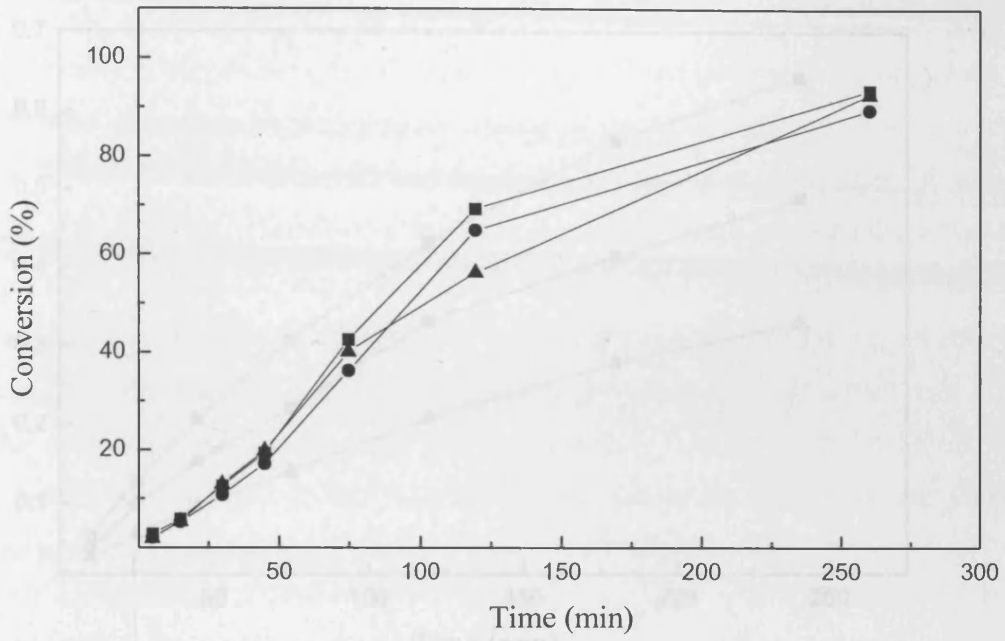


Figure 4.10 The Effects of NAD on Enzyme Activity

Bioconversion profile for reactions with a TTN of NAD of 20 (■), 10 (●), and 5 (▲). This graph illustrates how the amount of NAD (the oxidized form of NADH) affects the bioconversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol. The experiment was completed at NAD levels consistent with, and higher than, the regenerative bioconversions levels. The results demonstrate that NAD does not affect the bioconversion at the levels which will be employed in the bioreactor. (Reference Section 4.2.12 for methodology).

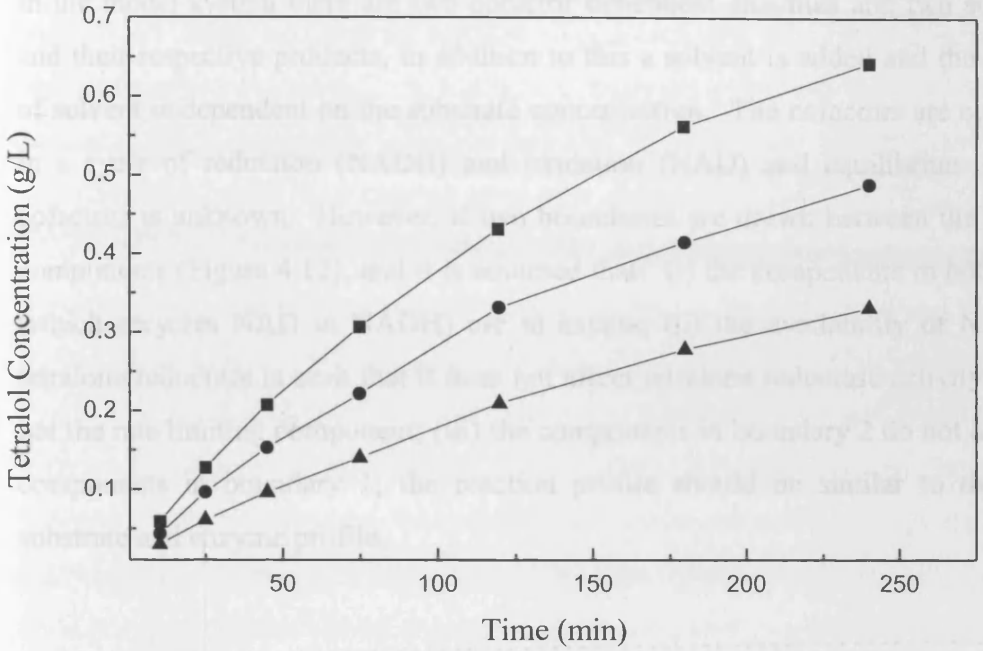


Figure 4.11 The Effects of Tetralone Reductase on the Rate of Reaction

Bioconversion profile for reduction of 6-bromo-β-tetralone with the concentration of tetralone reductase at 0.8g/L (■), 0.6g/L (●), 0.4g/L (▲). This graph illustrates how the concentration of tetralone reductase affects rate of production of 6-bromo-β-tetralol. The experiment was completed at enzyme levels consistent with the regenerative bioconversions levels. The results demonstrate that the concentration of tetralone reductase considerably affects the rate of reaction and will be the rate-limiting component in the bioreactor. (Reference Section 4.2.13 for methodology).

4.3.8 Two Enzyme Cofactor-Requiring Bioreduction

In the model system there are two cofactor dependent enzymes and two substrates and their respective products, in addition to this a solvent is added and the quantity of solvent is dependent on the substrate concentration. The cofactors are constantly in a cycle of reduction (NADH) and oxidation (NAD) and equilibrium of these cofactors is unknown. However, if two boundaries are drawn between the reaction components (Figure 4.12), and it is assumed that: (i) the components in boundary 2 (which recycles NAD to NADH) are in excess; (ii) the availability of NADH to tetralone reductase is such that it does not affect tetralone reductase activity yet it is not the rate limiting component; (iii) the components in boundary 2 do not affect the components in boundary 1; the reaction profile should be similar to the single substrate and enzyme profile.

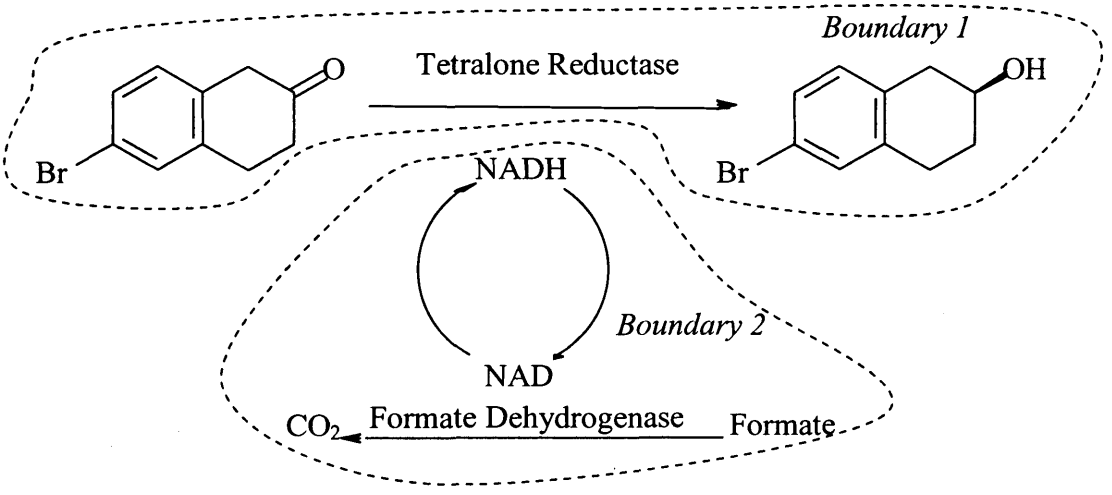


Figure 4.12 Boundary Interface to Determine Kinetics

If the components of the two enzyme cofactor-requiring system may be separated into two distinct boundaries, adding excess of the components in boundary 2 into the reaction will mean that the reaction should follow the single substrate profile.

However, this does not take into consideration the decrease in activity due to increasing the solvent quantity with increasing substrate concentration and since there is an effect on enzyme activity with increasing solvent concentration the reaction parameters will only be similar for reactions which use the same volume of solvent. This experiment was carried out to determine whether an efficient bioconversion could be achieved using low volumes of methoxyethanol but high concentrations of 6-bromo-β-tetralone. The regenerating enzyme and cosubstrate were both added to the reaction mixture in excess. 6-bromo-β-tetralone was added to the reaction mix at 1g/L (highest initial enzyme reaction rate determined from Figure 4.5), 5g/L and 10g/L. Results show that the recycling enzyme system works effectively (Figure 4.13). However, the effects of inhibition by the synthetic substrate and solvent when these initial concentrations are increased are evident. For example, the rate of reaction when an initial substrate concentration of 5g/L is used is approximately half of the reaction rate compared to when an initial substrate concentration of 1g/L is used, the final conversions, however, are similar. When the initial substrate concentration is 10g/L, the initial rate of reaction is half of the reaction rate when 5g/L is used, and the final conversion is less than half. These results show that an efficient bioconversion can be achieved at low substrate concentrations. Using the initial substrate concentration of 1g/L gives a high conversion and a high initial activity for the enzyme. It is this initial substrate concentration (1g/L) which will be employed in subsequent bioreactor experiments to prove the theory that product can be removed *in situ* whilst maintaining the enzyme and cofactor in a bioreactor for recycling.

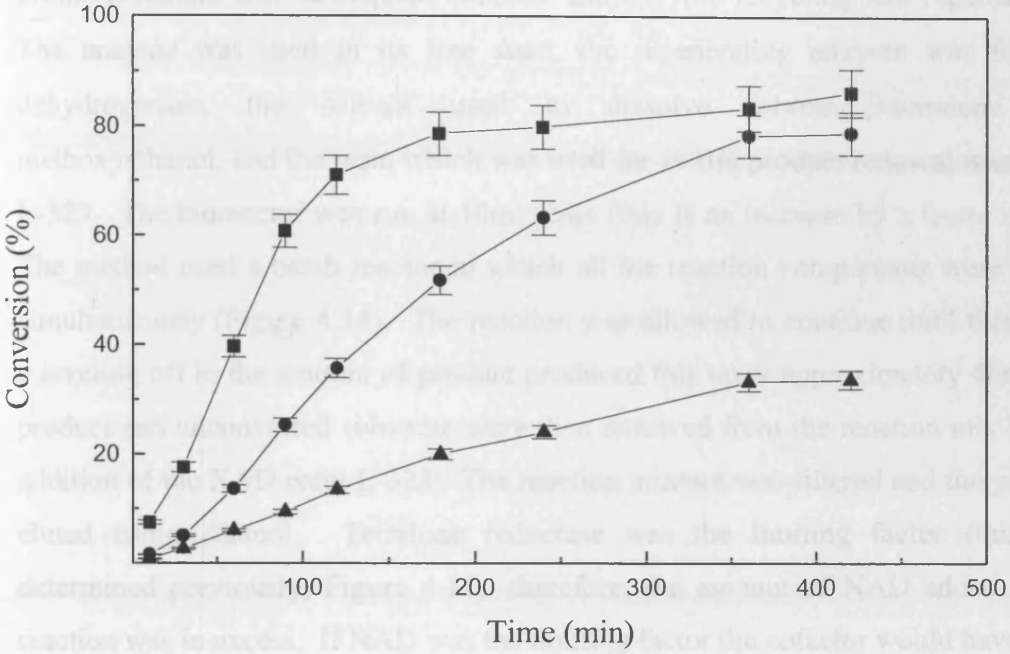


Figure 4.13 Regenerative Bioconversion

Bioconversion profile of 6-bromo-β-tetralone to 6-bromo-β-tetralol using methoxyethanol as the solvent with an initial substrate concentration of 1g/L (■), 5g/L (●), and 10g/L (▲). In this experiment NADH was regenerated by formate dehydrogenase using formate as the regenerative substrate. The graph illustrates the change in the rate of reaction when different substrate concentrations are used. At 1g/L the conversion is significantly faster than when the substrate concentration is 5g/L, the conversions, however, are not too dissimilar at approximately 80%. When a substrate concentration of 10g/L is employed there is a low rate of reaction and only a conversion of approximately 30%. (Reference Section 4.2.14 for methodology).

4.3.9 Scaled Bioreactor

The following experiments were carried out to prove that a bioreactor configuration can be established where there is *in situ* removal of 6-bromo-β-tetralone and 6-bromo-β-tetralol and subsequent cofactor and enzyme recycling and regeneration. The enzyme was used in its free state, the regenerating enzyme was formate dehydrogenase, the solvent used to dissolve 6-bromo-β-tetralone was methoxyethanol, and the resin which was used for *in situ* product removal was XAD L-323. The bioreactor was run at 10ml scale (this is an increase by a factor of 10). The method used a batch reactor in which all the reaction components were added simultaneously (Figure 4.14). The reaction was allowed to continue until there was a leveling off in the amount of product produced this takes approximately 4hr. The product and unconverted substrate were then removed from the reaction mix by the addition of the XAD resin L-323. The reaction mixture was filtered and the product eluted using ethanol. Tetralone reductase was the limiting factor (this was determined previously, Figure 4.11), therefore, the amount of NAD added to the reaction was in excess. If NAD was the limiting factor the cofactor would have been recycled 14 times, but theoretically a much higher number was achieved by the enzyme. The results show that this method can be used for the bioreduction of 6-bromo-β-tetralone to 6-bromo-β-tetralol, and the enzymes and cofactors can also be reused (Figure 4.15). In the second pass, the same quantity of substrate was added to the reaction mixture (the product and unconverted substrate was removed from pass 1 by *in situ* product adsorption and removal). Compensation was made for the loss of cofactors and enzymes during the assay process. It is evident from the results that the enzyme and cofactors can be used in multiple passes (Figure 4.16). The second pass follows a similar reaction profile to the first pass. Complete conversion was not met in either case, with the second pass showing lower conversion than the first pass. This could be because the scale of the experiments was small and protein could adhere to surfaces such as the reaction vessel or pipette tips. Compensation was also not made for the loss of reaction components in the dead space of the XAD resin in the initial pass and also losses in the filtering process. The results show,

under the operating system used, that the enzyme was stable enough in its free form to perform a multiple batch reaction; the batch reactor configuration employed also indicates that there was successful recycling of the enzymes and regeneration of NADH.

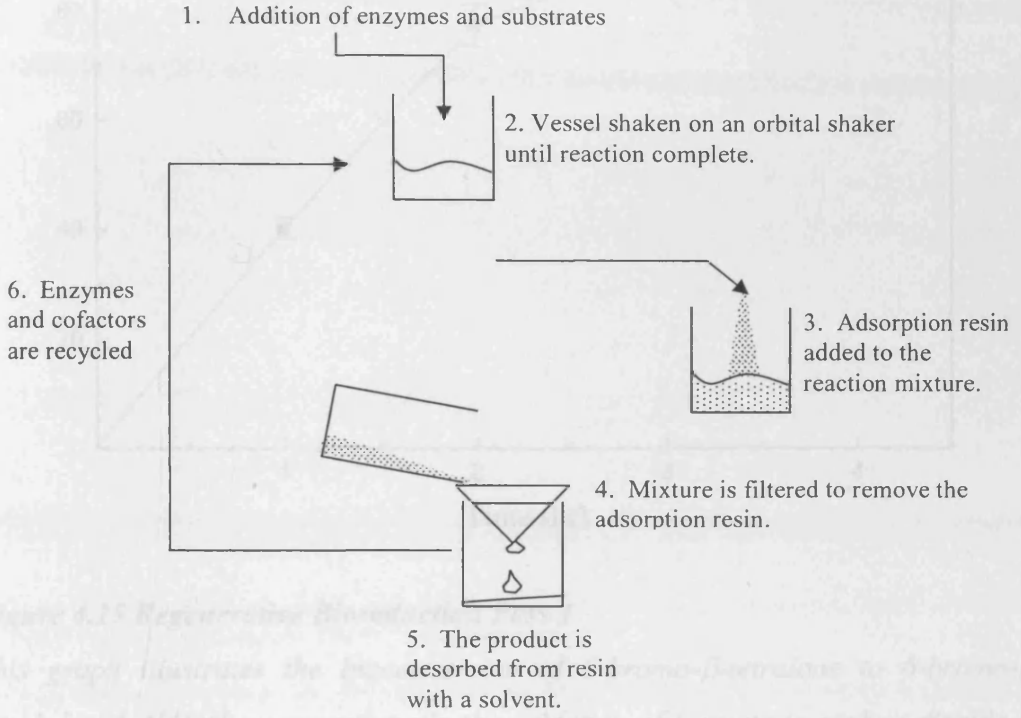


Figure 4.14 Reaction Methodology

Reaction methodology for the batch production of 6-bromo-β-tetralol with in situ product removal. The enzymes, substrates, and cofactors are added to the reaction vessel and mixed until the reaction reaches completion. The polymeric resin is then added to the vessel and the product and unconverted substrate adhere to the resin. The resin is then filtered and the product is desorbed from the resin by a solvent. The enzymes and cofactors are then recycled and used in another reaction.

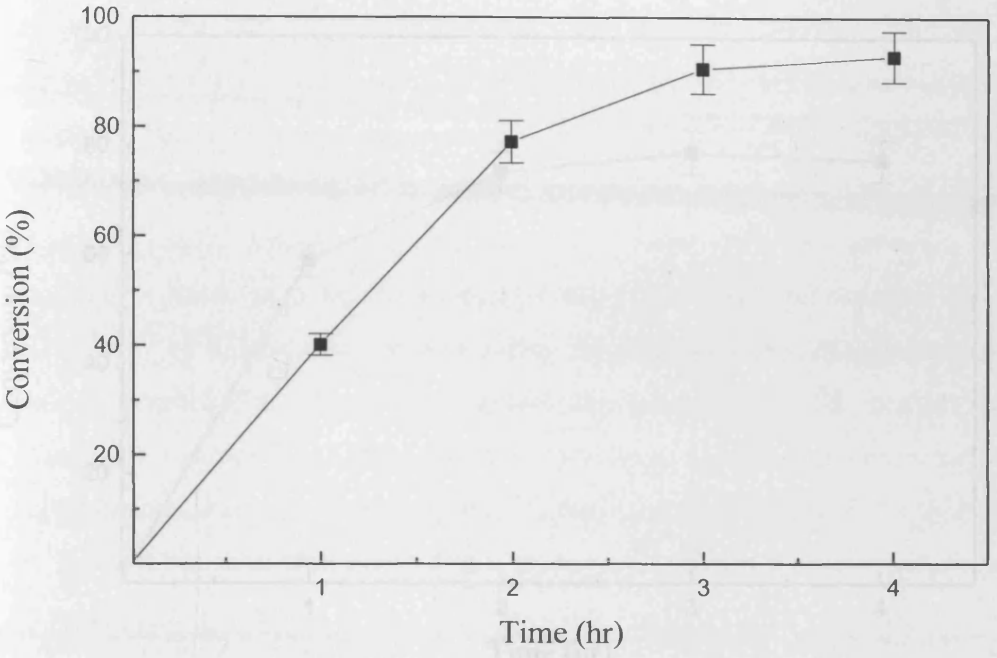


Figure 4.15 Regenerative Bioreduction Pass 1

This graph illustrates the bioconversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol with NADH regeneration via the oxidation of formate to carbon dioxide by formate dehydrogenase and NAD. The concentration of 6-bromo-β-tetralone is 1g/L, and tetralone reductase and NAD(H) are recycled after 4hr using in situ product removal. The reaction was completed in batch operation with all components in the same reaction vessel. The results demonstrate that an effective free enzyme bioconversion with cofactor regeneration can be applied for the reduction of 6-bromo-β-tetralone. After 4hrs the product and remaining substrate were removed using the previously screened XAD resin L-323, thus, separating the enzymes and cofactors from the product and remaining substrates. (Reference Section 4.2.15 for methodology).

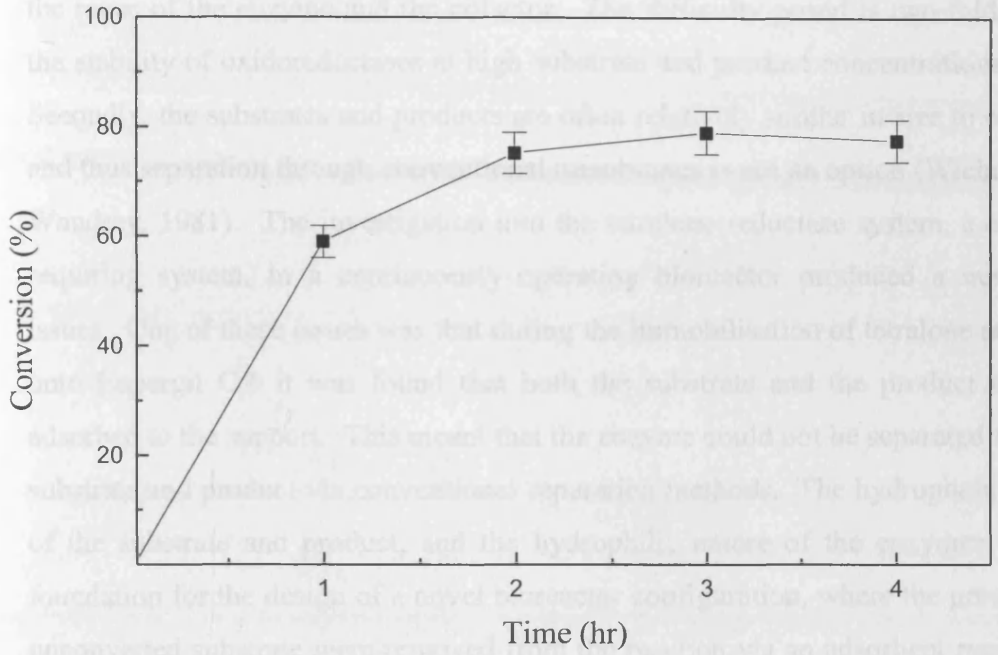


Figure 4.16 Regenerative Bioconversion Pass 2

This graph illustrates the bioconversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol with NADH regeneration for the second batch. The product and remaining substrate are separated from the enzymes and cofactors which have already completed a first batch reaction. A further quantity of substrate is added to the reaction mixture for the second pass. This graph shows that an isolated enzyme cofactor regenerating system can be used in a multiple pass system and the isolation of the product and substrate via a polymeric resin can be used as a separation method. (Reference Section 4.2.16 for methodology).

4.4 Discussion

Continuously operating bioreactors for large-scale isolated oxidoreductase bioreductions with cofactor regeneration requires biotechnology which can ensure the reuse of the enzyme and the cofactor. The difficulty posed is two-fold, firstly, the stability of oxidoreductases at high substrate and product concentrations is low. Secondly, the substrates and products are often relatively similar in size to cofactors and thus separation through conventional membranes is not an option (Wichman and Wandrey, 1981). The investigation into the tetralone reductase system, a cofactor-requiring system, in a continuously operating bioreactor produced a number of issues. One of these issues was that during the immobilisation of tetralone reductase onto Eupergit C® it was found that both the substrate and the product had also adsorbed to the support. This meant that the enzyme could not be separated from the substrate and product via conventional separation methods. The hydrophobic nature of the substrate and product, and the hydrophilic nature of the enzymes was the foundation for the design of a novel bioreactor configuration, where the product and unconverted substrate were removed from the reaction via an adsorbent resin. This design exploits product (and unconverted substrate) immobilisation methods rather than enzyme immobilisation methods. It is this type of bioreactor design which was deemed most appropriate for the bioreduction of 6-bromo-β-tetralone by tetralone reductase with cofactor regeneration via the enzymatic oxidation of formate by formate dehydrogenase. This method of product removal, and cofactor and enzyme reuse, was proved for a number of batches, where, the cofactors and free enzymes upon completion of the bioreduction were available to be used in further reactions. The principles of this type of bioreactor configuration were proven at scale; however, in the tetralone reductase case, the low potency of the enzyme was the limiting component in the reaction. The low potency of tetralone reductase meant that the bioreduction of 6-bromo-β-tetralone to 6-bromo-β-tetralone was the rate-limiting step. The bioreactor system may have been improved by increasing the amount of tetralone reductase available; increasing the enzyme concentration could potentially be achieved through over-expression of the enzyme into a

microorganism. This would most likely increase the efficiency of the purification and thus produce greater amounts of tetralone reductase. A higher tetralone reductase concentration in the reaction would mean that the bioreduction of 6-bromo-β-tetralone would not necessarily be the rate-limiting step.

A large-scale generic method for the application of this type of two enzyme cofactor-requiring system has been configured (Figure 4.17).

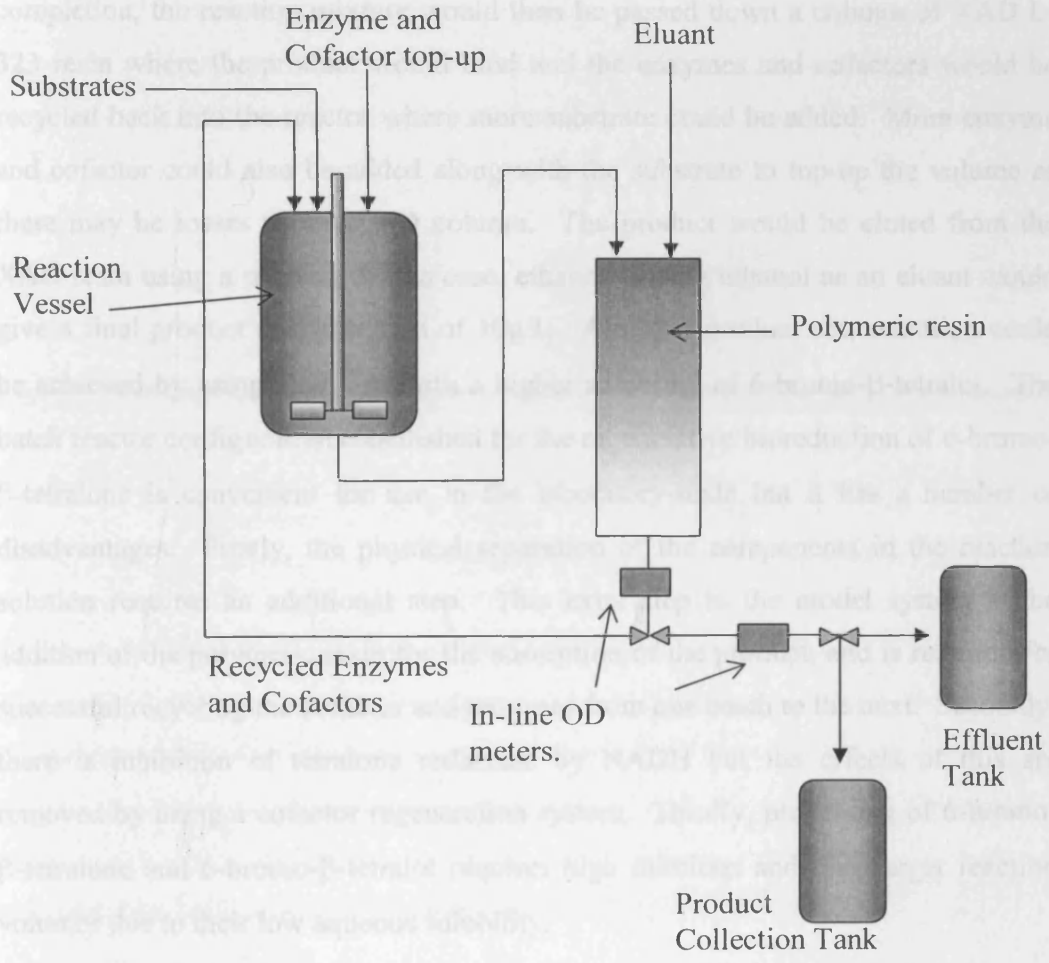


Figure 4.17 Theoretical Design of a Bioreactor with in situ Product Removal
The bioreactor configuration shows the reaction vessel which would contain all the substrate, enzymes, and cofactors; after the reaction had reached final conversion the reaction mix would pass though a polymeric resin column where the product and

remaining substrate would adhere to the column. The remaining enzymes and cofactors would pass though the column into the reaction mixture where the quantities could be topped up by exogenous amounts. The product adsorbed onto the resin could be eluted by a solvent wash and collected in an appropriate vessel.

This would work by adding the substrates, enzymes and cofactors together into the bioreactor to carry out the asymmetric synthesis. After the reaction reached completion, the reaction mixture would then be passed down a column of XAD L-323 resin where the product would bind and the enzymes and cofactors would be recycled back into the reactor where more substrate could be added. More enzyme and cofactor could also be added along with the substrate to top-up the volume as there may be losses in the XAD column. The product would be eluted from the XAD resin using a solvent, in this case, ethanol. Using ethanol as an eluant would give a final product concentration of 10g/L. A higher product concentration could be achieved by using a solvent with a higher solubility of 6-bromo-β-tetralol. The batch reactor configuration established for the regenerative bioreduction of 6-bromo-β-tetralone is convenient for use in the laboratory-scale but it has a number of disadvantages. Firstly, the physical separation of the components in the reaction solution requires an additional step. This extra step in the model system is the addition of the polymeric resin for the adsorption of the product, and is required for successful recycling the cofactor and enzymes from one batch to the next. Secondly, there is inhibition of tetralone reductase by NADH but the effects of this are removed by using a cofactor regeneration system. Thirdly, processing of 6-bromo-β-tetralone and 6-bromo-β-tetralol requires high dilutions and thus larger reaction volumes due to their low aqueous solubility.

Improvements to the current system would include increasing tetralone reductase concentration and thus reducing the reactor volumes. This could be achieved through over-expression of the enzyme into a microorganism. Currently, the enzyme concentration in *Trichosporon capitatum* (MY 1890) is assumed to be in the

region of 1 x 10^{-2} % (g/g), increasing this to 10% of the cell protein may have a number of consequences. It would potentially increase enzyme stability (Johnson *et al.*, 1978), the purification stages may be easier and the reaction rates would potentially increase.

The reaction configuration discussed previously is not limited to free two-enzyme systems with cofactor regeneration. For example, in the model system if both the enzymes (FDH and tetralone reductase) could be immobilized using a resin which 6-bromo-β-tetralone and 6-bromo-β-tetralol did not adhere onto, the same bioreactor methodology could still be used (Figure 4.18).

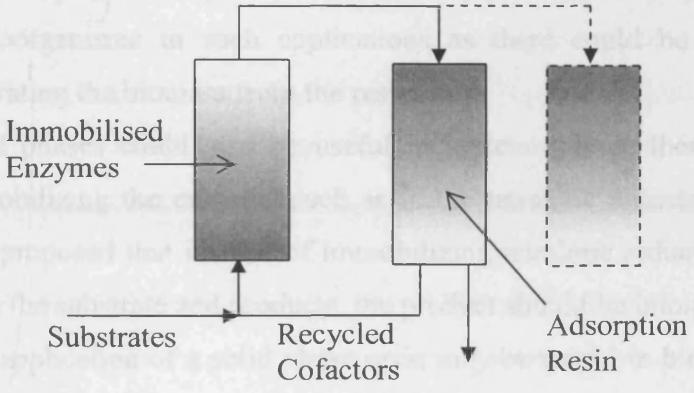


Figure 4.18 Theoretical Method for the Use of Resins in Continuous Operation. *The synthetic and regenerative enzymes are immobilized onto a resin in a column, the substrate is passed through the enzyme column and converted, the product is then passed through an adsorption column along with the cofactors. The product binds to the adsorption resin but the cofactors pass through the column and are recycled back into the enzyme column with more substrate. The columns could be duplicated, thus allowing the adsorption onto one column whilst the elution of the product from the other column was occurring.*

The immobilized enzymes could be retained in a column where the cofactor and other reaction components could be passed through the immobilized enzyme column

where the reaction would take place. The reacted components would then be passed through an XAD column where the product (and unconverted substrate would adsorb), recycled cofactors, and new substrate would be recycled back into the immobilized enzyme bioreactor column in continuous operation

Solid phase components applied for *in situ* product adsorption have the potential to be used in a number of other biocatalytic systems (not just for cofactor requiring enzyme applications) including:

1. Whole cell biocatalysis, where the product can be removed from the reaction solution onto a resin, however, there may be difficulties in using whole cell microorganisms in such applications as there could be the difficulty in separating the biomass from the resins.
2. Solid phases could also be useful in systems where there is difficulty in immobilizing the enzyme, such as in the tetralone reductase case, where it was proposed that instead of immobilizing tetralone reductase to separate it from the substrate and products, the product should be immobilized instead.
3. The application of a solid phase resin may be useful in biocatalytic systems which have compounds which exhibit low solubility in aqueous environments. Instead of using a solvent, the substrate could be pre-bound onto the resin and then added to the reaction solution. This removes the need for adding a third component such as a solvent to increase the solubility of the substrate.
4. There may also be the need to control the amount of substrate and product delivered if the components are toxic to the biocatalyst, and this could be achieved using the substrate adsorbed to a solid phase.

A common problem of many oxidoreductase processes is the substrate and product toxicity to the cell. Methods to deliver substrates via an adsorption resin to control the addition of the substrate and also to use the same resin to capture the product are

relatively new. An early use of a resin to enhance a bioreduction was for the reduction of a methyl benzyl ketone via Baker's yeast (Anderson *et al.*, 1995). The substrate was adsorbed onto XAD 7 and added to the reaction mix. The substrate diffused into the solution, was reduced by the microorganism, and then re-adsorbed back onto the resin. The adsorbent resin, XAD 7, has also been used to reduce the toxicity of a substrate and its respective product to a microorganism in a whole cell stereoselective ketone reduction (Vicenzi *et al.*, 1999). In this system, XAD 7 was used as a method of substrate delivery and product removal. 3,4-methylene-dioxyphenyl acetone was reduced to the corresponding *S*-3,4-methylene-dioxyphenyl isopropanol in greater than 95% isolated yield and 99.9% enantiomeric excess. The supply of the substrate and the removal of the product via a polymeric hydrophobic resin allowed the reaction concentration to be increased from 6g/L to 40g/L, with an overall productivity of 75g/L/day. This method of substrate delivery and product removal has also been described for the Baeyer-Villiger oxidation, where the yield was increased from 1g/L to 20g/L (Simpson *et al.*, 2001). The Baeyer-Villiger oxidation has also been applied in a bubble column reactor with substrate delivery and product adsorption using a polymeric resin (Hilker *et al.*, 2004). The results gave similar productivities to previous experiments by the same group. More recently, resins have been applied in immobilised enzyme systems where the substrate was delivered using Amberlite (IRA 400) resin (Rojanarata *et al.*, 2004). The methods, which had not been optimised, improved the product concentration by 4-fold. Enhanced enantioselectivity, chemoselectivity and space-time yields for biotransformations of α,β-unsaturated carbonyl compounds through the use of absorbing resins have also been reported (D'Arrigo *et al.*, 1997, 1998) (Nakamura *et al.*, 2002) (Houng and Liau, 2003). In cases as just described, the properties of the substrate and the product must be such that the substrate can be easily desorbed from the adsorbent resin and the product easily adsorbed back onto the resin. The limitations of this method would depend upon the affinity the substrate and product have for the resin. This theory has recently been confirmed (Straathof, 2003) where it is suggested that the charge and the solubility of the

substrates and products are important factors in confirming the usefulness/limitations of adsorption resins for substrate delivery and removal.

The employment of solid phase resins in biocatalytic reactions has served to: (1) control the movement of substrates and products, and (2) enhance enantioselectivity. The discovery of resins for these applications is in its infancy and may have wide-ranging possibilities in biocatalytic reactions for whole cell biocatalysts, immobilised enzymes, and free enzymes. A solid phase method of substrate delivery and product removal removes the requirement for the addition of solvents in these reactions and, thereby, removing any compromise between enzyme stability and solvent type and volume. If 6-bromo-β-tetralone could be delivered to the aqueous enzyme solution and 6-bromo-β-tetralol extracted from the aqueous solution by the use of adsorbent resins, this would potentially have a great impact on the reaction efficiency. The removal of solvent completely from the reaction solution would serve to maintain enzyme activity; delivery of the substrate via the use of an adsorbent resin would control the amount of substrate in the reaction at any one time and limit substrate inhibition. Removal of the product by selecting a resin with a high affinity for the product would ensure that there is limited product inhibition. However, 6-bromo-β-tetralol has a higher solubility in aqueous solutions than 6-bromo-β-tetralone, and therefore the resin selected to deliver the substrate may not be appropriate to remove the product from the aqueous solution. In this case it may be appropriate to use two types of resin, where one which exhibits a high affinity for 6-bromo-β-tetralone is used to deliver the substrate, and one, which exhibits a high affinity for 6-bromo-β-tetralol is used to extract the product. A method which uses two different resins for the substrate delivery and product removal would still be applicable to cofactor requiring enzymes. It may, however, be necessary to screen the adsorbent resins, in particular, the resin which is used for the adsorbance of the lower solubility compound for the adsorbance of the hydrophilic cofactors and enzymes to ensure that the cofactors and enzymes do not adsorb. If either the cofactor or the enzyme adsorbed onto the resin then it would be

less practical to recycle the enzymes and cofactors for further reactions and thus the reaction due to the high cost of cofactors would not be efficient.

4.5 Summary

Tetralone reductase can be used as a free enzyme in a low solvent single phase system for the bioreduction of 6-bromo-β-tetralone to 6-bromo-β-tetralol, with cofactor regeneration. The immobilisation of tetralone reductase onto a solid support to facilitate a continuously operating bioreactor was not possible due to the subsequent adherence of 6-bromo-β-tetralone and 6-bromo-β-tetralol to the immobilisation support (Eupergit C™). This adsorbance phenomenon was employed instead to promote *in situ* removal of the product and unconverted substrate. An alternative bioreactor configuration was established for the bioreduction of 6-bromo-β-tetralone by tetralone reductase. In the bioreactor configuration, the reaction components were added together in batch, and the regeneration of NAD(H) was completed by the addition of formate dehydrogenase (FDH) and formate. The bioreduction was monitored and after the reaction showed completion, XAD L-323 was added to the reaction mixture and the product adsorbed from the reaction mix onto the resin. The resin was filtered and the product was eluted from the XAD resin in a highly pure form using a solvent. The remaining components (tetralone reductase, FDH and NAD(H)) were recycled back into the reactor, where more substrates were added and the reaction continued. This method was successful for at least two reaction passes. It is possible that the system could be improved further by the application of resins to introduce the substrate as well as separate the enzyme from the product.

In the following chapter, the whole cell biocatalyst, *Trichosporon capitatum* (MY 1890), will be characterised with respect to the substrate delivery, solvent tolerance, and the process requirements; this will then allow a comparison of the isolated enzyme and whole cell biocatalysts systems.

The Bioreduction of 6-Bromo-β-Tetralone by the Whole Cells of *Trichosporon capitatum* (MY 1890)

5.1 Introduction

The vast majority of enantioselective bioreductions for the synthesis of chiral alcohols are conducted using intact whole cells (Straathof *et al.*, 2002). This is because firstly, the fermentation technology is available and already in place, and secondly, no external cofactor regeneration is required as the regeneration is carried out as part of the cells own metabolism. Whole cell oxidoreductases have been used for both laboratory and large scale syntheses of pharmacologically valuable compounds, many of which have been reviewed (Zaks and Dodds, 1997). The bakers' yeast *Saccharomyces cerevisiae* is one of the favoured model organisms for basic biological research and was the first eukaryote whose genome was entirely sequenced (Dujon, 1996). As a consequence of this, the potential for this microorganism as a valuable biocatalyst has greatly improved. *Saccharomyces cerevisiae* has also been has been successfully exploited to carry out a large-scale bioreductions (Kometani *et al.*, 1993). The difficulties associated with whole cell biocatalysis include; competing reactions, mass transfer limitations and difficult downstream processing. Some competing enzymes may be eliminated by media optimisation, which can alter the cells metabolic pathway (Reddy *et al.*, 1996). Stereoselectivity can also be improved by genetic engineering. This has been observed in bakers' yeast where reductases were either knocked out or overexpressed (Rodriguez *et al.*, 1999). Mass transfer limitations through the cell membranes mean that catalytic activities of many intracellular enzymes are not seen to be adequately exhibited by the whole cells. This may be due to the permeability barrier of the cell membrane to the substrates and products. Mass transfer limitations can also occur because lipophilic substrates and products can incorporate themselves into the cell membranes (Angelova *et al.*, 1999). In the case of the hydroxylation of steroids, the hydroxylated product exhibits a higher solubility than

the product so that the transport out of the cell may be facilitated (Angelova *et al.*, 1999). This, however, is only the case for steroids, and products with lower aqueous solubilities may accumulate in the membrane and other cell surfaces causing irreparable cell damage. The accumulated lipophilic products can only usually be removed by the addition of a solvent therefore preventing the reuse of the cells. Lipophilic compounds such as steroids and their problems have been reviewed (Angelova *et al.*, 1999). Whole cell biocatalysis in organic media has also been reviewed (León *et al.*, 1998). The objectives of this Chapter are to investigate the bioreduction of 6-bromo-β-tetralone by the whole cells of a soil organism *Tricosporon capitatum* (MY1890). To determine the potential for using the cells in the bioreduction the following factors will be investigated: bioreduction methodology (e.g. growing cells/ resting cells), the effects of solvent on the bioreduction, the effects of substrate on the bioreduction, also potential improvements to the whole cell biocatalytic system will be discussed.

5.2 Analytical Techniques

5.2.1 Extraction of Substrate and Product from the Cell

The substrate and product were extracted from the cell suspension by addition of the same volume of solvent to cells. The solutions were vortexed to mix and then centrifuged for 10min at 10000rpm. The supernatant was used to determine the quantities of 6-bromo-β-tetralone and 6-bromo-β-tetralol using the HPLC assay (Chapter 2.8.2).

5.3 Materials and Methods

5.3.1 Production of Trichosporon capitatum (MY 1890)

1ml of frozen *Trichosporon capitatum* (MY 1890) stock was used to inoculate 50ml of media, the cells were incubated at 28^0C for 48hr. The final dry cell concentration was 18g/L.

5.3.2 Whole Cell Bioconversion of 6-bromo-β-tetralone

50ml of cells were grown to a dry cell weight of 18g/L in a 250ml Erlenmeyer flask. 5ml and 10ml of a 10g/L 6-bromo-β-tetralone in ethanol solution were added to the 50ml cell solutions. The cells were agitated on an orbital shaker. The bioconversions were monitored by assaying for 6-bromo-β-tetralone and 6-bromo-β-tetralol using the HPLC assay (Chapter 2.7.2).

5.3.3 Product Recovery Methods

Cells were grown to a dry cell weight of 18g/L; 9 × 10ml were aliquoted into 50ml reaction vessels. 1ml of a 10g/L 6-bromo-β-tetralone in ethanol solution was added to the cells (final substrate concentration 1g/L). The bioconversion was allowed to proceed for 2hrs. Nine methods of product (and unconverted substrate) extraction techniques were employed:

a. The first method measured the amount of 6-bromo-β-tetralone and 6-bromo-β-tetralol in the supernatant after the reaction mix had progressed for 2hrs.
b. The second method left the reaction to progress for 2hrs, sonicated the cells for 5 passes (10secs on, 10secs off at 8MHz), and then the supernatant was assayed for 6-bromo-β-tetralone and 6-bromo-β-tetralol.
c. The third method was as the method 2, but the cells were sonicated for 10 passes.
d. After the cells had been left to progress for two hours, 1ml of the cells was aliquoted into an Eppendorf and 1ml of ethanol added, and then the supernatant was measured for 6-bromo-β-tetralone and 6-bromo-β-tetralol.
e. The fifth method was the same as the fourth method except 1ml of acetone was used instead of ethanol.
f. This method used sonication as 2 and then 1ml of ethanol was added as 4.
g. As 6, but acetone instead of ethanol was used.
h. As 4, but 2ml of ethanol was used in 1ml of cells.
i. As 8, but acetone was used instead of ethanol.

The supernatant from each method was assayed by vortexing and then centrifugation of the cells for 10min at 10000rpm. The assay for 6-bromo-β-tetralone and 6-bromo-β-tetralol was completed by HPLC (Chapter 2.7.2).

5.3.4 Bioconversion with Substrate and Product Extraction

50ml of cells were grown to 18g/L (dcw); 10ml of the cells were aliquoted into 4 × 50ml reaction vessels. Volumes of 250µl, 500µl, 1ml, 1.5ml of a 10g/L 6-bromo-β-tetralone in ethanol were added to the cell solutions. The reaction vessels were shaken on an orbital shaker. The bioconversions were monitored by assaying for 6-bromo-β-tetralone and 6-bromo-β-tetralol using the HPLC assay (Chapter 2.7.2).

5.3.5 The Effects of Removing the Growth Media

50ml of cells were grown to 18g/L (dcw) and harvested by centrifugation (Beckman Coulter, J2 M1). The pellet was re-suspended in 50ml of 10mM Tris pH7, re-centrifuged and re-suspended in 50ml 10mM Tris pH7. 10ml of the washed cells were aliquoted into 4 × 50ml Sterelin™ tubes and then 250µl, 500µl, 1ml, and 1.5ml of a 10g/L 6-bromo-β-tetralone in ethanol solution were added to the cell solutions. The Sterelin™ reaction vessels were shaken continuously on an orbital shaker. The bioconversions were monitored by assaying for 6-bromo-β-tetralone and 6-bromo-β-tetralol using the HPLC assay (Chapter 2.7.2).

5.3.6 The Effects of Ethanol and Methoxyethanol

50ml of cells were grown to 18g/L (dcw); 10ml of the cells were aliquoted into 8 × 50ml reaction vessels. 6-bromo-β-tetralone in ethanol was added to 4 × cell solutions to give initial substrate concentrations of 0.5g/L, 1g/L, 1.5g/L and 2g/L; the respective volumes of solvent were 5% 10% 15% and 20%. This was achieved using a stock solution of 10g/L. 6-bromo-β-tetralone in methoxyethanol was added to 4 × cell solutions to give initial substrate concentrations of 0.5g/L, 1g/L, 1.5g/L

and 2g/L; the respective volumes of solvent were 0.4%, 0.8%, 1.25% and 1.6%. This was achieved using a stock solution of 120g/L. The reaction was run for 7hr and the samples were taken and assayed by HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone and 6-bromo-β-tetralol at defined time points.

5.3.7 The Effects of Solvent Volume

50ml of cells were grown to 18g/L (dcw) and 10ml aliquoted into 5 × 50ml Sterelin™ tubes. Stock solutions of 50.5g/L, 25.5g/L, 13g/L, 6.75g/L and 4.7 g/L of 6-bromo-β-tetralone in methoxyethanol solution were added to the cells in 100µl, 200µl, 400µl, 800µl, 1200µl volumes respectively. This produced an initial substrate concentration of 0.5g/L in each tube. The reaction was run for 6.5hr and the samples were taken and assayed by HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone and 6-bromo-β-tetralol at defined time points.

5.3.8 The Effects of Initial Substrate Concentration

50ml of cells were grown to 18g/L (dcw) and 10ml aliquoted into 6 × 50ml Sterelin™ tubes. 6-bromo-β-tetralone was dissolved in 100µl of methoxyethanol and added to the cells to give final substrate concentrations of 0.5g/L, 1g/L, 1.5g/L, 2g/L, 2.5g/L, 3g/L. The reaction was run for 7.5hr and the samples were taken and assayed by HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone and 6-bromo-β-tetralol at defined time points.

5.3.9 The Effects of Exogenous Quantities of NADH

50ml of cells were grown to 18g/L (dcw) and 10ml aliquoted into 4 × 50ml Sterelin™ tubes. NADH was added to the cells with 1ml of 0g/L (control), 0.25g/L, 0.5g/L, 0.75g/L NADH in 10mM Tris buffer pH7. The reactions were initiated using 100µl of a 100g/L tetralone in methoxyethanol. The bioconversions were

monitored by assaying for 6-bromo-β-tetralone and 6-bromo-β-tetralol using the HPLC assay (Chapter 2.7.2).

5.3.10 Growth of E. coli

0.5ml of frozen *E. coli* JM107 pQR711 stock was used to inoculate 50ml of LB media (10g/L bactotryptone, 10g/L NaCl, 5g/L yeast extract). The cells were left to grow at 37^0C for 16hr on an orbital shaker.

5.3.11 The Effects of 6-bromo-β-tetralone on E. coli JM107 pQR711

Cells of *E. coli* were aliquoted into 5 × 10ml samples, where 1ml, 0.75ml, 0.5ml or 0.25ml of a 10g/L 6-bromo-β-tetralone in ethanol solution was added to the cells. The cells were mixed on an orbital shaker and the supernatant assayed after 1hr and 3hr. The cells were vortexed in ethanol, centrifuged and the supernatant assayed by HPLC (Chapter 2.7.2) for 6-bromo-β-tetralone and 6-bromo-β-tetralol.

5.4 Results

5.4.1 Whole Cell Bioconversion of 6-bromo-β-tetralone

A whole cell bioconversion was carried out to show that *Trichosporon capitatum* (MY1890) can convert 6-bromo-β-tetralone into 6-bromo-β-tetralol. Analysis of the quantities of 6-bromo-β-tetralone and 6-bromo-β-tetralol in the reaction mix during the course of the bioconversion showed that (under the conditions used in the bioreduction) a full mass balance of the reaction components was not obtained. The results showed that 6-bromo-β-tetralone was removed from the reaction mixture within 1hr of the reaction starting but a concurrent increase of product was not visible (Figure 5.1). After 1hr when an initial 6-bromo-β-tetralone concentration of 0.5g/L was used, there was approximately 0.15g/L of 6-bromo-β-tetralol and 0.02g/L of 6-bromo-β-tetralone in solution. Thus, 0.33g/L of either substrate or product was not measured in the supernatant. Similar results can also be observed at

different substrate and solvent concentrations (Appendix B1.3 and B1.4). Under the conditions used for the bioreductions and the associated methods it currently does not prove that an efficient bioreduction is possible. One reason that the substrate and product were not accounted for may be that they degraded into components which did not show up during the analysis. Another reason that the components were not accounted may be because the substrate and product were not secreted into the cell environment and became associated within the cell membrane. Diffusion of a substrate across the cell membrane is strongly correlated with the molecule's solubility in lipids (Bailey and Ollis, 1986), and the model substrate and product are lipophilic compounds. It is hypothesized that the mass balance can be completed by analysing the cell debris.

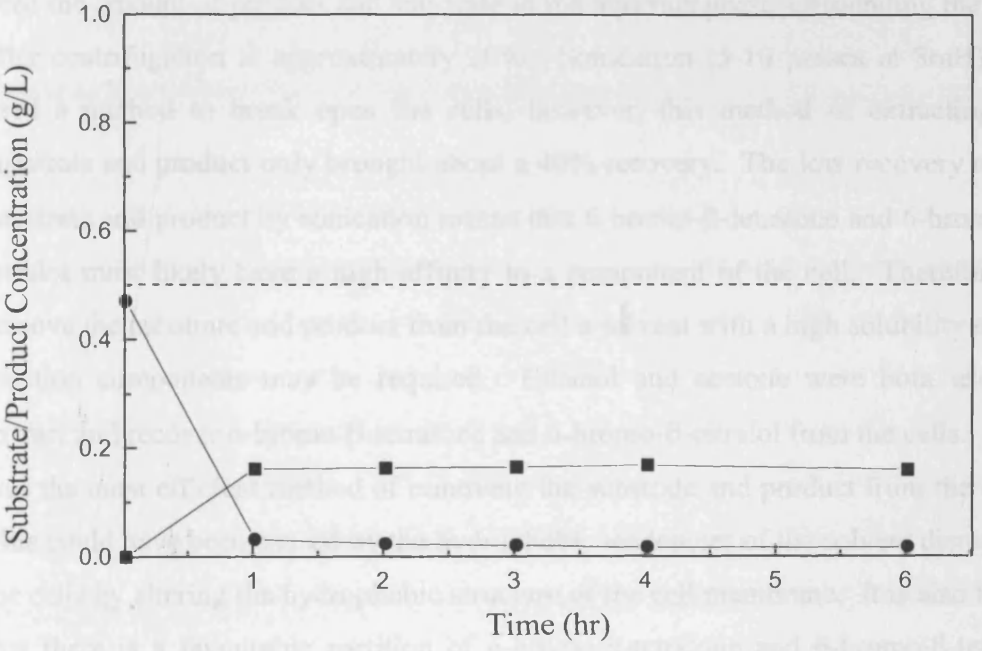


Figure 5.1 Whole Cell Bioconversion

A whole cell bioconversion of 6-bromo-β-tetralone (●) to 6-bromo-β-tetralol (■). Initial substrate concentration 0.5g/L (---) with a solvent concentration 5% (v/v). The graph illustrates how the substrate is rapidly removed from the aqueous media but a concurrent increase in product is not observed. Results using higher levels of

6-bromo-β-tetralone are shown in Appendix B 1.3 and 1.4. (Reference Section 5.3.2 for methodology)

5.4.2 Product Recovery Methods

The results from Section 5.4.1 demonstrated that a high percentage of the product and unconverted substrate is not in the aqueous phase surrounding the cells, but was most likely associated with the cells. Experiments were set up to show that a full mass balance could be achieved by extracting the substrate and product from the centrifuged cell debris. A number of different techniques were employed to try to discover which method would be the most effective. Cells were incubated with 1g/L of 6-bromo-β-tetralone for 2hr; the cells were then put through a number of different disruption techniques. The results show (Figure 5.2) that when 1g/L of substrate is used the amount of product and substrate in the aqueous phase surrounding the cells after centrifugation is approximately 20%. Sonication (5-10 passes at 8mH) was used a method to break open the cells, however, this method of extracting the substrate and product only brought about a 40% recovery. The low recovery of the substrate and product by sonication means that 6-bromo-β-tetralone and 6-bromo-β-tetralol most likely have a high affinity to a component of the cell. Therefore, to remove the substrate and product from the cell a solvent with a high solubility of the reaction components may be required. Ethanol and acetone were both used to extract and recover 6-bromo-β-tetralone and 6-bromo-β-tetralol from the cells. This was the most efficient method of removing the substrate and product from the cells. This could have been caused by the hydrophobic tendencies of the solvent disrupting the cells by altering the hydrophobic structure of the cell membrane. It is also likely that there is a favourable partition of 6-bromo-β-tetralone and 6-bromo-β-tetralol into the solvents. Extraction by the addition of a solvent was also the simplest and most effective method to obtain a mass balance. These results show that a full mass balance can be achieved for the substrate and product, but the extraction has to be done via a solvent (not sonication), since it is likely that the substrate and product accumulate in the cell, probably on the cell membrane.

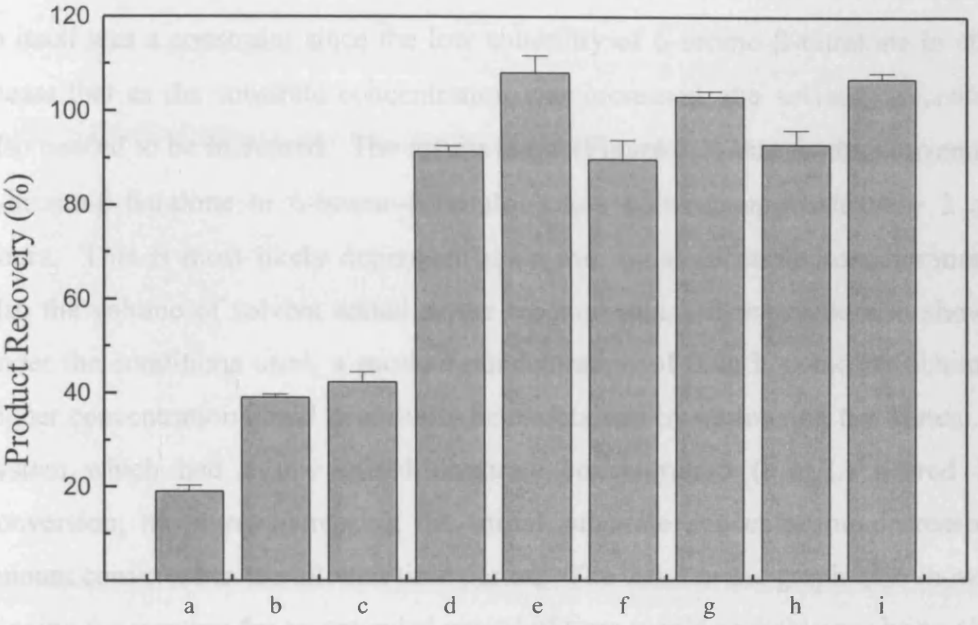


Figure 5.2 Methods of Substrate and Product Recovery

Methods of substrate and product recovery: (a) Concentration in the supernatant, (b) Concentration in supernatant after a 5 pass sonication (10 seconds on, 10 seconds off at 8mH) and centrifugation, (c) Concentration in supernatant after a 10 pass sonication (10 seconds on, 10 seconds off at 8mH) and centrifugation, (d) Extraction with 1ml ethanol, (e) Extraction with 1ml acetone, (f) Extraction as (b) and then addition of 1ml ethanol then centrifugation, (g) Extraction as (c) and then addition of 1ml acetone then centrifugation, (h) Extraction with 2ml ethanol, (i) Extraction with 2ml acetone. (Reference Section 5.3.3 for methodology).

5.4.3 Whole Cell Bioconversion of 6-bromo-β-tetralone with Recovery

Four bioconversions were run using substrates at different concentrations to investigate the whole cell bioreduction. Solvent extraction was used to remove the product and unconverted substrate. Ethanol was used to solubilise the substrate; this in itself was a constraint since the low solubility of 6-bromo-β-tetralone in ethanol meant that as the substrate concentration was increased, the solvent concentration also needed to be increased. The results show (Figure 5.3) that the bioconversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol takes between approximately 2 and 6 hours. This is most likely dependent upon the initial substrate concentration and also the volume of solvent added to the reaction mix. The results also show that under the conditions used, a product concentration of 0.8g/L could be obtained; a higher concentration could potentially been obtained by optimising the system. The system which had a low initial substrate concentration (0.2g/L) neared 100% conversion; however, increasing the initial substrate concentration decreased the amount converted in the allotted time period. The trend in the graph also shows that running the reaction for an extended period of time would probably not bring about a 100% conversion in the other systems. This reduction in conversion is probably caused by the inhibitory effects of high substrate and solvent concentrations causing irreparable damage to the cell metabolism. At the higher substrate concentrations there may also be effects of product inhibition which are not seen at the lower substrate concentrations. The effects of substrate and solvent concentration on the biocatalytic reaction rates and conversions are discussed in greater detail in this chapter. In general, these results suggest that there are a number of components in the reaction which constrain the bioconversion (even at low levels of substrate). This could be the effects of solvent, substrate or product on the enzyme. It is also probable that there is not one component which limits the bioreduction; it is likely that there is a combination of effects which limit the bioreduction. Further investigation is required into the effects on the biocatalyst using each of the components individually. This can be accomplished by maintaining either the

substrate or solvent concentration at a set value and varying the other component; this is discussed later in this chapter.

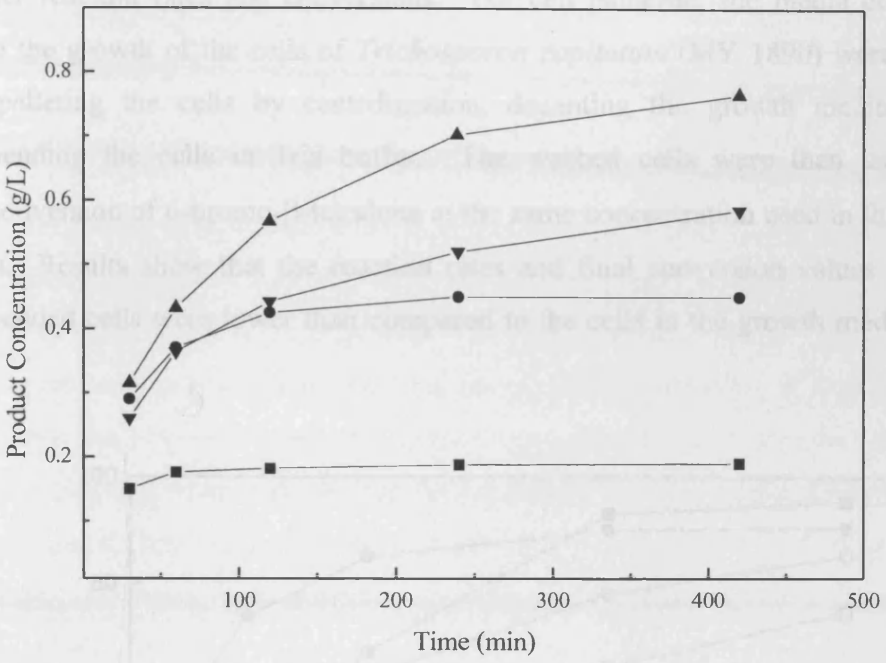


Figure 5.3 Whole cell Bioreduction of 6-bromo-β-tetralone to 6-bromo-β-tetralol

A 10g/L solution of 6-bromo-β-tetralone in ethanol was added to 10ml of cells in volumes of 2.4%, 0.5%, 9.0%, 13.0% to give respective substrate concentrations of 0.2g/L (■), 0.5g/L (●), 0.9g/L (▼), 1.3g/L (▲). 6-bromo-β-tetralone and 6-bromo-β-tetralol were extracted from the cells via the addition of a solvent, centrifugation was used to pellet the cell debris. The supernatant was assayed for 6-bromo-β-tetralone and 6-bromo-β-tetralol. The graph shows that the bioreduction of 6-bromo-β-tetralone to 6-bromo-β-tetralol can be achieved by the addition of 6-bromo-β-tetralone in ethanol, however, there are effects on the rates of reaction and the conversions by increasing either the substrate and/or the solvent concentration. (Reference Section 5.3.4 for methodology).

5.4.4 *Comparison of Growing and Resting Cells*

A comparison of the growing cells of *Trichosporon capitatum* (MY 1890) was investigated to determine which of the reaction systems would have potentially higher reaction rates and conversions. For cell isolation, the media components from the growth of the cells of *Trichosporon capitatum* (MY 1890) were removed by pelleting the cells by centrifugation, decanting the growth media, and re-suspending the cells in Tris buffer. The washed cells were then used in the bioconversion of 6-bromo-β-tetralone at the same concentration used in the growing cells. Results show that the reaction rates and final conversion values of the re-suspended cells were lower than compared to the cells in the growth media (Figure 5.4).

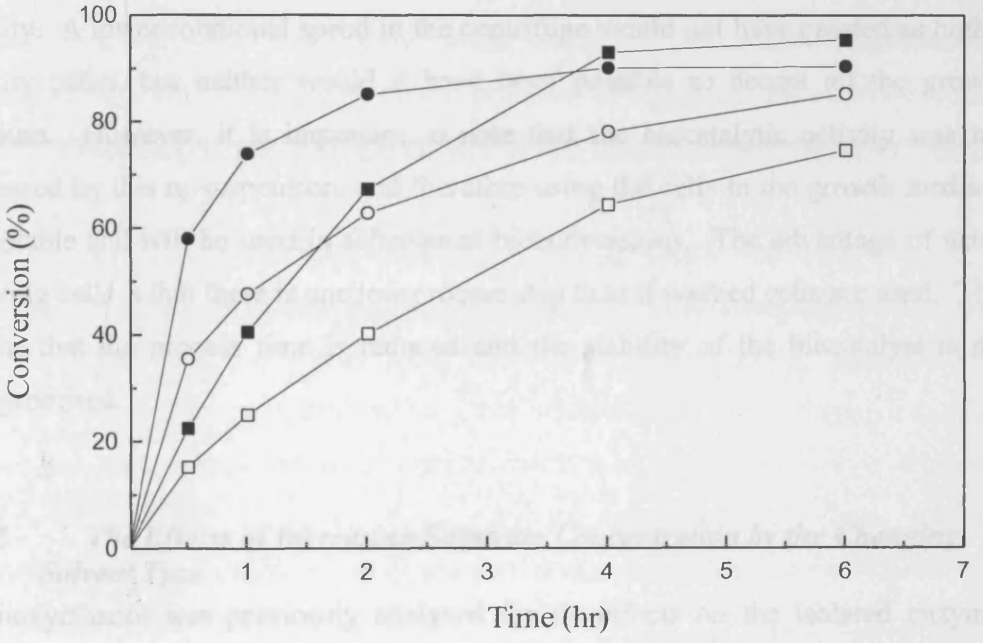


Figure 5.4 Comparison of Resting and Growing Cell Biocatalytic Activity

The bioreduction of 6-bromo-β-tetralone to 6-bromo-β-tetralol using resting cells (■□) and growing cells (●○). A 10mg/ml stock solution of 6-bromo-β-tetralone in ethanol was added to the cells at volumes of 1ml (○□) and 0.5ml (●■). The

results show that when the cells are washed and re-suspended in Tris buffer they exhibit lower initial activities and lower conversions than for the growing cells. The results also show that the reaction rates at the higher substrate concentration (and higher solvent concentrations) are lower for both the growing and resting cells. (Reference Section 5.3.5 for methodology).

The results suggest that centrifugation of the cells and re-suspension in Tris buffer has a detrimental effect on the activity of the cells. This may be due to disruption of the cell components during centrifugation or it may be due to the difficulty in the re-suspension of the cells after pelleting. The re-suspended cells may have had lower surface areas if the cells had not been re-suspended properly. It was difficult to resuspend the cells since the centrifugation process produced a pellet of high cell density. A lower rotational speed in the centrifuge would not have created as high a density pellet, but neither would it have been possible to decant all the growth medium. However, it is important to note that the biocatalytic activity was not increased by this re-suspension, and therefore using the cells in the growth media is acceptable and will be used in subsequent bioconversions. The advantage of using growing cells is that there is one less process step than if washed cells are used. This means that the process time is reduced and the stability of the biocatalyst is not compromised.

5.4.5 The Effects of Increasing Substrate Concentration by the Changing Solvent Type

Methoxyethanol was previously analysed for its effects on the isolated enzyme, where tetralone reductase had previously shown comparable reaction rates in ethanol and methoxyethanol (Chapter 3). The following experiment was carried out to determine whether the addition of high levels of ethanol limits the whole cell bioconversion, and whether if methoxyethanol was employed (in lower amounts – with a higher substrate concentration) the whole cell would benefit by showing increased reaction rates and higher conversions. The main critical difference in the

characteristics of the solvents is their solubility of 6-bromo-β-tetralone; methoxyethanol dissolves 6-bromo-β-tetralone to a concentration of 250g/L, where ethanol dissolves 6-bromo-β-tetralone to a concentration of 10g/L. The results show (Figure 5.5) that when methoxyethanol was used to dissolve 6-bromo-β-tetralone the initial biocatalytic activity increases with increasing concentration of solvent, however, at higher solvent concentrations there is a decrease in the rate of increase. The initial activity of the whole cell increases with a methoxyethanol content of up to 1.6% (it was not measured after this point) (Figure 5.5). The effects of ethanol on the biocatalytic activity were significantly different to the effects of methoxyethanol. There was no dramatic increase in biocatalytic activity; however there was a small increase, followed by a small decrease in activity at solvent concentrations greater than 10%. The results are comparable in that an increase in solvent increases the initial rate of reaction, although this increase was less so for ethanol. A decrease in activity was not seen when using methoxyethanol, however it is expected that as the solvent volume increases, perhaps up to 10% a decrease would be seen. To get comparable substrate concentrations in ethanol a solvent amount 25 times greater than methoxyethanol needs to be used. It is this high volume of solvent which most likely had an adverse effect on the biocatalytic activity. The conversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol using the whole cell biocatalyst decreases with increasing solvent concentration when either ethanol or methoxyethanol is used as the solvent, the decrease in substrate conversion is more pronounced when ethanol is used as the solvent (Figure 5.6). At the substrate concentration of 0.5g/L (5%(v/v) ethanol, 0.4%(v/v) methoxyethanol) the conversion is approximately 90% at both the concentrations. A decrease from 90% to 70% when the ethanol concentration was doubled is observed, whereas a doubling of the methoxyethanol did not see a decrease within the limits of error. At an ethanol concentration of 20% the cells did not convert any substrate; this is consistent with the biocatalytic activity which was negligible at this solvent level. This is consistent with the hypothesis that a solvent with a higher solubility of substrate is less detrimental to the biocatalytic activity of

the cells. This is demonstrated via the higher reaction rates and conversions which were achieved when methoxyethanol (not ethanol) was used.

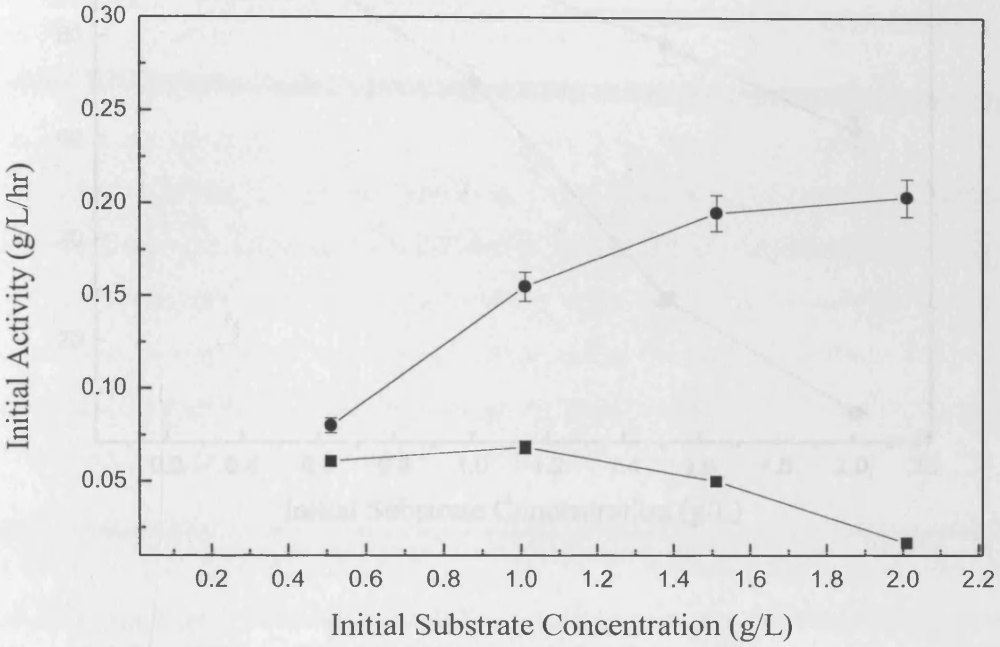


Figure 5.5 The Effects of Type of Solvent on the Biocatalytic Activity

Initial activity of the whole cells when ethanol is used as the solvent (■). Ethanol dissolves 6-bromo-β-tetralone to a concentration of 10g/L. Methoxyethanol dissolves 6-bromo-β-tetralone to a concentration of 250g/L, but used here at 120g/L. The solvent volumes for ethanol are 5%, 10%, 15% and 20% respectively. Initial activity of the whole cell biocatalyst when methoxyethanol is used as the solvent (●). The solvent volumes for methoxyethanol are 0.4%, 0.8%, 1.25% and 1.6% respectively. The graph shows that the preferred solvent is methoxyethanol as the initial activities increase with increasing solvent. (Reference Section 5.3.6 for methodology).

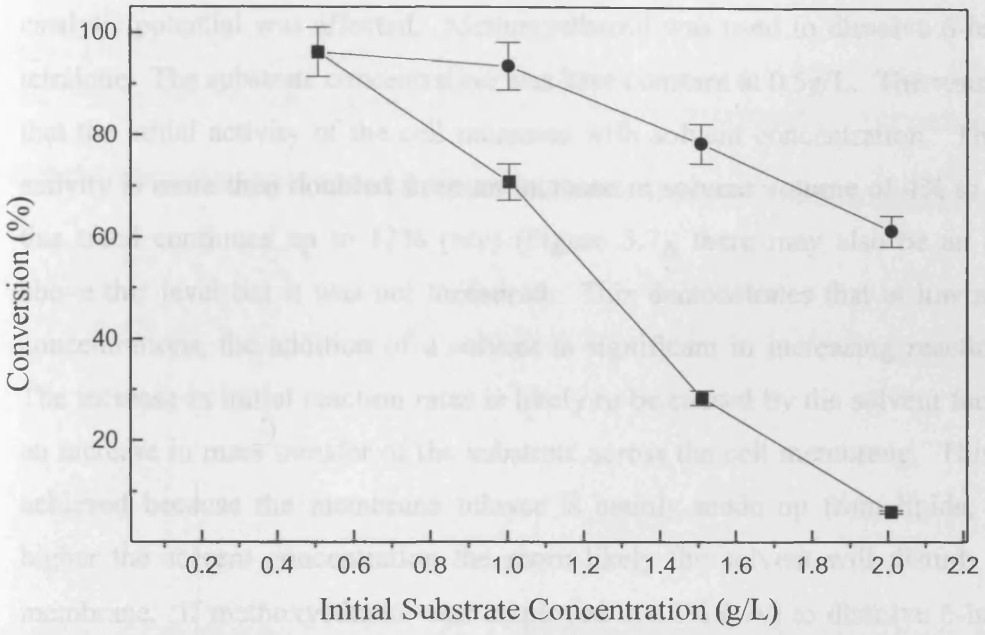


Figure 5.6 The Effects of Type of Solvent on the Conversion

Final conversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol using whole cells and ethanol as the solvent (■). Ethanol dissolves 6-bromo-β-tetralone to a concentration of 10g/L. The solvent volumes for ethanol are 5, 10, 15 and 20% respectively. Final conversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol using whole cells and methoxyethanol as the solvent (●). The solvent volumes for methoxyethanol are 0.4, 0.8, 1.25 and 1.6% respectively. The results show that the final conversions are detrimentally affected by the addition of either solvent, however, the reduction in conversion is more pronounced for ethanol. (Reference Section 5.3.6 for methodology).

5.4.6 The Effect of Solvent Volume on the Whole Cells

The effect of the amount of solvent on the biocatalytic activity was measured to determine the levels of solvent in solution which could be used before the cells catalytic potential was affected. Methoxyethanol was used to dissolve 6-bromo-β-tetralone. The substrate concentration was kept constant at 0.5g/L. The results show that the initial activity of the cell increases with solvent concentration. The initial activity is more than doubled from an increase in solvent volume of 4% to 8% and this trend continues up to 12% (v/v) (Figure 5.7), there may also be an increase above this level but it was not measured. This demonstrates that at low substrate concentrations, the addition of a solvent is significant in increasing reaction rates. The increase in initial reaction rates is likely to be caused by the solvent facilitating an increase in mass transfer of the substrate across the cell membrane. This can be achieved because the membrane bilayer is mainly made up from lipids, and the higher the solvent concentration the more likely the solvent will disturb the cell membrane. If methoxyethanol was employed at 12% (v/v) to dissolve 6-bromo-β-tetralone at the limit of solubility (250g/L) in a reaction solution the amount of substrate supplied could be up to 30g/L. This would be a significant increase compared to the isolated enzyme productivity. In contrast to the reaction rates, the final conversions decreased with solvent volume (Figure 5.8). At high levels of solvent a decrease from 92% conversion in 1% solvent to 86% conversion in 12% solvent can be observed. This reduction in conversion is most likely associated with the solvent since all the other components were kept constant. It may be that the solvent brought about longer term detrimental effects on the cell, possibly a slow degradation of the cell metabolism, which was not seen during the early stages of the reaction. In comparison to the previous experiments (Chapter 5.5.4), the rates of reaction from this experiment are higher at the same solvent volumes used. However, the substrate concentration tested in this experiment (0.5g/L) was lower than the concentrations used previously. This probably means that the substrate concentration also has a significant effect on the cells of *Trichosporon capitatum*

(MY 1890) at levels above 0.5g/L. Therefore, it is likely that the addition of a solvent increases reaction rates, but only at low substrate concentrations.

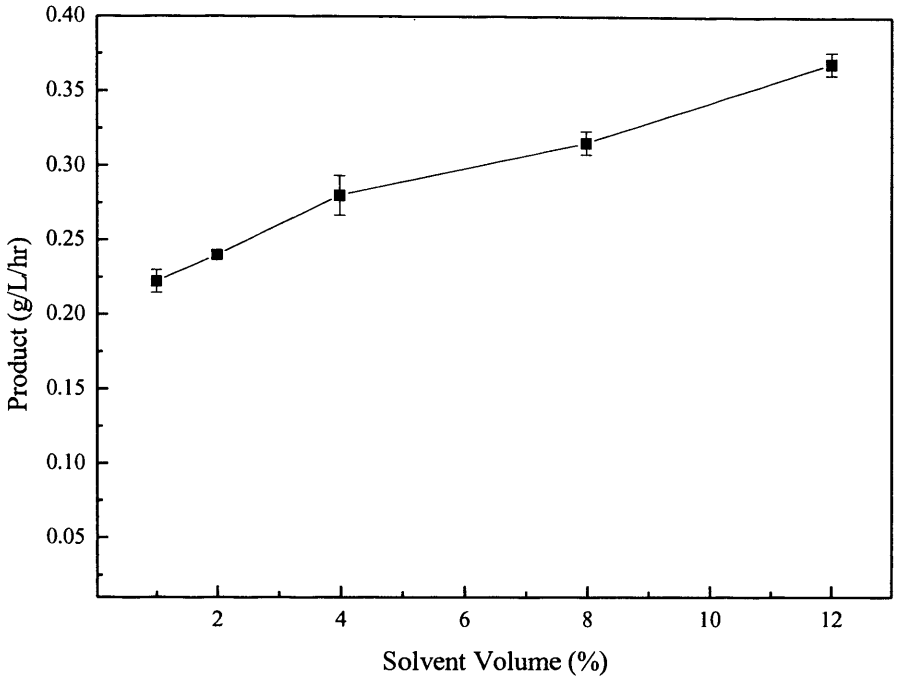


Figure 5.7 The Effects of Methoxyethanol on Biocatalytic Activity

The effects of methoxyethanol concentration on the biocatalytic activity of the whole cells of Trichosporon capitatum (MY 1890). The substrate concentration was maintained constant at 0.5g/L. The solvent concentration employed was 1%, 2%, 4%, 8% and 12%. The results show that at low solvent concentrations the cells exhibit low catalytic activity; however, as the volume of solvent increases the initial activity of the cells also increases. (Reference Section 5.3.7 for methodology) (See Appendix B1.5 for raw data).

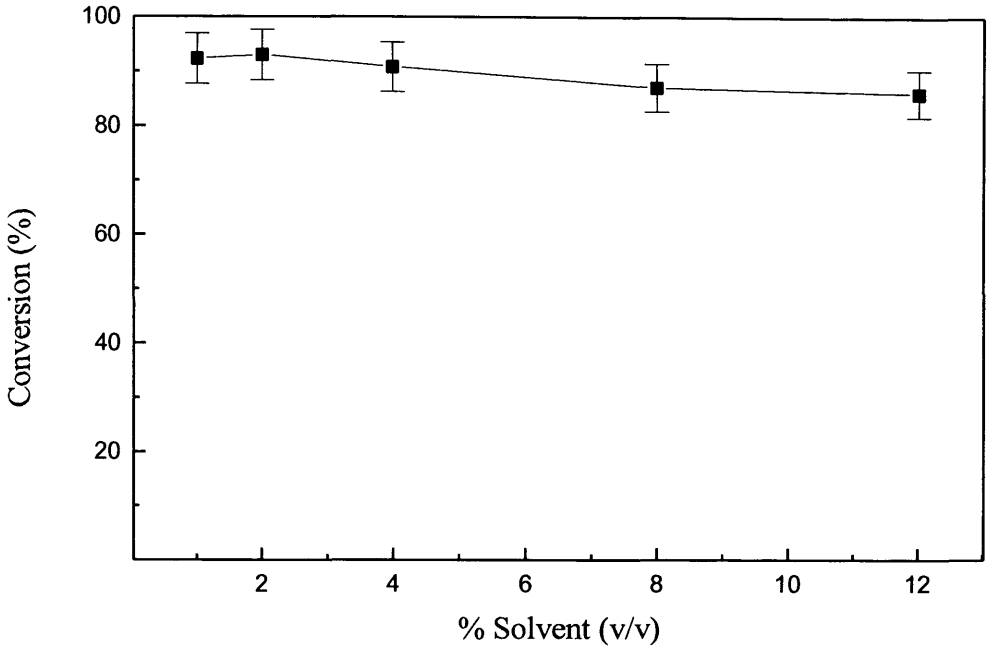


Figure 5.8 The Effects of Methoxyethanol Concentration on Conversion

The effects of methoxyethanol concentration on the conversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol by the whole cells of Trichosporon capitatum (MY 1890). The substrate concentration was maintained constant at 0.5g/L. The solvent concentration employed was 1%, 2%, 4%, 8% and 12%. The results show that final conversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol was not significantly affected by methoxyethanol up to concentrations of 12% (v/v). (Reference Section 5.3.7 for methodology) (See Appendix B1.5 for raw data).

5.4.7 The Effect of Initial Substrate Concentration on the Whole Cells

The initial substrate concentration was investigated to determine the effects of substrate concentration on the biocatalytic activity of the cells. The volume of solvent was kept constant throughout the investigation at a concentration of 0.1%

(v/v). This constant volume of solvent was used to differentiate the effects of the solvent on the biocatalytic activities from the effects of the substrate. The results show that the initial biocatalytic activity increases until an initial substrate concentration of 1g/L (Figure 5.9), after this point the reaction rates remain at a constant level. This is likely to be the concentration at which all of the active sites on the enzymes are used in the bioconversion. The results also show that the final conversions are significantly affected by an increase in initial substrate concentration (Figure 5.10). Up to 1g/L there is a slow drop off in conversion, but as the substrate concentration is increased to 2g/L there is a much more rapid decrease in conversion. The rate of decrease then slows again up to an initial substrate concentration of 3g/L, where the conversion after 7.5hr is approximately 38%. The conversion at the initial substrate concentration of 0.5g/L at 7.5hr is 80%, but by increasing the substrate concentration to 3g/L the conversion decreases to 38%. This reduction in the conversion was most likely caused by the substrate and product adhering either on or inside the lipid bilayer membrane, and this may in turn have caused: (1) substrate and/or product inhibition. It is likely that there was product inhibition since Figure 5.11 shows that the amount of substrate added increases but the amount of product produced maintains at a similar level. Product inhibition was not tested to confirm this since there was not enough of the product to run the experiment. (2) A disturbance to the cell metabolism and therefore the normal cell pathways were not maintained. This could have meant that the enzyme could not reduce the substrate. (3) Proteolysis of the cell, also causing detrimental effects to the metabolic pathway. (4) Changes in enzyme conformation, thus the substrate is unable to bind to the active site. The reason why there is a much quicker decrease in conversion after 1g/L may be down the combined effects of the substrate and solvent. At low substrate concentrations, the solvent is helpful in increasing the rate of mass transfer across the cell membrane, thus increasing the availability of the substrate to the enzyme. As the substrate and solvent are increased a faster rate of degradation is potentially caused through the combined effects of the solvent, substrate, and product inhibition. The results also show that the amount of substrate

converted into product is between 1g/L and 1.2g/L for all initial substrate concentrations above 1.5g/L (Figure 5.11) and in all cases the quantity of substrate available to the cell is significantly greater than the amount converted.

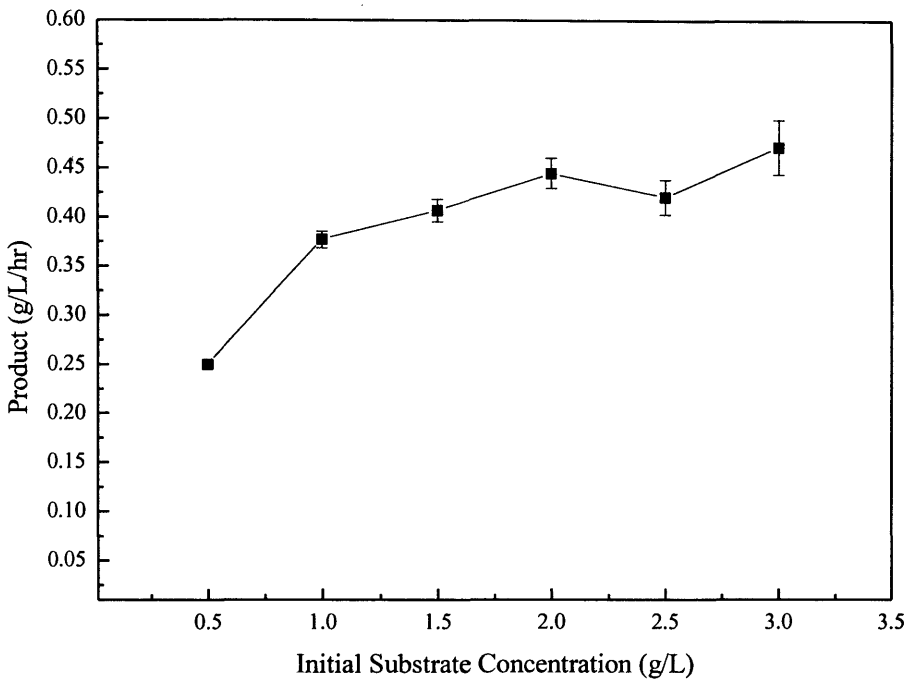


Figure 5.9 The Effects of Initial Substrate Concentrations on Biocatalytic Activity

The effects of initial concentrations of 6-bromo-β-tetralone on the biocatalytic activity of the whole cells of Trichosporon capitatum (MY 1890). The solvent concentration was maintained constant at 0.1% methoxyethanol (v/v). The substrate concentrations employed were 0.5g/L, 1g/L, 1.5g/L, 2g/L, 2.5g/L and 3g/L. The results show the increase in initial activity of tetralone reductase up to an initial substrate concentration of approximately 1g/L after this point the initial activity levels out. (Reference Section 5.3.8 for methodology) (See Appendix B1.6 for raw data).

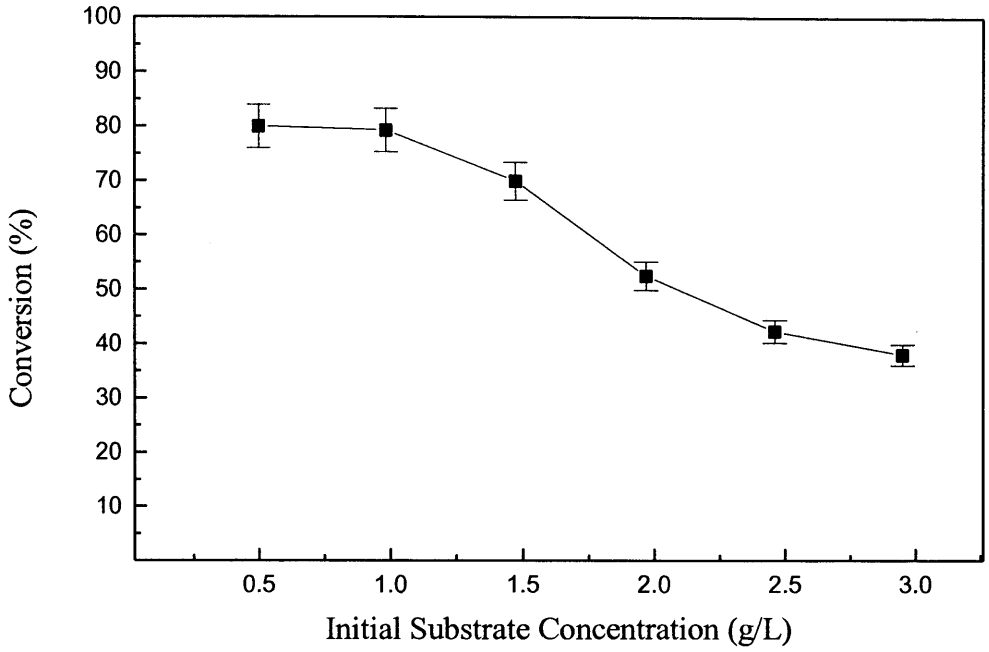


Figure 5.10 The Effects of Initial Substrate Concentration on Conversion

The effect of initial concentrations of 6-bromo-β-tetralone on the conversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol by the whole cells of Trichosporon capitatum (MY 1890). The solvent concentration was maintained constant at 0.1% methoxyethanol (v/v). The substrate concentrations employed were 0.5g/L, 1g/L, 1.5g/L, 2g/L, 2.5g/L and 3g/L. The results show that the initial substrate concentration has a significant effect on the final conversions and has a greater effect on the conversions than compared to the initial activities. A decrease in the final conversion can be observed after 1g/L is added to the reaction mixture. (Reference Section 5.3.8 for methodology) (See Appendix B1.6 for raw data).

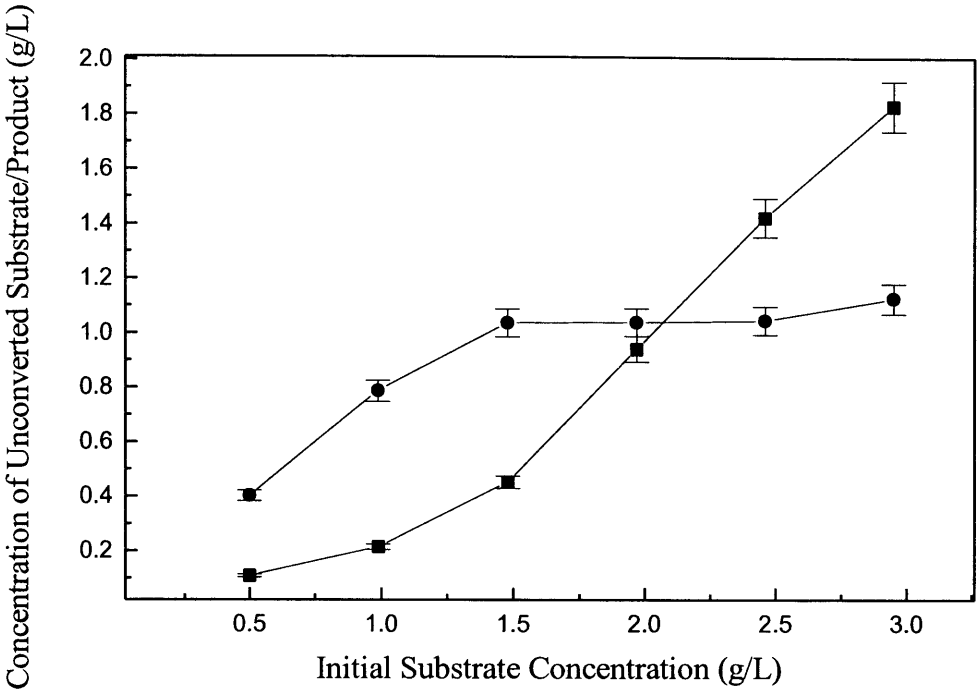


Figure 5.11 The Amount of Substrate Converted in a Reaction

This graph illustrates how the amount of product (●) formed remains constant at approximately 1g/L at concentrations of substrate added to the reaction at levels much higher than this (■). The solvent concentration was maintained constant at 0.1% methoxyethanol (v/v). The substrate concentrations employed were 0.5g/L, 1g/L, 1.5g/L, 2g/L, 2.5g/L and 3g/L. (Reference Section 5.3.8 for methodology).

5.4.8 The Effect of Exogenous Quantities of NADH

NADH is the cofactor which is required by tetralone reductase for the bioreduction of 6-bromo-β-tetralone to 6-bromo-β-tetralol. NADH was added to the cells to determine whether NADH was a limiting component in the reaction media and whether additional quantities of NADH in the reaction solution could increase the amount converted and also potentially increase the reaction rates. The results show

that there is no increase or decrease in reaction rates, or change in the final conversion values (Figure 5.12). This may mean that enzyme in the cell is already saturated with cofactor, or it may mean that the NADH was not adsorbed into the cells and, therefore, unavailable for the enzymes to use in a reaction. The results show that adding NADH to the reaction solution does not affect the current bioconversion.

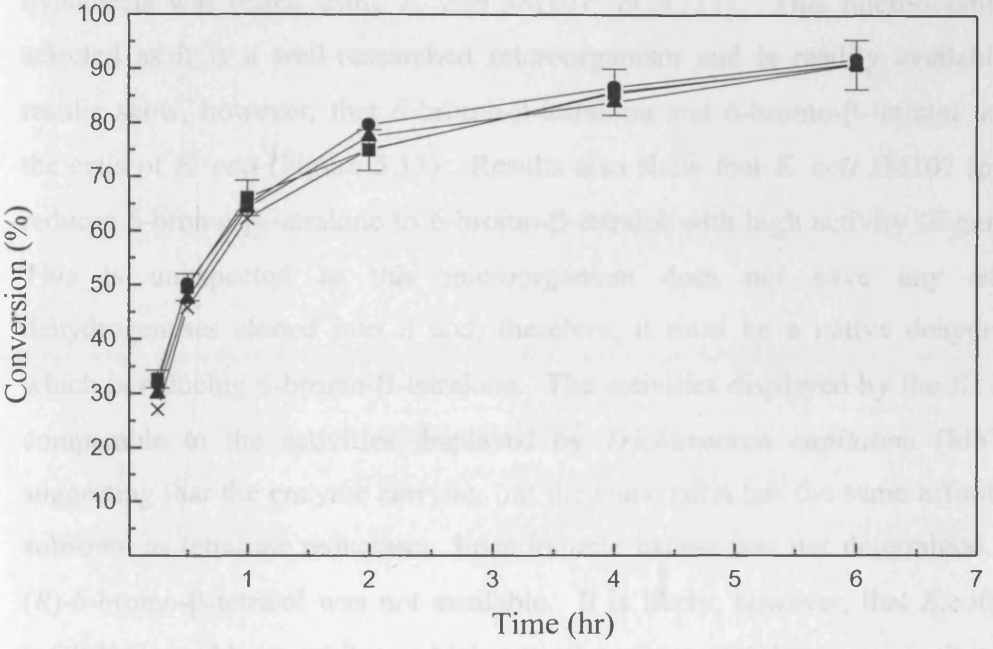


Figure 5.12 The Effects of Exogenous Quantities of NADH on the Bioreduction

The effect of adding exogenous quantities of NADH on the conversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol by the whole cells of Trichosporon capitatum (MY 1890). NADH concentrations employed were 2.5mg/ml (■) 5mg/ml (●), and 0.75mg/ml (▲). A control of 1ml of buffer was also run with no NADH (×). The results show that there is no significant difference in the rates of reaction at different levels of NADH. (Reference Section 5.3.9 for methodology).

5.4.9 Effect of 6-bromo-β-tetralone on E. coli JM107 [pQR711]

In the previous sections it was demonstrated that the substrate, 6-bromo-β-tetralone, adheres to the cells of *Trichosporon capitatum* (MY 1890). If tetralone reductase could be cloned into another type of cell, for example, *E. coli*, the whole cell might exhibit different adherence properties towards 6-bromo-β-tetralone. There is a possibility that different substrate and solvent tolerance might also be seen. This hypothesis was tested using *E. coli* JM107 [pQR711]. This microorganism was selected as it is a well-researched microorganism and is readily available. The results show, however, that 6-bromo-β-tetralone and 6-bromo-β-tetralol adhere to the cells of *E. coli* (Figure 5.13). Results also show that *E. coli* JM107 [pQR711] reduces 6-bromo-β-tetralone to 6-bromo-β-tetralol with high activity (Figure 5.14). This is unexpected as this microorganism does not have any additional dehydrogenases cloned into it and, therefore, it must be a native dehydrogenase which is reducing 6-bromo-β-tetralone. The activities displayed by the *E.coli* were comparable to the activities displayed by *Trichosporon capitatum* (MY 1890), suggesting that the enzyme carrying out the conversion has the same affinity to the substrate as tetralone reductase. Enantiomeric excess was not determined because (*R*)-6-bromo-β-tetralol was not available. It is likely, however, that *E.coli* JM107 [pQR711] would not exhibit as high optical purity as *Trichosporon capitatum* (MY 1890) since this microorganism is not currently used for asymmetric bioreductions. The enzyme in *E.coli* is more likely to be a competing enzyme which would need to be removed or suppressed from the bioreduction (in the selection of *Trichosporon capitatum* (MY 1890) the competing enzymes were removed via media optimization (Reddy *et al.*, 1996)). The aim of this experiment using *E.coli* was to determine whether the substrate and product had similar adherence to the *E.coli* cells; however, the results suggest more importantly, that if tetralone reductase was cloned into *E.coli* there would be a competing enzyme (from *E.coli*) which would have a similar catalytic activity towards 6-bromo-β-tetralone, therefore, to remove the impact of

competing enzymes on the enantiomeric selectivity of the product it may be more appropriate to use the native cells of *Trichosporon capitatum* (MY 1890) as the host microorganism.

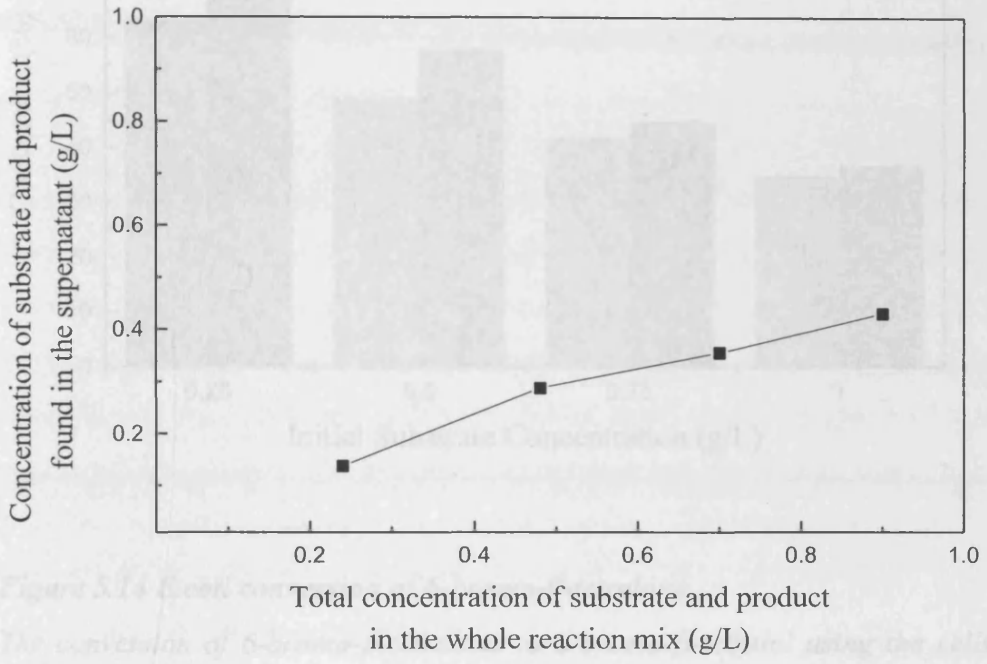


Figure 5.13 The Adherence of the Substrate and Product to E.coli

The adherence of 6-bromo-β-tetralone and 6-bromo-β-tetralol on the cells of E.coli. Solutions of 0.25ml, 0.5ml, 0.75ml and 1ml of a 10g/L 6-bromo-β-tetralone in ethanol were added to 10ml of cells. This gave an initial concentration of 6-bromo-β-tetralone of 0.24g/L, 0.48g/L, 0.70g/L and 0.91g/L. The results show that 6-bromo-β-tetralone and 6-bromo-β-tetralol adhere to the cells of E.coli, and at a initial substrate concentration of 9g/L, less than half of the substrate is observed in the supernatant. (Reference Section 5.3.11 for methodology).

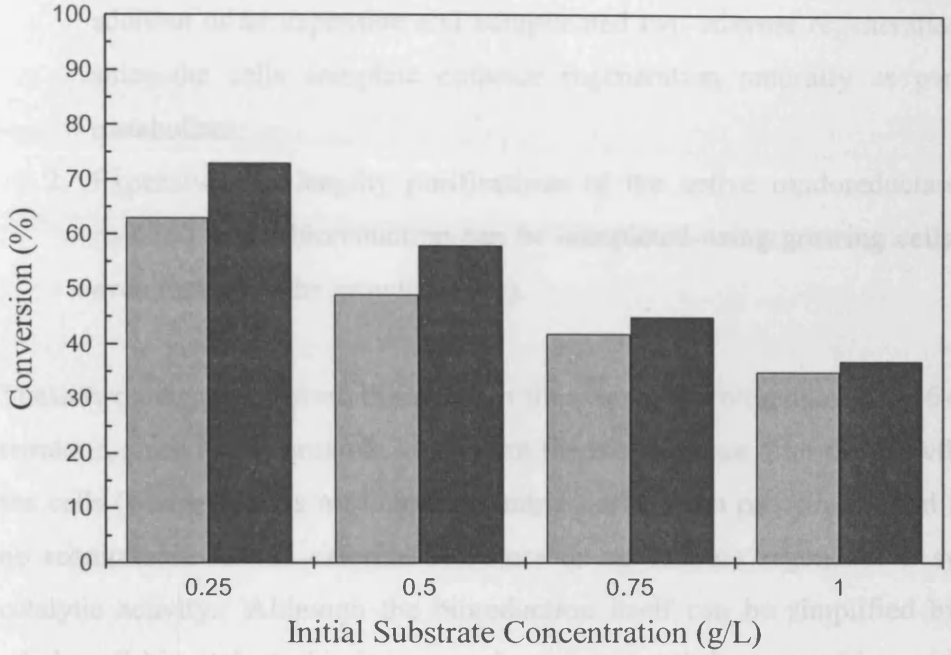


Figure 5.14 E.coli conversion of 6-bromo-β-tetralone

The conversion of 6-bromo-β-tetralone to 6-bromo-β-tetralol using the cells of E. coli JM107 [pQR711] after 1hr (■) and 3 hr (■). The graph shows that the cells of E.coli carry out the bioreduction effectively. After 3hr and a substrate concentration of 0.25g/L there is a 70% conversion. This is comparable to the bioreduction by Trichosporon capitatum (MY 1890). (Reference Section 5.3.11 for methodology).

5.5 Discussion

Whole cell biocatalysis is a more established technology than isolated oxidoreductase biocatalysis for enantioselective bioreductions in the synthesis of chiral alcohols. There are two main reasons for this:

1. Whole cells used in enantioselective bioreductions do not require the addition of stoichiometric quantities of cofactor, nor do they require the addition of an expensive and complicated two-enzyme regeneration system, since the cells complete cofactor regeneration naturally as part of cell metabolism.
2. Expensive and lengthy purifications of the active oxidoreductase are not required as the bioreduction can be completed using growing cells (without even removing the growth media).

These two advantages were observed in the whole cell bioreduction of 6-bromo-β-tetralone, since it was possible to perform the bioreduction after the growth stage of the cells (where there is no time-consuming purification procedure), and there was no requirement to add external cofactors or an enzyme regeneration system for catalytic activity. Although the bioreduction itself can be simplified by using a whole cell biocatalyst, this increases the number and the type of impurities in the solution compared to the isolated enzyme system. This consequently increases the amount of downstream processing required. Contamination from the crude biocatalytic system can potentially arise from:

1. The accumulation of by-products produced by competing enzymes, particularly problematical are competing enzymes which produce the opposite enantiomer to the active compound. This causes difficulties in downstream processing as the by-product and the active ingredient will be chemically similar.
2. The media used for the growth of the cells.
3. Cell debris including, other cell proteins.
4. The position on the growth curve may also produce difficult types of contamination. For example, if the cells are not grown for enough time there may not be sufficient enzymes to produce an efficient system, if the cells are

grown for too long then there may be more impurities through the degradation of the cell components.

All these types of contaminants will need to be separated from the product via suitable methods. The crude isolation of the product from the cell growth media and the cell debris could be made via solvent extraction. However, it is the purification of the product from molecularly similar compounds which is more difficult to achieve. In instances where there are chemically similar compounds in solution which are difficult to separate, it may be more practical to use a less crude form of biocatalyst; for example, an isolated and purified oxidoreductase. In a purified oxidoreductase system there would be very few other enzymes (depending on the purity of the oxidoreductase) which could potentially cause the build up of chemically similar compounds by competing with the active oxidoreductase. The relative purity of the whole cell biocatalyst could potentially be improved by overexpressing the active oxidoreductase, overexpression would likely increase the ratio of active enzyme to competing enzyme, thus also increasing the ratio of product to by-products; this is discussed in more detail later in this chapter.

The delivery of substrate and the removal of product from the cells of *Trichosporon capitatum* (MY 1890) was completed using a solvent. The use of a solvent serves to increase the solubility of the substrate to enable the bioreduction to advance at a faster rate than if the substrate was added as a solid of low aqueous solubility. Investigations using the *Trichosporon capitatum* (MY 1890) whole cell system demonstrated that there are issues associated with the delivery of the substrate to the cells and also the removal of the product from the cells. One of the main problems was that the substrate and the product accumulated inside the cell and could only be removed via the addition of a solvent (the substrate and product could not be disassociated from the cell components through sonication). In this case, the substrate and product are hydrophobic and it is this property which causes them to have a higher affinity for the cells than the aqueous environment surrounding the

cells. All cells have a membrane made up of a bilayer of lipids (Bailey and Ollis, 1986), and the permeability of substances through the membrane can depend upon the lipid solubility (partition coefficient). 6-bromo-β-tetralone is a lipophilic compound and most likely either diffuses through the cell membrane or becomes incorporated into the lipid bilayer (Angelova *et al.*, 1999). The varying amounts in the cell, in the bilayer, and in the aqueous environment may all depend upon one or a combination of:

- The amount of solvent in the reaction.
- The amount of substrate added to the reaction.
- The ratio of substrate and product in the cell.
- The solubility of the substrate and product in the solution

The acute adherence and accumulation of substrate and product in the cell is disadvantageous in the whole cell biocatalytic system as it means that the substrate and product will not be homogenous throughout the solution (in the isolated enzyme system the solution is homogeneous as there are no surfaces for substrate and product adherence). This means that there is a high risk that the substrate and product will be toxic to the cell even at low substrate concentrations, as the cell and consequently the enzyme inside the cell, will be subjected to higher overall concentrations of substrate than exhibited in a homogenous solution. This shows that even at low substrate concentrations there is the potential for only low productivities to be obtainable. The adherence of the substrate and product to the cells also means that the product will have to be recovered from the cells through a method such as solvent extraction. The addition of large volumes of solvent will generally cause a detrimental effect on the cell by disrupting the cell membrane, it will also cause the enzyme to become deactivated. This was observed in the whole cell bioreduction of 6-bromo-β-tetralone where the addition of equivalent parts of solvent for extraction to the reaction solution destroyed the cells so they could not be recycled. Solvent extraction has also been used in the extraction of the product from a benzylidene thiazolidinedione reduction (Heath *et al*, 1997). In this paper (Heath

et al, 1997) it was stated that such an extraction, although prevented the cells from reuse, was actually economically feasible and preferred.

The delivery of the substrate to the cells and removal of the product from the cells is not the only constraint in the application of an effective whole cell biocatalytic bioreduction. For oxidoreductases, it is necessary to ensure that the cofactor required for the reaction is not limiting the conversion. Cofactors are a low molecular weight, non-protein species that are essential participants in most isolated enzyme catalysed reactions (Chenault *et al*, 1988), they are either oxidised, donating a hydrogen ion, or reduced, accepting a hydrogen ion. In whole cell biocatalysis the cofactors are in a continuous loop of oxidation and reduction via the cells metabolic pathway. One way to speed up the whole cell oxidoreductase reaction could be to increase the amount of cofactor available in the cell, thus if the regenerating enzyme is the limiting parameter in the cell, the addition of further quantities of cofactor may increase the reaction rates. In the *Trichosporon capitatum* (MY1890) whole cell system, this theory was tested, where exogenous quantities of NADH were added to the whole cell reaction to determine whether these additional quantities of cofactor could increase the reaction rate. The results showed that there was no increase or decrease in activity and it was assumed that either, the NADH, which is highly soluble in aqueous environments was not taken up by the cells, and therefore could not be used in an enzymatic reduction by tetralone reductase; or tetralone reductase was the limiting enzyme and was already saturated in NADH. However, in the isolated enzyme system an increase above a certain level of NADH proved to be detrimental to the enzyme (see Figure 3.11). This result was not observed in the whole cell bioconversion, and therefore it was more likely that the cells did not take up the NADH. To increase the amount of NADH available to the enzyme in the cell, it may be necessary to permeabilise the membrane to enable the hydrophilic component to enter the cell. Permeabilisation of the cell membrane could cause the creation of a synthetic pathway in the membrane which the cofactor could simply diffuse through.

In the whole cell system it seems that there are two main parameters which control the rate of reaction. These are (1) the amount of substrate/product in the reaction, and (2) the amount of solvent in the reaction. It is evident from the results that the cells and their corresponding reaction rates are adversely affected by an increased amount of substrate/product in the reaction. In contrast, however, the amount of solvent used to deliver the substrate was significant in positively increasing the reaction rates. In the case of *Trichosporon capitatum* (MY1890) the results demonstrated that at low levels of substrate (0.5g/L) reaction rates were improved with increasing solvent concentration (up to 12%). This increase in biocatalytic activity may have been caused by a permeabilisation of the cell membrane by the solvent, thus facilitating transfer of the substrate across the cell membrane more readily. However, if the mass transfer rate of the substrate into the cell is increased, this increase in substrate is also likely to increase the rate of degradation of the cell membrane and most likely cause a reduction in the cell viability. Therefore, there the amount of solvent used to deliver the substrate must be optimized with respect to the amount of substrate required in the reaction and the associated effects on the biocatalytic activity and stability. The effects of cell permeabilisation have previously been reported where the yeast cells of *Saccharomyces cerevisae* were permeabilised with alcohol (Liu *et al.*, 1999). The treatment of cells with an alcohol significantly enhanced the biocatalytic activities cells. It was proposed that this method increased the membrane permeability to the substrate and product (Liu *et al.*, 1999). This effect has also been previously reported, where it was demonstrated that by reducing the permeability barrier of the cell membrane (using a solvent) caused an increase in reaction rates but also caused a reduction in the cell viability (Gowda *et al.*, 1991). This result was similar to behaviour of *Trichosporon capitatum* (MY1890) where there was an increase in the initial rate of reaction with higher solvent concentrations, but a decrease in conversion. This decrease in conversion was probably caused by a reduction in cell viability, potentially caused by an increased amount of solvent which had diffused through the cell membrane and had

become in contact with the active enzymes. This increase in solvent will most likely have caused the active site of the enzyme to become perturbed. Therefore, using a solvent to increase the reaction rates by facilitating an increase in mass transfer across the cell membrane must be balanced against cell viability, biocatalytic reaction rates and conversions. The correct choice of the type and amount of solvent is therefore critical in maintaining the biocatalytic viability and ensuring good reactions rates through appropriate levels of cell permeabilisation.

If the volume of solvent is trivial in a whole cell bioreduction at low substrate concentrations then it is the amount of substrate added to the reaction and the amount of product removed from the reaction which will need to be accurately monitored and controlled to maximize the biocatalytic activities of the cell. Adding a high level of substrate in batch and not removing the product will have two consequences. Firstly, in a whole cell system with a substrate of low aqueous solubility there will be a tendency for the substrate and converted product to adhere and accumulate onto the cell membrane. Secondly, the uncontrolled addition of substrate into the reaction will have an adverse effect on the cell and enzyme stability, as at high substrate/product concentrations they are likely to become toxic to the cell and corresponding enzyme. An increase in the initial substrate concentration was investigated in the whole cell bioreduction of 6-bromo-β-tetralone to determine the effects on the biocatalytic reaction rates and conversions. An increase of 6-bromo-β-tetralone caused the initial activity of the whole cell to increase up to approximately 1g/L. This is likely to be the concentration at which all the active sites of the enzyme are used; at substrate concentrations above this value the associated conversions were significantly affected. To improve the whole cell system, it seems that the method by which the substrate is delivered to the cell and the method by which the product is removed from the cell must be enhanced if the system is to be capable of producing significant amounts of product. In the isolated enzyme system it was suggested that the use of polymeric resins would be ideal, in that they could be employed instead of a solvent for the addition and removal of the

substrate/product. The use of resins in whole cell biocatalytic reactions for substrate delivery and product removal has been applied in a number of recent systems (these were reviewed in Chapter 4.4). Examples of the successful use of resins for substrate delivery and product removal include a ketone reduction using XAD-7 (Vicenzi, *et al.*, 1997), and the Baeyer-Villiger oxidation using Optipore L493 (Hilker *et al.*, 2004). In the case of *Trichosporon capitatum* (MY1890) and 6-bromo-β-tetralone, the use of resins alone to add the substrate to the reaction mix may not be appropriate as the substrate requires the permeabilisation of the cell membrane to increase the reaction rates. If resins were employed, it may be appropriate to use them in conjunction with a solvent. In such a case, the resin would need to have the appropriate affinity for the substrate to allow the correct equilibrium of substrate on the resin and substrate in the solution to ensure satisfactory reaction rates. If resins were employed for delivery and removal of the substrate and product, it is optimisation of the properties of the substrate, product, and resin, which would be critical in ensuring the correct equilibrium of the components in the reaction solution. This would need to be assessed before resins could be employed in a bioreactor (Straathof *et al.*, 2003). Two-phase (aqueous/organic) solutions could also potentially be used to control the delivery of the substrate. However, as discussed for the resins, the use of a secondary phase (whether the secondary phase is liquid or solid) for the delivery of the substrate may not cause permeabilisation of the cell and it may be necessary to use the combination of an immiscible solvent to control the substrate and a miscible solvent to permeabilise the cell.

Generally, for the application of whole cell oxidoreductase biocatalysts, the enzymes are purified from their native cells with the intent of overexpressing and cloning the enzyme into a microorganism. The selected microorganism is generally thoroughly characterized and has well-defined growth parameters. As a host microorganism, however, it must maintain the biocatalytic properties of the enzyme. To improve the biocatalytic reduction of 6-bromo-β-tetralone with respect to substrate and product

adsorbance a change the cell type was investigated. The experiment looked at whether the bioconversion could be improved by changing the cell type to *E. coli* JM107 [pQR711]. This microorganism (*E. coli* JM107 [pQR711]) expresses transketolase and is ampicillin resistant (however, it was only selected because it is a well-characterized microorganism). The results from the experiment show that not only does the substrate have a high affinity for the cells, but also and more significantly, is the fact that *E.coli* converts the substrate 6-bromo-β-tetralone into 6-bromo-β-tetralol. Cloning the active enzyme into a host cell which has a competing enzyme would be of little use if the native enzyme caused a reduction in the optical purity of the product. It is likely, however that if the active enzyme was overexpressed into a microorganism this would dramatically increase the ratio of overexpressed enzymes to native cell enzymes; it may also mean that the biocatalytic activity of the enzymes native to the host microorganism are not adequate to have an effect on the optical purity of the product. This would have to be tested and depending on the results it may be more appropriate to overexpress the active enzyme back into its own cell. Other options to improve the stereoselectivity of the whole cells could include knocking-out competing enzymes by genetic engineering, this has been accomplished in bakers' yeast where reductases were knocked out, the results demonstrated that it is possible to improve stereoselectivity of baker's yeast via genetic modification (Rodriguez *et al.*, 1999). The results from this chapter show that the cells of *Trichosporon capitatum* (MY 1890) can be employed for the bioreduction of 6-bromo-β-tetralone to 6-bromo-β-tetralol. However, the whole cells are limited by the accumulation of substrate and product inside the cell membrane causing a detrimental effect on reaction rates and productivities. A solvent is also required to extract the product from the cells and because of this the cells cannot be recycled. The bioconversion could not be improved by increasing the amount of NADH available to the cell, or by changing the cell type.

5.6 Summary

The whole cells of *Trichosporon capitatum* (MY 1890) can be employed for the bioreduction of 6-bromo-β-tetralone to 6-bromo-β-tetralol. However, a high percentage of 6-bromo-β-tetralone and 6-bromo-β-tetralol associates with the cell; either accumulating inside the cell or becoming associated to the hydrophobic cell membrane. To deliver the substrate and also to increase the substrate concentration in the reaction mix two options were analysed. Firstly increasing the solubility of the substrate by changing the solvent type, and secondly, increasing the volume of solvent. The results show that higher reaction rates and higher conversions can be achieved by changing the solvent from ethanol to methoxyethanol. Results also show that at low substrate concentrations (0.5g/L), the higher the solvent concentration (up to 12%) the higher the initial biocatalytic activities; the respective conversions are also high at all solvent concentrations investigated. This was attributed to the solvent facilitating an increase in the rate of mass transfer of 6-bromo-β-tetralone through the lipophilic membrane. As there was little detrimental affect on the cells at high solvent concentrations the effects on the biocatalytic activity of the cells at different substrate concentrations were investigated. Results show that the initial activity increases up to substrate concentrations of 1g/L. Conversions are significantly affected at substrate concentrations above 1g/L and a decrease of 50% is observed between an initial substrate concentration of 0.5g/L and 3g/L, the results indicate that the amount of substrate is more detrimental to the biocatalytic activity of the whole cells than the concentration of the solvent. It is hypothesised that the substrate due to its hydrophobicity adheres to the cell membrane and somehow causes degradation of the cell components, whereas the solvent increases the reaction rates by permeabilising the cell membrane and allowing easier transfer of the substrate into the cell through the lipophilic membrane. Further investigation in the rate-limiting step demonstrates that exogenous quantities of NADH have no affect on the whole cell biocatalytic activities. Results also show that if *Eschericha coli* JM107 [pQR711] is used as a cell type to host tetralone reductase, it is limited due to a similar association of 6-

bromo-β-tetralone with the cells, and it is also limited as there is a native enzyme in *E.*coli which reduces 6-bromo-β-tetralone to 6-bromo-β-tetralol.

The following chapter will be used to compare the whole cell biocatalytic system to the isolated oxidoreductase system for comparison of: (1) the method of bioconversion of 6-bromo-β-tetralone, including the type and purity of the oxidoreductase; (2) the delivery of the substrate and the consequences of using a solvent; and (3) potential improvements to the biocatalytic systems, including improvements arising from recombinant technology.

An NADH dependent Reductase for Isolated Enzyme and Whole Cell Catalysis

6.1 Introduction

NAD(P)H dependent oxidoreductases are one of the most important class of enzyme as they have the ability to produce compounds of high optical purity (Anthonsen, 2000). One way of achieving this is via the stereospecific reduction of prochiral substrates, in this case, the formation of a chiral alcohol ((*S*)-6-bromo-β-tetralol) from a cyclic ketone (6-bromo-β-tetralone) by tetralone reductase. The problems associated with implementing such oxidoreductases at large scale are dependent on whether isolated enzymes or whole cell biocatalysts are employed for the bioreduction. The most documented advantage of using whole cell biocatalysts over isolated enzymes is that whole cells contain all the necessary cofactors and metabolic pathways for their regeneration, also the enzymes and cofactors are protected in their natural environments (Faber, 1997). Yet, for whole cells to be used at scale there are a number of significant drawbacks. Firstly, their productivity is generally low since the majority of non-natural substrates are toxic to living organisms. Secondly, large amounts of biomass cause low overall yields and make product recovery hard, particularly when the product is stored inside the cell and not secreted into the medium. Lastly, the formation of by-products causes difficulties in reaction monitoring (Faber 1997). The use of isolated enzymes for bioreductions can overcome the difficulty with respect to product recovery as the large amounts of biomass have already been removed and discarded during the enzyme isolation without the use of large volumes of solvents. The use of isolated enzymes also reduces the number of competing enzymes which also potentially limits the formation of by-products. However, on isolation of oxidoreductases from their natural cell environment, there is then the requirement to add stoichiometric quantities of the associated cofactor. Currently, the addition of stoichiometric quantities of cofactor is not suitable for large-scale processes due to the high costs

which would be incurred, thus methods must be employed for the regeneration and retention of these cofactors (Chenault and Whitesides, 1987; Hummel, 1997). There is also the problem of enzyme inhibition by the cofactors, as demonstrated in Chapter 3 (Figure 3.11).

There are currently few systematic comparisons of isolated enzymes and whole cells, examples include: a comparison of pig liver esterase and *Bacillus subtilis* as catalysts for the hydrolysis of menthyl acetate in stirred two-liquid phase reactors (Williams *et al.*, 1990). In this system, the results demonstrated a number of differences between the isolated enzyme and the whole cell including, the whole cell requirement for much higher agitator speeds to maximize productivity (approx 4000rpm) whereas the esterase reaction was maximized at about 1000rpm. Results also demonstrated that the optimized agitator speed was dependent on the phase ratio (defined as the volume fraction of reaction liquor occupied by organic phase), where the enzyme was much more susceptible to inactivation at the liquid/liquid interface. Lastly, the results showed that higher enzyme concentrations could increase reaction rates although this was probably due to the high concentration masking the decay of enzyme over time. Pigs liver esterase for benzyl acetate hydrolysis and a whole cell (*Pseudomonas putida)* for toluene oxidation have been employed in Lewis cell studies to determine reactor design data for two-phase bacterial and enzymatic reactions (Woodley *et al.*, 1990); however, this was a non-direct comparison as neither the enzyme origin or the reaction was the same. Cyclohexanone monooxygenase (CHMO) and *E.coli* overexpressing CHMO have also been used for the comparison of a biocatalytic reaction (Zambianchi *et al.*, 2004), in this case the reaction was an enantiomeric oxidation. Results from the comparison demonstrated that productivity was similar in both cases; however, the optical purity was much higher with the isolated enzyme than the whole cell. It was concluded that in this instance the isolated enzyme appeared to be preferable. There has also been an assessment of the impact of biocatalyst selection on the design of aqueous-organic biphasic biocatalytic processes (Woodley *et al.*, 1990). There are currently,

however, no systematic comparisons of oxidoreductases and whole cells used for bioreductions, and neither are there any comparisons of isolated oxidoreductases which have not been overexpressed and native whole cells. The objective of this chapter is to compare tetralone reductase and *Trichosporon capitatum* (MY1890) for the bioreduction of 6-bromo-β-tetralone. Initially, it was hypothesized that the whole cell would be robust and simple to employ, since whole cells (in general) have been previously used for bioreduction reactions. Also, there is no requirement for the addition of cofactors. In contrast, the isolated enzyme would be difficult to employ mainly due to the requirement for a cofactor recycling system to be set up. The isolated enzyme would also need to be purified; and the enzyme may be difficult to use in a process due to instabilities caused by removing the enzyme from its natural cell environment. In this Chapter, the findings from Chapters 3, 4 and 5 will be used to compare the whole cell and isolated enzyme against these general hypotheses. This will include comparisons of the biocatalyst type, biocatalyst purity, bioreduction methodology, productivity, methods of substrate delivery and methods of extracting the product from the reaction solution. Hypothetical potential improvements to the isolated enzyme and whole cell systems via recombinant technology will also be discussed.

6.2 General Considerations Required for Biocatalyst Comparisons

In the comparison of an isolated enzyme or whole cell biocatalyst for the application of a particular bioreduction there should be, as a minimum, consideration of the following parameters:

Biocatalyst Characteristics

1. Biocatalyst purity, reaction materials and related downstream processing (including the separation of compounds of similar chemical properties and structures).
2. The type of biocatalyst, biocatalyst stability, and their substrate specificities.

3. Cofactor regeneration to ensure that the maximum activity of the enzyme is achieved due to the associated cost implications.
4. Biocatalyst recycling including any recycling of associated regenerating enzymes.
5. Potential improvements to the biocatalytic systems via recombinant technology.

Substrate/Product Characteristics

6. Delivery of the substrate to the biocatalyst.
7. Recovery of the product from the biocatalytic reaction mix.
8. The effect of the substrate and product on the biocatalyst (e.g. inhibition and stability).

Other

9. Bioreduction methodology including ease of processing and robustness of the process.
10. Equipment and materials.
11. Process costs.

All these listed parameters were proven to be important issues to consider in the production of 6-bromo-tetralol by isolated tetralone reductase and *Trichosporon capitatum* (MY1890). Also, and just as importantly, was the consideration of the effects these parameters had upon each other, since in this particular process the characteristics of the biocatalyst were driven by the composition/characteristics of the substrate(s), product(s) and the reaction medium which came into direct contact with the biocatalyst. This is illustrated in Figure 6.1.

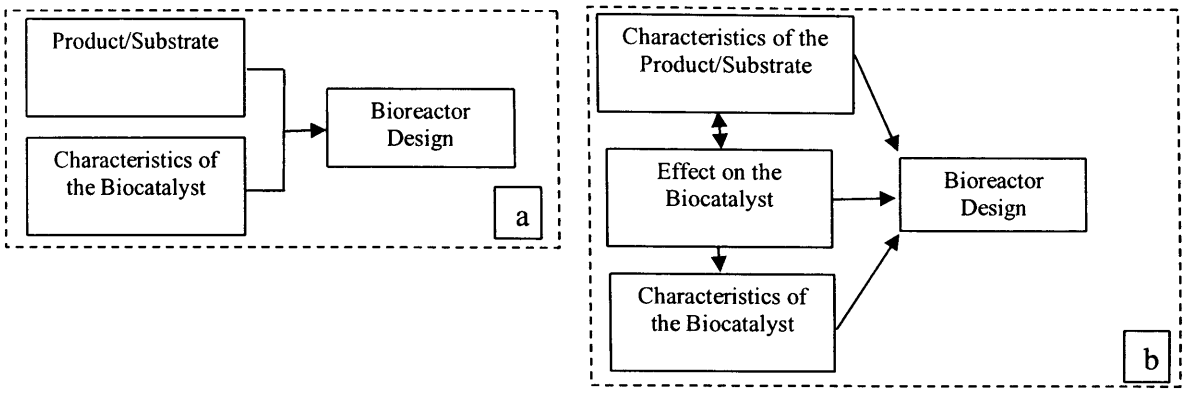


Figure 6.1 Effects of Reaction Components on Reactor Design

Figure 6.1a shows both the biocatalyst and the substrates/products must be considered to enable the design of an efficient bioreactor. For example, in isolated enzyme cofactor requiring reactions the configuration of the cofactor must be maintained and the enzyme activity must be retained, so the reaction environment must be such that it is suitable for both components (e.g. conventional filtration cannot be used to retain the cofactor, therefore there must be the adaptation of the cofactor or retention method). Figure 6.1b is an adaptation of Fig 6.1, it shows that the substrate/product and biocatalyst should not be investigated independently and the effects of one upon the other should be considered. For example; in the isolated enzyme reaction the addition of the cofactor must be controlled or recycled and not added stoichiometrically in batch. This is because high concentrations of cofactor cause enzyme inhibition (see Figure 3.11) (i.e. not only does the cofactor need to be retained, it also needs to be regenerated).

It is important to characterise the biocatalyst for a number of reasons; however, it is also important to characterise the substrate and product. An example of the importance of this is described in Chapter 4 where the hydrophobic nature of the substrate and product was only detected once immobilisation of the enzyme onto a support was attempted. Characterisation of the substrate and product could have potentially detected this inherent property at an early stage. This characterisation should have also prevented the initial confusion on running a whole cell bioreduction, where the substrate and product seemed to degrade, but were actually

accumulating in the cell. This illustrates the importance of initial characterisation work, where the reaction components and surrounding environments should be investigated individually first of all and then again once the reaction components are put together. Only then should a satisfactory comparison of the reaction systems be considered.

6.3 Biocatalyst Purity

The purity of the biocatalyst employed in a reaction has a number of consequences on the purity and the enantiomeric excess of the final product and also the amount of downstream processing which is required. To achieve a high conversion in a bioreduction it is generally considered important to have a high purity enzyme.

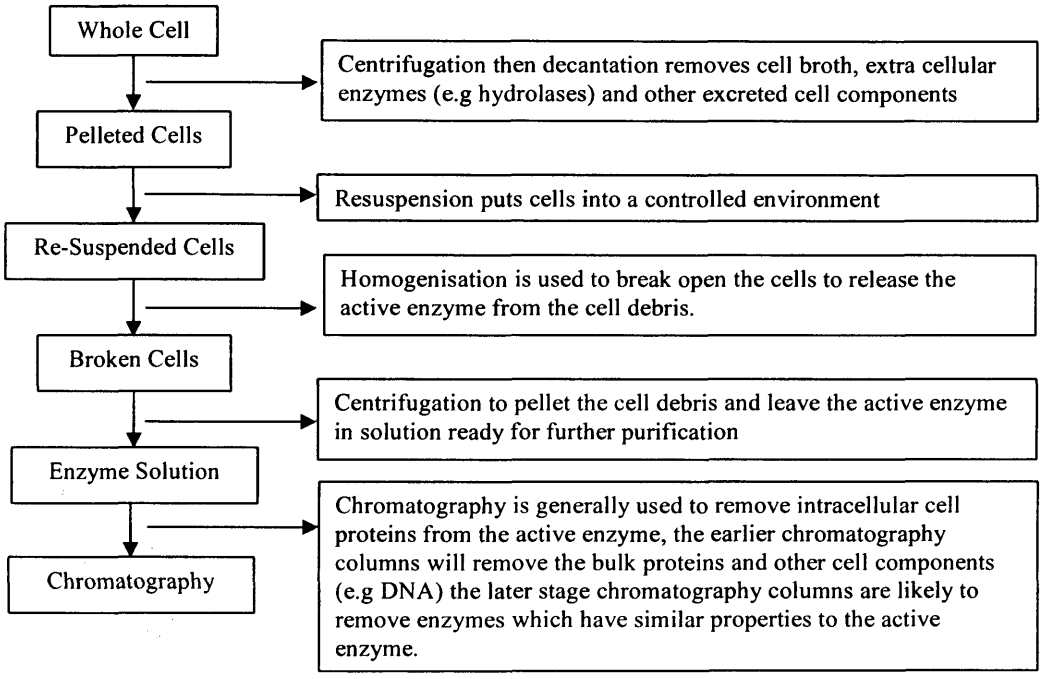


Figure 6.2 Isolation Flow Diagram

This flow diagram shows a simplified isolation of tetralone reductase, it is easy to see how many more impurities the whole cell system has ranging from biomass, to reaction medium, to contaminating proteins.

In whole cell bioreductions, there are significantly more contaminants in the initial stages of the reaction when compared to isolated enzymes including, growth media, competing enzymes and proteolytic enzymes. These contaminants need to be removed from the active ingredient via extractive processes which could potentially also separate the biocatalyst for reuse. If the contaminants from the whole cell system are easily extracted from the product then the whole cell process may be the optimal choice for the type of biocatalyst. However, as the contaminants start to resemble the product itself (due to competing enzymes catalyzing a change in the substrate) the purification of the active compound during the downstream processing becomes more difficult to achieve; and depending on the ease of separation of the similar compounds it may be more appropriate to use a biocatalyst which is in a more pure form. Isolated enzymes which have undergone a number of chromatography purification stages should contain very few contaminants either from the isolation (e.g. growth media) or from the cell (e.g. competing enzymes). In such a scenario, the product of the reaction should be relatively pure as there should be limited competition for the substrate, and the amount downstream processing should be relatively simple. The question of how pure the biocatalyst needs to be is really dependent of the end reaction mixture and how easy it is to separate the product from the other reaction components. If an isolated enzyme is employed, there is a choice in the level of enzyme purity since the number of chromatographic stages can be selected and optimized as appropriate, it may even be suitable to use a low purity enzyme, possibly the use an enzyme from a one-stage chromatography purification. This would decrease the associated costs with respect to fully purifying the enzyme.

6.4 Biocatalyst Substrate Specificity

Selection of the most appropriate type of biocatalyst for a particular process may be based upon its ability to accept a diverse number of substrates. The substrates accepted by the active site of an enzyme in a reaction are known as the enzymes substrate specificity. The more diverse the type of substrate accepted by the

biocatalyst the more potential the biocatalyst has for further application; and it is of particular relevance that the biocatalyst accepts compounds of pharmaceutical importance. Horse liver ADH is a very catholic enzyme with a broad substrate specificity (including aldehydes and cyclic ketones) and a narrow stereospecificity and because of this is probably the most widely used dehydrogenase in biotransformations (Faber, 1997). In contrast, yeast ADH (in general) only accepts aldehydes and methyl ketones and is therefore of limited use. The specificity of oxidoreductases has recently been reviewed (Hummel, 1999). There have also been reports of mass transfer problems for whole cell systems; for example, large and/or hydrophilic substrates may not be easily transferred through the lipophilic cell membrane. The cell membrane acts as a barrier to the substrate entering the cell, and therefore if the substrate cannot diffuse through the membrane the contact of the substrate with the enzymes active site is not made and the product cannot be formed. In examples such as this, it is expected that the isolated enzyme would have a higher substrate specificity than the whole cell enzyme as there is no barrier surrounding the enzyme. In the final experimental work, substrates were investigated to compare the isolated enzyme and whole cell substrate specificity.

The substrates examined were pharmaceutically important precursors ranging from cyclic ketones to small molecules to long chain molecules. The substrates were either solubilised in ethanol or were themselves organic liquid substrates. The results show that the whole cell system and the respective isolated enzyme system can both carry out a variety of bioconversions for a range of pharmacologically important substrates (Figure 6.3), it also shows that the substrate specificity is the same for the whole cell as for the isolated enzyme (the assays used for the substrates and product are described in Appendix C). The results also show the high biocatalytic potential of tetralone reductase, since its specificity is not just limited to a single bioreduction.

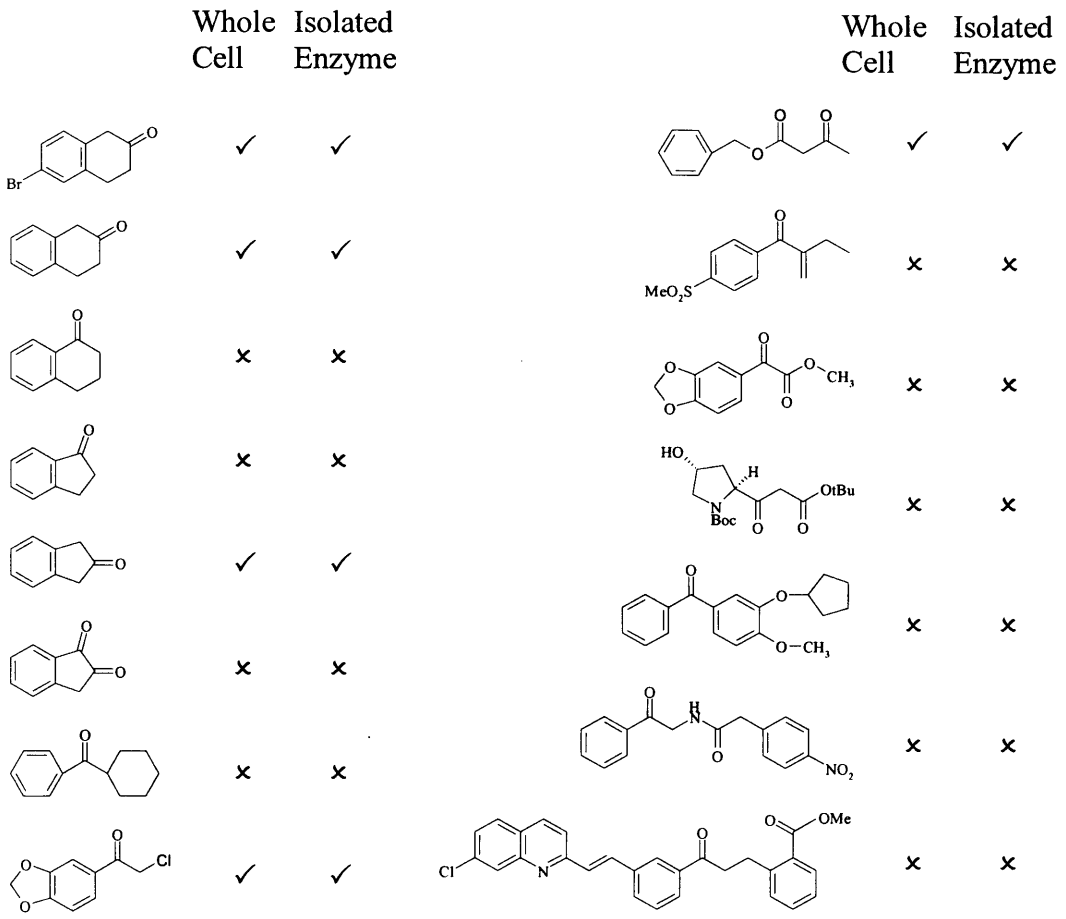


Figure 6.3 Substrate Specificity

A comparison of the substrate specificity between the isolated enzyme and the whole cell biocatalyst shows that both types of catalyst carry out the same type of reaction.

Structurally, tetralone reductase seems to need space to the right hand side of the oxygen component, and therefore compounds which have rings on both sides of the reducing centre are not capable of being reduced. The enzyme is also able to reduce the oxygens at the end of straight line chains, such as the chloroketone (chlorine is a relatively small molecule) and benzylacetoacetate. Tetralone reductase could also easily reduce β-tetralones with or without a bromine molecule; however, the α-tetralone molecule could not be reduced even though the only change was a small

positional change of the oxygen. The fact that the whole cell catalyst has the same specificity as the isolated enzyme means that bioconversion of a substrate to its respective product by a whole cell may rely more upon the substrate hydrophobicity and its interaction with the lipophilic membrane, than the relative size of the substrate.

6.5 Methodology and Productivity

The effective use of isolated tetralone reductase requires the *in-situ* regeneration of NADH, this can be achieved by the addition of formate and formate dehydrogenase (FDH). This additional cofactor regeneration system increases the complexity of the isolated enzyme reaction methodology and also increases the process costs. The cofactor can not be added stoichiometrically to the reaction mixture as tetralone reductase is inhibited at high levels of NADH, and therefore cofactor regeneration is essential to maintain enzyme activity (not just to decrease the cost of the process). The FDH cofactor regeneration system was selected for the regeneration of NADH on the basis that the enzyme and its associated regeneration components have previously been employed as a secondary enzyme and substrate for cofactor regeneration; they are also reasonably well-defined (Chenault and Whitesides, 1987). This is particularly true in comparison to other synthetic cofactor regeneration systems for example, chemical and electrochemical cofactor regeneration methods produce cofactors which exhibit low specificity and are inactive when applied to an enzyme reaction (see Chapter 1.6). In contrast to the isolated enzyme system, the whole cell regenerates NADH as part of its own cell metabolism and thus there are no implications on the associated methodologies (by adding further components) and neither is there a change in the costs.

The reaction methodology selected for the biocatalytic reaction will affect the efficiency of the process; this can be measured using productivity. Conversion should be considered concurrently with productivity, as a high conversion will ultimately affect the purity and the level of downstream processing required. In the

isolated enzyme bioreduction of 6-bromo-β-tetralone to 6-bromo-β-tetralol, 3.9g/L (Figure 4.13) could be produced, in the whole cell bioreduction of 6-bromo-β-tetralone, 1.2g/L (Figure 5.10) could be produced. These results show that under the conditions applied, the isolated enzyme biocatalyst has a productivity 3 times greater than the whole cell biocatalyst (these results do not include any bioreactor recycling as developed in Chapter 4).

Table 6.1 Effect of Substrate Concentration on Reaction Parameters

This table shows how much of the initial substrate has been converted after the allocated time. In all cases, as the initial amount of substrate is increased, the amount converted has decreased.

Substrate Concentration (g/L)	Isolated Enzyme at t = 420min		Whole Cell at t = 450 min	
	g/L Product	Conversion	g/L Product	Conversion
0.5	0.46	92	0.40	79
1	0.86	86	0.79	79
1.5	1.25	83	1.08	72
2.0	1.64	82	1.06	53
2.5	2.03	81	1.03	41
3.0	2.31	77	1.17	39

For an initial substrate concentration of 1g/L the isolated enzyme also achieves a higher conversion of 86% after 420min, where the whole cell biocatalyst exhibits a final conversion of 79% after 450. Neither the isolated enzyme nor the whole cell system reached 100% conversion within the experimentation time at any of the substrate concentrations employed. There are a number of reasons which could explain the cause of the low productivities and conversions. One of which may be the effects of the method used for the delivery of the substrate and also the method used for the removal of the product; these are discussed later in this chapter (Chapter 6.6). Other explanations include the method of running the bioreduction, for example, a number of the reaction parameters were not monitored and controlled. In the isolated enzyme system, the quantity of regenerating enzyme, substrate, and the cofactor was calculated and added to the reaction mix in quantities which would not affect the isolated enzyme; however, the affect these substrates had on the pH was

not monitored. The mixing of the reaction was not optimised (although was maintained constant for comparison) neither was the temperature of the reaction optimised. The most logical reason the whole cell productivity is lower than the isolated enzyme productivity is probably due to the hydrophobicity of the substrate and product. This hydrophobicity was discussed in Chapter 5.4 where it was hypothesized that as the substrate and product accumulate in the cell, the components are no longer homogenous in solution, this then causes the cells to be at higher substrate/product concentrations then if the solution was homogeneous. In the isolated enzyme system, the enzymes, substrates and the products are homogeneous throughout the reaction mixture, thus the effects of small intense areas of substrate/product were not an issue; however, the isolated enzyme was directly impacted by the addition of any components including mediums used to deliver substrates of low water solubility.

6.6 Substrate Delivery and Product Removal

It is not unusual for synthetic biocatalytic substrates to be highly insoluble in aqueous environments (Angelova *et al.*, 1999). To increase the availability of low aqueous solubility substrates to the active site of the enzyme requires an increase in the availability of the substrates surface area. If the substrate is added as a solid and is not soluble in aqueous environments then the molecule will not be able to bind to the active site of the enzyme. One method to increase the availability of the surface area of the substrate to the enzyme is to increase the solubility of the substrate by dissolving the substrate in a solvent. The substrate, which has been solubilised in a solvent, can then be delivered homogenously to the biocatalytic solution. The optimal method of delivering the substrate to the biocatalyst will depend mainly on the stability of the biocatalyst under the given conditions and also the properties of the substrate and product. In an isolated enzyme system, the enzyme is homogenous in solution and will be impacted directly by the addition of other compounds/solutions; therefore the addition of a substrate must be carefully controlled particularly if the compound is toxic to the enzyme. In the case of

tetralone reductase, the effects of using a solvent for substrate delivery were observed immediately by a decrease in catalytic activity as the amount of solvent was increased. In the isolated enzyme system, the enzyme comes into direct contact with the solvent and the carefully folded structure is perturbed by the solvent disturbing the interactive forces which maintain the enzyme in its folded state in an aqueous solution (Klysov *et al.*, 1975). Therefore, to maintain a high enzyme activity and stability the amount of solvent added to the reaction solution should be minimised to reduce the enzyme/solvent contact. In the case of the whole cells of *Trichosporon capitatum* (MY1890) the effects were somewhat different in that an increase in solvent used to deliver the substrate actually increased the biocatalytic reaction rates. This was only true for low levels of substrate (0.5g/L), and substrate concentrations at levels higher than this had a much more detrimental effect.

Table 6.2 Effect of Solvent Concentration on Reaction Parameters

This table shows that the isolated enzyme reaction rate decreases with increasing solvent concentrations. The whole cell reaction rate increases with reaction rate. The data was taken from results observed after 90 minutes. (Substrate concentration was 0.5g/L).

Isolated Enzyme at t = 90min			Whole Cell at t = 90min		
Solvent Concentration (%)	g/L Product	Conversion (%)	Solvent Concentration (%)	g/L Product	Conversion (%)
1	0.40	80	1	0.23	46
2	0.37	74	2	0.25	50
3.8	0.35	70	4	0.29	58
7.4	0.33	66	8	0.32	64
10.7	0.25	50	12	0.35	70

For the whole cell system, it is assumed that at high substrate levels, and therefore high solvent levels (as substrate concentration increases, the volume of solvent required to dissolve the substrate also increases), the solvent increases the rate of mass transfer of substrate across the cell membrane, which in turn increases the rate of accumulation of substrate and product in the cell. This then increases the rate of enzyme activation until a point at which the enzyme becomes inactivated by the high

concentration of substrate/product available in the cell. If lower volumes of solvent are used there is less interaction with the membrane, and thus the rate of mass transfer across the membrane is reduced. In the whole cell system, whether a solvent which exhibits a high solubility of the substrate is used or whether a solvent which exhibits a lower solubility is used is important in determining the rate of transfer of substrate across the cell membrane. However, it likely the interaction and accumulation of the substrate and product within the cell membrane which is most significant in determining conversions. It is therefore important in the cell solution to control both the addition and the removal of the substrate and the product respectively. It can also be important to control the amount of substrate in the reaction due to potential unwanted changes in the enantiomeric excess. In a recent example, the enantiodivergent oxidation of a racemic bicyclohept-2-en-6-one by cyclohexanone monooxygenase from *Acinetobacter calcoaceticu* showed that substrate concentration and the degree of conversion significantly affected the enantiomeric excess of one of the lactones (Zambianchi *et al.*, 2000).

The main problem with the whole cell biocatalytic system was not the biocatalyst itself but the interaction of the hydrophobic substrate and product with the cell membrane, and for the isolated enzyme it was a combination of the substrate and product and the use of a solvent to deliver the components. Therefore, to increase the substrate concentration in the reaction solution for both systems most likely requires different process methodologies. For the whole cell system, there needs to be a method of supplying the substrate yet avoiding the accumulation of the substrate and product in the cells. For the isolated enzyme it is important that there is controlled substrate addition and product removal in an environment which is aqueous rich. One method to increase the substrate concentration may be to employ a different type of medium which would maintain enzyme activity and stability. Mediums could include two-phase systems (León *et al.*, 1998; Carrea *et al.*, 1988) or new types of media for lipophilic compounds such as ionic liquids (Kragl *et al.*, 2002) may be more appropriate to use. The use of resins as described in Chapters 4

and 5 may also be an appropriate method of substrate delivery and product removal. However, for whole cell systems, two-phase systems, ionic liquids, and resins may only be available as a delivery system if a high enough affinity is not available for the product to diffuse back into the medium. The whole cell system and also the isolated enzyme system could potentially both benefit from increasing the solubility of the substrate, perhaps even by chemically changing the structure of the substrate into a more soluble form. However, a less water soluble compound may decrease the conversion in the whole cell system as a substrate soluble in an aqueous environment may have problems in diffusing across the cell membrane without the use of a solvent.

If the converted substrate causes a detrimental effect on the biocatalytic activities and conversions, or the build-up of product has the potential to have a negative affect on the enantiomeric excess, then the product should be removed from the reaction solution. In the bioreduction of 6-bromo-β-tetralone the removal of the product from the two biocatalytic systems was achieved using two different methods. The product from the isolated enzyme system was removed by adsorbing the product onto a cheap hydrophobic resin, this meant that the enzymes could be recycled. The adsorbed product was then removed from the resin by a solvent wash. In the whole cell biocatalytic system, the product was removed by the addition of a volume of solvent equivalent to the reaction solution volume; the addition of such high volumes of solvent destroyed the cells so they could not be reused/recycled. This product solution was then centrifuged to remove the cell debris and finally a two-phase solvent would be employed to remove any media components found in the supernatant. The amount of downstream processing required for both of the biocatalytic systems is hard to quantify, although it would be fair to say that the whole cell system requires much more. The isolated enzyme system is a much cleaner system as much of the clean-up work is completed during the enzyme isolation and purification.

6.7 Hypothesis for Overexpression

An enzyme cloned from its native cell and overexpressed into a well-defined microorganism can appear advantageous in comparison to the native cell biocatalyst. This is because the host cell will contain a higher ratio of overexpressed enzymes to host cell enzymes than the native cell, the host cell is also generally better defined than the native microorganism. Therefore, overexpressing tetralone reductase into a well-defined microorganism gives the potential for producing a biocatalyst with greater reaction rates and potentially higher tolerances to components such as hydrophobic substrates and solvents. If tetralone reductase could be overexpressed to a level of about 10% of the total protein in the cell, this would potentially have significant effects on the productivity of the enzyme. A higher level of about 40% overexpression could possibly be achieved but this could lead to the formulation of inclusion bodies, which are not an active form for biocatalysis (Violand, 2001), so a more realistic level of 10% will be used to predict the potential of the isolated enzyme and the whole cell biocatalyst.

The results from Chapter 5.4.9 suggest that if an oxidoreductase is cloned and overexpressed in a new microorganism, then there are a number of complications which can arise. One of which is that the enzymes which are naturally available in the host cell may compete with the cloned enzyme for the synthetic substrate and thus may cause a reduction in the amount of active product produced. Competing enzymes may cause a reduction in the optical purity of the substrate, or may even bind to a separate part of the molecule to create an altogether different molecule. For example, the compound may have two reducing centres in different locations on the molecule; a competing enzyme may reduce the incorrect centre creating a molecule which may be difficult to separate from the active molecule. This is similar to the results from Chapter 5 (Figure 5.14), where it was observed that 6-bromo-β-tetralone was reduced by an active reductase from *E.coli*, this reduction caused by a competing enzyme could contaminate the active product and cause difficulties in downstream separation. The productivity of the cells for the

enantiomeric bioreductions of pharmaceutically important precursors is likely to be unaffected through recombinant technology. This is because although the sequencing, cloning, and overexpression of the gene which codes for tetralone reductase into another microorganism (or *Trichosporon capitatum* MY 1890) would theoretically increase the quantity of active protein in the cell, it would not guarantee a parallel increase in the amount of product produced. There are two principle reasons for this; firstly, the model substrate 6-bromo-β-tetralone adheres to the cells of *Trichosporon capitatum* (MY 1890), (whether this was inside the cell, in the membrane, or elsewhere is unknown and more research needs to be done on the transport of lipophilic compounds into and out of the cell before this can be determined). In Chapter 5, (Figure 5.13) the substrate and product were also shown to adhere to *E.coli* JM107 pQR711, even though this was a completely different type of cell. The adherence of 6-bromo-β-tetralone to the whole cell was caused by its hydrophobicity, and therefore the substrate is highly likely to adhere to any other type of microorganism. This argument is obviously only valid for hydrophobic compounds, however, many important pharmaceutical precursors are highly insoluble in aqueous environments (Angelova *et al.*, 1999). The second reason an increase in enzyme in the whole cell may not increase the amount of product produced is because the enzyme tetralone reductase is an oxidoreductase which requires the nicotinamide cofactor, NADH. Overexpressing the enzyme in *E.coli* may cause NADH limitation as the overexpression of the enzyme may not automatically cause an increase in the quantity of cofactor available or quantity of the regenerating enzyme. The reaction would therefore become limited by the units of cofactor, or the units of regenerating enzyme, rather then as at present, the enzyme. It may, however, be possible to overexpress another enzyme in the same microorganism to reduce the cofactor, but again, would not increase the initial amounts of cofactor, and low cofactor levels could still limit the system. This problem of cofactor depletion has previously been described (Boonstra *et al.*, 2001), whereby the depletion was overcome by the expression of a third enzyme into *E. coli*, a soluble pyridine nucleotide transhydrogenase. The transhydrogenase

catalysed the transfer of reducing equivalents between the cofactor pools of NAD and NADP without the requirement for any other substrate. This increased the productivity when compared to the system lacking the soluble transhydrogenase. This result did not, however, increase the amount of available cofactor. It was the oxidised/reduced form of the cofactor, which had been altered.

Overexpressing and cloning tetralone reductase to level of 10% of the total protein in a well-defined cell (100 times greater than previous level) would most likely be more advantageous for the isolated enzyme. The same size fermentations would create significantly more active enzyme, the amount of chromatography resin required for purification would be less due to the decreased binding of other cell proteins. However, although providing more enzyme, there would probably be the same number of purification steps to remove the residual cell debris and unwanted enzymes, this is because resins of differing properties are used to separate the diverse cell components and this would still be required albeit in smaller amounts. The purification steps may be more efficient due to the higher concentration of active enzyme, and the enzyme may be more stable because of this high active protein concentration, high protein content can maintain activity this has previously been reported (Johnson *et al.,* 1978; see Chapter 1.11). The cloning of tetralone reductase into a well-defined microorganism would potentially increase the fermentation efficiencies due to enhanced characterized fermentation methods, this would mean an improvement for both the isolated enzyme and whole cell systems. However, in the recombinant whole cell system, the competing enzymes would need to be identified and quantified for the potential detrimental effects on the active substrate. The affects of substrate and product hydrophobicity of the transfer of these components across the cell membrane in the recombinant microorganism will also need to be determined. The ideal scenario would be the overexpression of tetralone reductase into a well-defined microorganism, in a system where there are the same number of units of enzyme as there are units of cofactor, and where the kinetic parameters of the regenerating enzyme are comparable (whether this was in

the cell or in a synthetically built environment). The substrate would be delivered and the product removed in a controlled manner. In a system like this, although the use of whole cells seems appealing, the reality of co-overexpressing two enzymes, one from a filamentous yeast, into *E.coli* with correct refolding of the enzymes and concurrent increase in NADH may not currently be a viable option.

6.8 Discussion

NADH dependent isolated enzymes and whole cell biocatalysts can be used for the reduction of carbonyl groups in the production of various chiral compounds. In general, it is the whole cell biocatalysts which are selected to perform such reactions. The rationale for employing whole cells include: the general consensus that whole cell biocatalysts are easy to use, expensive isolation of the desired enzyme is avoided, and cofactor regeneration is completed via the cells' own metabolic pathway. However, cells frequently contain enzymes which interfere with the desired reaction so that a low yield and low enantiomeric excess of the product can result (Hummel, 1997). Isolated enzymes, in contrast, perform with higher selectivity, have a higher space time yields, but require the addition of a cofactor regeneration system. They also require purification which is generally considered as a highly complex and time-consuming activity. In this thesis, the comparison of an isolated reductase and a whole cell biocatalyst demonstrated that the common assumptions regarding the employment of biocatalysts were generally true. However, there were also a number of other significant differences between each biocatalyst type, and these differences were generally a consequence of the model reaction system which was employed. The model reaction system that was used resulted in a number of operating constraints which were unavoidable due to the biocatalyst and reaction component characteristics. The constraints for each of the operating systems are summarised below in Table 6.3

Table 6.3 Operating Constraints

The physical state of a biocatalyst used in a bioreaction and the characteristics of the reaction components can influence the method by which the reaction needs to be run due to the constraints of the system.

Isolated Enzyme Tetralone Reductase Operating Constraints	
1	Tetralone reductase needed to be purified; this added extra time, complexity and expense to the overall reaction. Also, the fact that tetralone reductase is not over-expressed meant that only small quantities of the enzyme were produced at the end of the purification.
2	Tetralone reductase and its cofactor were immediately affected by changes to the environment, this meant that good control of the reaction was required and minimal addition of other compounds was essential.
3	NADH needed to be recycled for two reasons: (1) it was expensive; (2) it caused substrate/product inhibition. Thus, the requirement to use a secondary enzyme made the reaction more complicated than if just a single enzyme was used.
4	Since NADH needed to be recycled this increased the processing costs as both NADH and FDH were expensive components, this also meant that much of the experimentation had to be completed in small volumes.
5	Immobilisation of Tetralone Reductase was not possible since the substrate and product adhered to the available surfaces. This meant that removal of the substrate and product from the enzyme required another method.
6	Tetralone reductase was adversely affected by relatively large amounts of substrate.
7	Miscible solvents had a detrimental effect on tetralone reductase activity. This reduction in activity drives towards the use of two-phase solvents or other types of medium. Two-phase solvents, however (in general) did not solubilise 6-bromo-β-tetralone.

Whole Cell Biocatalyst *Trichosporon capitatum* (MY1890) Operating Constraints	
1	The initial growth of the cells required sterile technology and the reaction required aseptic techniques. However, such methods and equipment are well defined and readily available.
2	The reaction was run with growing cells, this meant that the final reaction solution had a large amount of biomass and growth medium. This caused difficulties in the handling and downstream processing.
3	The substrate and product adhered to the cell (probably the cell membrane); this meant that an extraction method was required to isolate the product and unconverted substrate from the cells.
4	The accumulation of substrate and product in the cells caused a reduction in cell

	viability.
5	*Trichosporon capitatum* (MY1890) was adversely affected by relatively large amounts of substrate.
6	At high substrate concentrations the addition of miscible solvents caused a reduction in the biocatalytic reaction rates.

The general assumption for the isolated enzyme system was that the isolated enzyme system would be difficult to employ (mainly due to the requirement for a cofactor recycling system to be set up). It was also suggested that the isolated enzyme would be unstable (caused by removing the enzyme from its natural cell environment). The methodologies and results from this thesis suggest that there are similarities and differences between the results and general assumptions. Contrary to the general assumptions, the isolated enzyme system was easy to employ and the enzyme retained much of its activity for a long period of time. The set up of a cofactor regeneration system was reasonably complicated but easily repeated; the only key problem would be the expense of the regenerating enzyme at large scale. The main difficulty was the isolation and purification which were considerably complex and time-consuming to develop, although once developed were robust and reproducible. The enzyme was unstable when removed from the cell environment, but this was due to the isolation buffer containing a certain protease inhibitor which was not necessary. This problem was rectified by removing the protease inhibitor from the reaction buffer. The enzyme also became unstable in the reaction solution due to the addition of solvents and substrates. This means that the enzyme must be protected as much as possible from the reaction components without causing a significant reduction on the reaction rates. Based on the observations taken from this thesis, the main consideration when employing isolated enzymes currently is the expense caused by using NAD(H) and FDH at large scale.

The general assumptions for the whole cell system suggested that whole cell systems are robust and simple to employ, since whole cells (in general) have been previously used for bioreduction reactions. The results from this thesis agree that the whole cell system was relatively easy to employ for the bioreduction. However, there were

considerably difficulties caused by the use of a substrate and product of low aqueous solubility. The operating constraints caused by using a substrate and product which were hydrophobic meant that they adhered to the cell membrane and caused enzyme inhibition /cell toxicity at very low levels of substrate. This meant the whole cell was difficult to employ and optimise as a biocatalyst in an efficient bioreactor, and only low levels of product could be obtained. There were also difficulties in recovering the product since it was retained by the cell. The only method for product extraction was via the addition of a solvent which in turn destroyed the cells for future use.

This thesis employed *Trichsporon capitatum* (MY1890) and tetralone reductase (isolated from this microorganism) for the bioreduction of 6-bromo-β-tetralone to 6-bromo-β-tetralol. This allowed a comparison to be made between two types of biocatalyst. The results from this thesis give good direction into the problems associated with using both types of systems. However, the comparison only deals with the model reaction system (low aqueous soluble substrate and product); and it is very likely that if a similar biocatalysts were employed with substrates of high aqueous solubility the results would be significantly different. In the use of biocatalysts for a long term applications, the comparison should look at all of the substrates and products which will be employed. The results from this thesis demonstrate that the isolated enzyme is a preferable system (it is cleaner, has higher reaction rates and higher conversions, the isolation is also easy to perform and robust) to the whole cell system. But, before tetralone reductase is used at an industrial scale it is essential that further investigation into the systematic regeneration and retention of cofactors is carried out.

6.9 Summary

The objective of the work described in this thesis was to compare the application of an isolated enzyme and a whole cell biocatalyst for the stereospecific bioreduction of a poorly soluble ketone. The results demonstrated that the isolated enzyme

exhibited higher conversions and higher reaction rates than the whole cell biocatalyst. Both of the biocatalytic systems were constrained by the substrate and product which exhibited low solubility in aqueous environments. In the isolated enzyme system, the enzyme activity and related conversions were directly affected by the addition of a solvent (required to solubilise the substrate) which most likely perturbed the active site of the enzyme. In the whole cell system, the substrate and product accumulated in the cell causing a decrease in the cell viability resulting in lower substrate conversions and reaction rates. Both systems were similar in that their substrate specificities were comparable; this suggests that the mass transfer through the cell membrane may be more reliant upon substrate hydrophobicity than molecule size. Recombinant technology is potentially more applicable to the isolated enzyme system. This is because an increase in the amount of active enzyme in the whole cell will potentially increase the purification efficiency. However, for the whole cell there would still be the problems of the substrate and product accumulating inside the cell. The application of the isolated enzyme system is preferable to the use of whole cell system; however, further work is required into the efficient regeneration of cofactors to reduce process costs at large scale.

7. Conclusions

1. A stable form of tetralone reductase (a novel NADH-requiring oxidoreductase) can be purified from the cells of *Trichosporon capitatum* (MY 1890) via homogenisation, centrifugation, and chromatography.

2. The stability of tetralone reductase is dependent upon the buffer conditions selected for isolation, purification and storage.

3. Removal of the protease inhibitor cocktail containing EDTA from the isolation and purification buffers increased tetralone reductase stability from 7hr at 22^0C to 92hr at 22^0C.

4. Tetralone reductase is inhibited by EDTA and requires a metal cation in its active site. However, tetralone reductase has a high enough affinity for the metal cation that extra amounts are not required in the buffers, and tetralone reductase is actually inhibited by heavy metal cations at levels of greater than 5mM.

5. Tetralone reductase exhibits its highest activity at the pH value of 6.5. There is an essential arginine residue and an essential sulfhydryl group in the active site of tetralone reductase. Tetralone reductase is also inhibited by levels of NADH greater than 0.4g/L.

6. Tetralone reductase activity is dependent on the type of solvent used. Methoxyethanol was selected as the most appropriate solvent as it maintains tetralone reductase activity and solubilises 6-bromo-β-tetralone to 250g/L.

7. Tetralone reductase could not be applied as an immobilised enzyme using Eupergit C™ because the substrate and product have a high affinity to this

immobilisation resin, and thus separation of the substrate and product from the support could not be performed.

8. The maximum productivity achieved by tetralone reductase using a cofactor regeneration system was 3.9g of product per g of cell mass.

9. An alternative bioreactor was developed using an adsorbent resin for *in situ* removal of the product. The bioreactor allows the free enzyme to be used and NADH to be recycled via the oxidation of formate by formate dehydrogenase and reused in at least a 2-pass reactor; tetralone reductase was the rate limiting factor in the reactions.

10. The whole cells of *Trichsporon capitatum* (MY 1890) can be used in the bioreduction of 6-bromo-β-tetralone to 6-bromo-β-tetralol.

11. The substrate and product due to their inherent hydrophobicity adhere to the whole cells and extraction of the substrate and product can only be achieved via solvent extraction

12. Increasing the quantity of substrate serves to increase this accumulation of substrate/product inside the cell, which in turn causes a detrimental affect on the biocatalytic potential of the cells.

13. Increasing the amount of solvent increases the rate of reaction (at low substrate concentrations) in the whole cell system due to the interactions between the solvent and the lipophilic membrane; this facilitates an increase in the rate of mass transfer of substrate across the membrane. The maximum product achieved in the whole cell system was 1.2g of product per g of cell mass.

14. Overexpression of the enzyme in either the native cell or a host cell is probably more useful, currently, for the isolated enzyme due to the issues of substrate/product accumulation in the whole cell.

8. Future Work

- Fully purify, sequence, and overexpress tetralone reductase into a host cell. The high level of overexpressed active enzyme in the cell will potentially enable a more efficient isolation and purification of tetralone reductase to be achieved. Tetralone reductase can then be used to perform the bioreduction of 6-bromo-β-tetralone, where tetralone reductase is no longer the rate-limiting factor. This will enable an accurate determination of the TTN (total turnover number) of the cofactor, as the cofactor will not be added in excess. This number can then be used to establish the cost of the process relative to the cost of the cofactor and the enzymes used. The host microorganism containing the overexpressed enzyme could also be used to determine whether there would be an increase in the activities and conversions of the whole cell biocatalyst or whether the adherence of the substrate to the cell would as hypothesized be the limiting factor.

- The addition and removal of the substrate and product respectively via polymeric resins is currently in its infancy and further research into such systems, particularly for oxidoreductases (whole cell and isolated enzyme) could provide answer to substrate and product toxicity. The use of resins to deliver the substrate and remove the product in the reaction solution could also eliminate the need for a solvent, and therefore remove the detrimental effects these solvents have on the enzyme. However, future work should also focus the transport of lipophilic compounds into and out of cells and the position of the compounds in

the cell (Angelova, 1999). The addition of a resin with the substrate adsorbed in a whole cell system, in some systems, may not desorb into solution and similarly may not adsorb back onto the resin, and research is required to understand the transport of the substrate and product through the cell membrane using resins. In the event that resins may not be applicable to all bioconversions and microorganisms it would also be appropriate to investigate novel approaches to increase the bioavailability of the substrate; for example, ionic liquids (Kragl *et al.*, 2002).

- Scale-up of a bioreactor with substrate delivery and product removal in a column reactor in an optimised and controlled environment with re-use of the enzyme and cofactor. This would give a greater degree of assurance, in that not only can an oxidoreductase can be used in such a manner with cofactor recycling, but also that significant quantities of product are obtainable from such a system. It would also be of use to compare the isolated enzyme bioreactor to a whole cell bioreactor. Substrate delivery and product removal has been previously described for whole cells in batch reactors (Vicenzi *et al.*, 1997) and for column reactors (Hilker *et al.*, 2004), but neither has been described for isolated oxidoreductases. Column reactors using whole cell biocatalysts would, however, have to be fluidized to some degree to prevent the column from blocking. Although, the whole cells do not have to be reused (as they do not suffer the problems of the high cost of cofactors), and therefore it may be more appropriate to run the whole cell reduction in a batch reactor.

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APPENDIX A: Calibrations

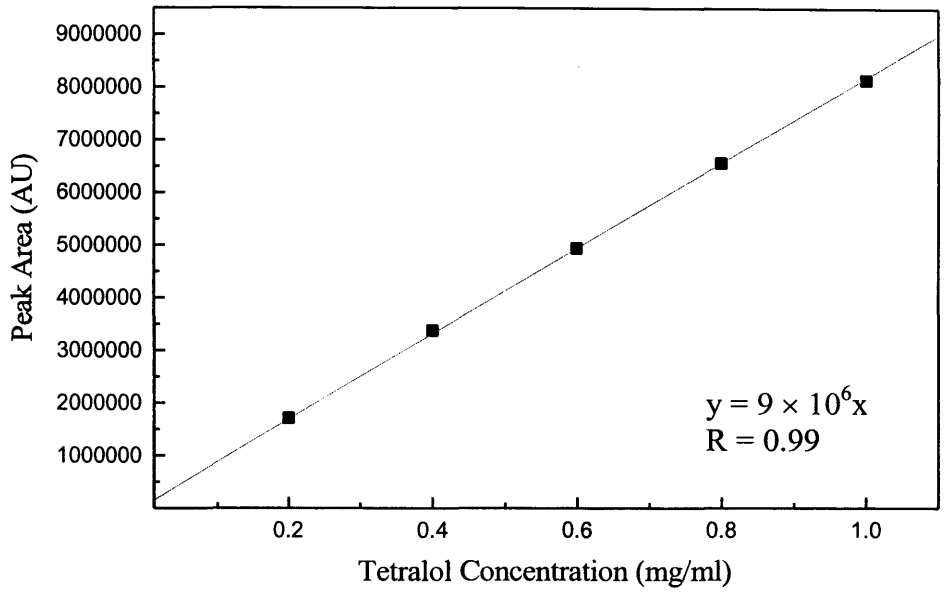

Figure A1.1 Calibration Curve of 6-bromo-β-tetralol

Analysis of predetermined quantities of 6-bromo-β-tetralol, solubilised in ethanol via the HPLC method defined in Chapter 2.8.2, to determine the peak area in absorbance units (AU). The AU of an unknown sample concentration can then be employed to determine the actual sample concentration.

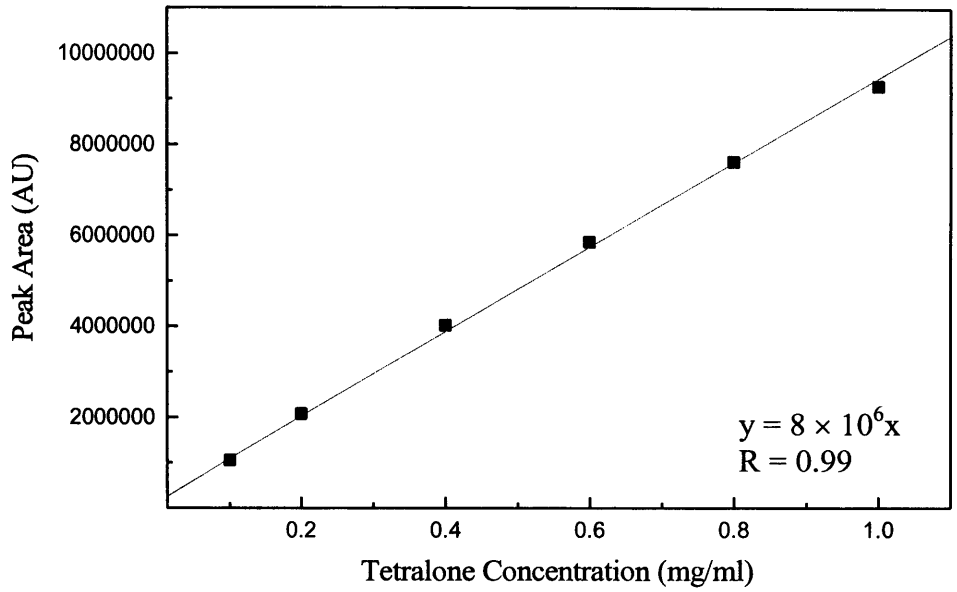

Figure A1.2 Calibration Curve of 6-bromo-β-tetralone

Analysis of predetermined quantities of 6-bromo-β-tetralone, solubilised in ethanol via the HPLC method defined in Chapter 2.8.2, to determine the peak area in absorbance units (AU). The AU of an unknown sample concentration can then be employed to determine the actual sample concentration.

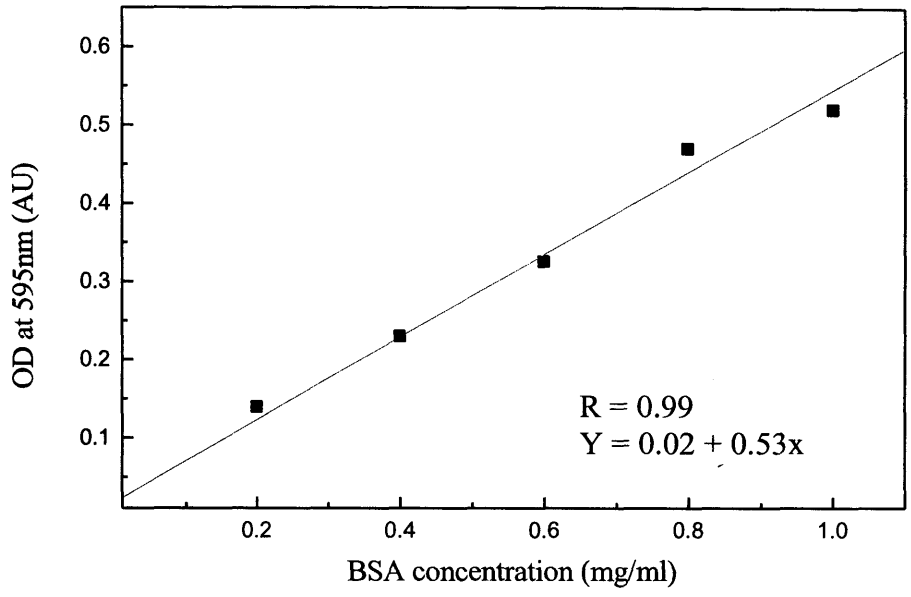

Figure A1.3 Calibration Curve of Total Protein

Analysis of predetermined quantities of BSA via the spectrophotomeric method defined in Chapter 2.8.5 to determine the absorbance units. The optical density of an unknown protein concentration can then be employed to determine the actual protein concentration in mg/ml.

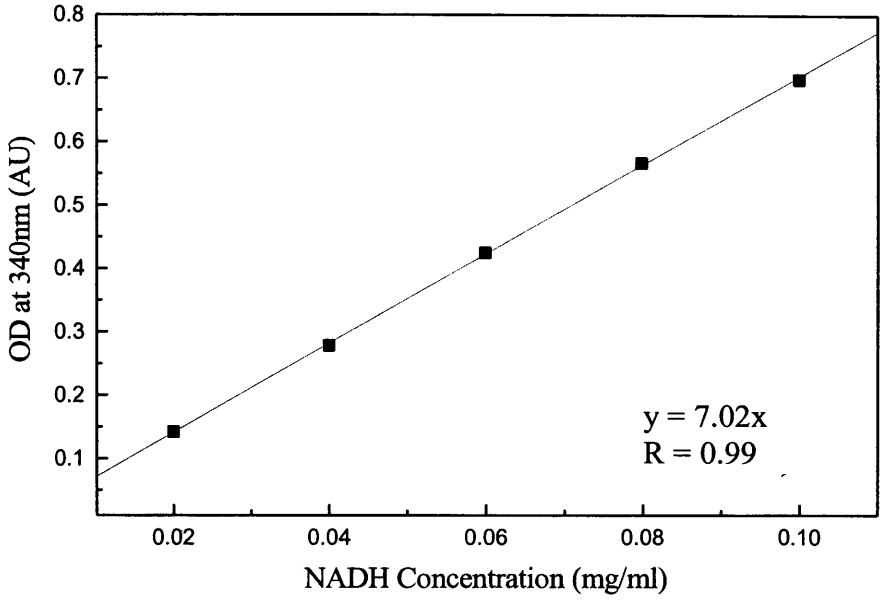

Figure A1.4 Calibration Curve of NADH

Analysis of predetermined quantities of NADH via the spectrophotomeric method defined in Chapter 2.8.3 to determine the absorbance units. The optical density of an unknown NADH concentration can then be employed to determine the actual NADH concentration in mg/ml.

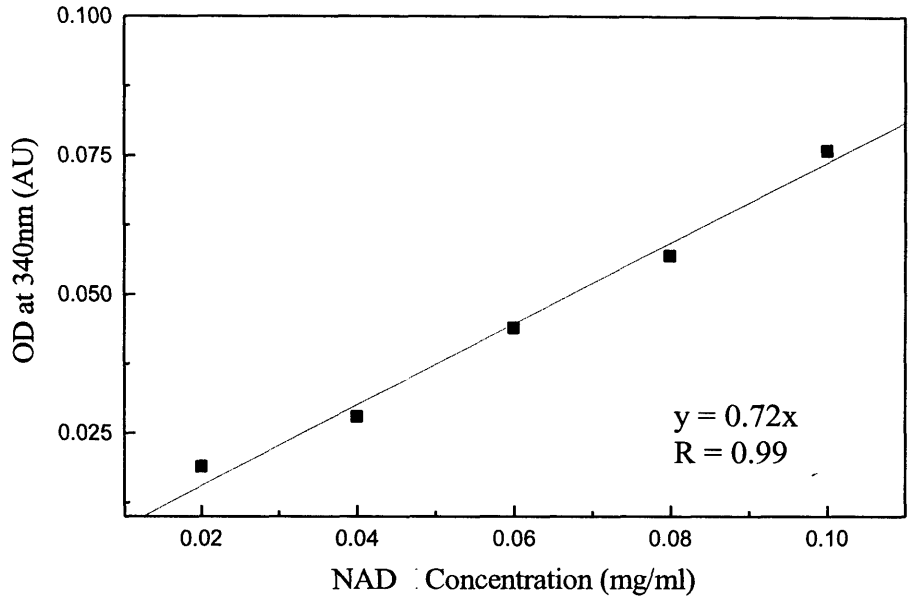

Figure A1.5 Calibration Curve of NAD

Analysis of predetermined quantities of NAD via the spectrophotomeric method defined in Chapter 2.8.3 to determine the absorbance units. The optical density of an unknown NAD concentration can then be employed to determine the actual NAD concentration in mg/ml.

APPENDIX B: Raw Data

B1.1 Raw Data Isolated Enzyme Bioconversion - Change in Substrate Concentration (see Figure 4.5)

Raw Data Isolated Enzyme Bioconversion									
Substrate (g/L)									
Time (min)	10	30	60	90	120	180	240	360	420
	0.46	0.36	0.25	0.11	0.05	0.04	0.04	0.04	0.04
	0.47	0.39	0.27	0.14	0.08	0.05	0.05	0.05	0.05
0.5	0.48	0.38	0.26	0.12	0.07	0.04	0.04	0.03	0.03
	0.92	0.78	0.57	0.37	0.20	0.18	0.17	0.16	0.14
	0.95	0.79	0.58	0.41	0.22	0.19	0.21	0.20	0.18
1	0.98	0.81	0.59	0.40	0.25	0.23	0.19	0.18	0.17
	1.42	1.29	1.10	0.91	0.65	0.53	0.42	0.29	0.26
	1.43	1.30	1.09	0.91	0.64	0.51	0.40	0.26	0.24
1.5	1.44	1.31	1.08	0.89	0.67	0.55	0.46	0.34	0.29
	1.93	1.78	1.58	1.37	0.16	0.73	0.64	0.44	0.36
	1.94	1.80	1.61	1.40	1.18	0.76	0.68	0.46	0.41
2	1.95	1.82	1.60	1.39	1.20	0.81	0.72	0.52	0.46
	2.44	2.29	2.08	1.65	1.36	1.00	0.76	0.52	0.48
	2.44	2.28	2.10	1.70	1.39	1.04	0.83	0.57	0.58
2.5	2.45	2.31	2.09	1.67	1.33	0.93	0.67	0.50	0.47
	2.93	2.80	2.59	2.09	1.75	1.26	1.05	0.75	0.70
	2.95	2.80	2.57	2.08	1.74	1.21	1.02	0.69	0.59
3	2.94	2.80	2.59	2.03	1.69	1.18	1.14	0.98	0.82
Product (g/L)									
Time (min)	10	30	60	90	120	180	240	360	420
	0.04	0.13	0.25	0.39	0.45	0.46	0.46	0.46	0.46
	0.03	0.12	0.24	0.37	0.43	0.45	0.46	0.45	0.45
0.5	0.02	0.12	0.24	0.36	0.42	0.46	0.46	0.46	0.47
	0.07	0.21	0.42	0.63	0.80	0.83	0.83	0.84	0.86
	0.06	0.21	0.42	0.59	0.78	0.80	0.79	0.80	0.81
1	0.02	0.20	0.41	0.60	0.76	0.77	0.81	0.82	0.83
	0.07	0.21	0.41	0.62	0.84	0.98	1.08	1.22	1.25
	0.07	0.20	0.41	0.59	0.86	1.00	1.09	2.24	1.26
1.5	0.06	0.19	0.42	0.59	0.82	0.94	1.03	1.16	1.18
	0.07	0.21	0.42	0.63	0.84	1.26	1.36	1.57	1.64
	0.06	0.20	0.40	0.61	0.83	1.24	1.32	1.54	1.59
2	0.06	0.20	0.40	0.60	0.80	1.20	1.28	1.49	1.54
	0.07	0.21	0.41	0.82	1.13	1.50	1.74	1.98	2.03
	0.06	0.22	0.40	0.80	1.12	1.45	1.65	1.94	1.92
2.5	0.06	0.19	0.41	0.83	1.17	1.57	1.83	2.00	2.12
	0.07	0.20	0.40	0.91	1.25	1.75	1.95	2.25	2.30
	0.06	0.20	0.42	0.92	1.26	1.78	1.97	2.30	2.41
3	0.06	0.20	0.40	0.96	1.30	1.82	1.86	2.02	2.17

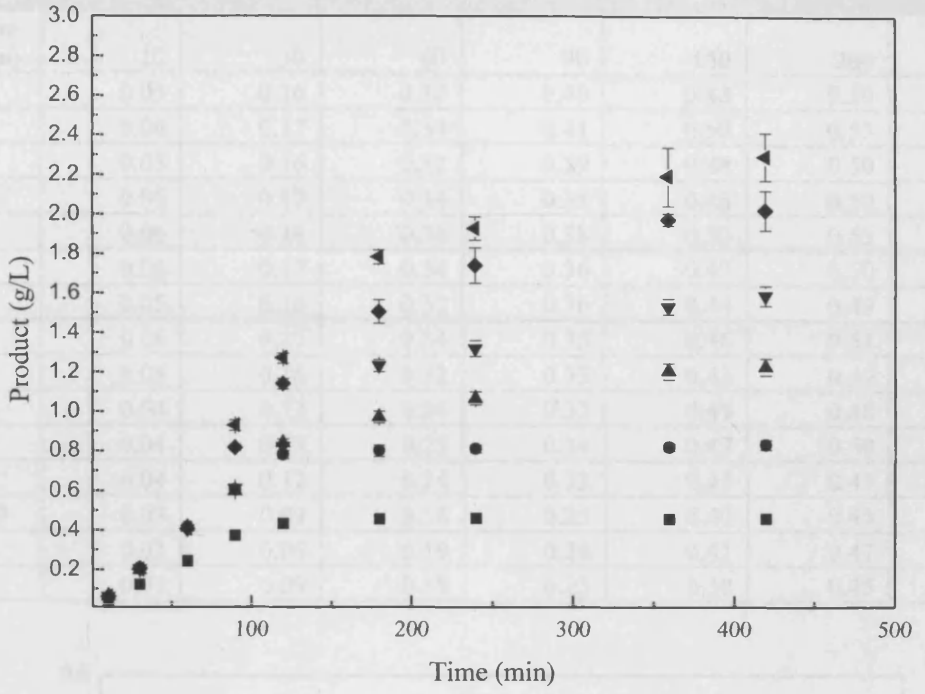

Figure B1.1 Isolated Enzyme - Reaction Profile
Isolated enzyme bioconversion reaction profile with substrate concentration as the experiment variable. The substrate concentrations used were as follows: ■ (0.5g/L) ● (1g/L) ▲ (1.5g/L) ▼(2g/L) ◆ (2.5g/L) ◀ (3g/L) - See Figure 4.5 for more detail.

B1.2 Raw Data Isolated Enzyme Bioconversion - Change in Solvent Concentration (see Figure 4.6)

Raw Data Isolated Enzyme Bioconversion							
Substrate Concentration (g/L)							
Time (min)	10	30	60	90	150	260	400
1	0.47	0.36	0.20	0.11	0.04	0.02	0.01
	0.47	0.36	0.20	0.13	0.04	0.02	0.02
	0.46	0.35	0.18	0.11	0.02	0.02	0.02
2	0.46	0.34	0.16	0.14	0.02	0.02	0.02
	0.46	0.35	0.18	0.14	0.05	0.02	0.02
	0.46	0.35	0.18	0.16	0.05	0.02	0.02
3.8	0.46	0.36	0.20	0.15	0.07	0.03	0.03
	0.46	0.34	0.18	0.17	0.06	0.02	0.02
	0.48	0.37	0.20	0.19	0.10	0.03	0.03
7.4	0.48	0.40	0.29	0.21	0.08	0.05	0.03
	0.48	0.39	0.26	0.18	0.04	0.02	0.02
	0.47	0.41	0.28	0.20	0.07	0.05	0.03
10.7	0.49	0.43	0.34	0.27	0.13	0.07	0.05
	0.50	0.43	0.33	0.25	0.11	0.05	0.02
	0.49	0.43	0.33	0.27	0.11	0.06	0.05

Raw Data Isolated Enzyme Bioconversion							
Product Concentration (g/L)							
Time (min)	10	30	60	90	150	260	400
1	0.05	0.16	0.32	0.40	0.48	0.50	0.50
	0.06	0.17	0.34	0.41	0.50	0.53	0.53
	0.05	0.16	0.32	0.39	0.48	0.50	0.50
2	0.06	0.17	0.34	0.38	0.48	0.50	0.50
	0.06	0.18	0.36	0.38	0.50	0.53	0.53
	0.06	0.17	0.34	0.36	0.47	0.50	0.50
3.8	0.05	0.16	0.32	0.36	0.44	0.49	0.50
	0.06	0.17	0.34	0.35	0.46	0.51	0.52
	0.05	0.16	0.32	0.33	0.43	0.49	0.49
7.4	0.04	0.12	0.24	0.32	0.45	0.48	0.49
	0.04	0.13	0.25	0.34	0.47	0.50	0.51
	0.04	0.12	0.24	0.32	0.45	0.47	0.49
10.7	0.03	0.09	0.18	0.25	0.40	0.45	0.48
	0.03	0.09	0.19	0.26	0.41	0.47	0.50
	0.03	0.09	0.18	0.25	0.39	0.45	0.47

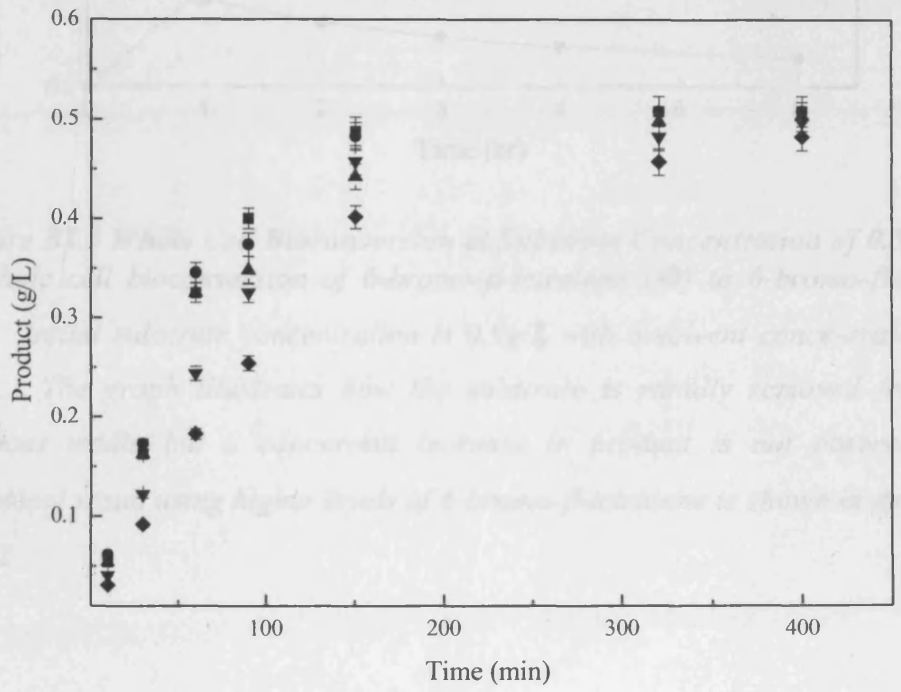

Figure B1.2 Isolated Enzyme - Reaction Profile
Isolated enzyme bioconversion reaction profile with solvent concentration as the experiment variable. The solvent concentration used are as follows: ■ (1%) ● (2%) ▲ (3%) ▼ (8%) ◆ (10.7%) solvent volume - See Figure 4.6 for more detail.

B1.3 Whole Cell Bioconversion without Recovery

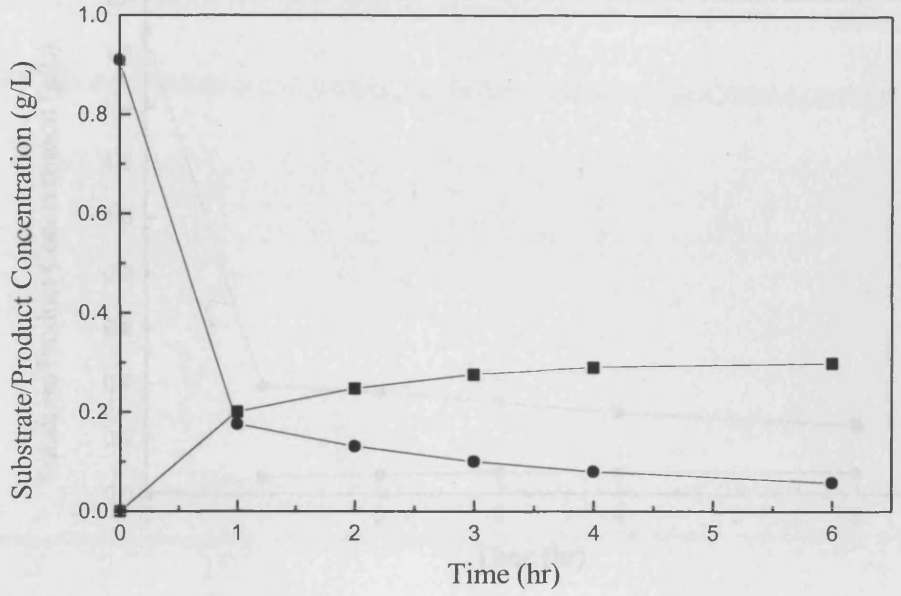

Figure B1.3 Whole Cell Bioconversion at Substrate Concentration of 0.5g/L
A whole cell bioconversion of 6-bromo-β-tetralone (●) to 6-bromo-β-tetralol (■). Initial substrate concentration is 0.9g/L with a solvent concentration 9% (v/v). The graph illustrates how the substrate is rapidly removed from the aqueous media but a concurrent increase in product is not observed. A graphical result using higher levels of 6-bromo-β-tetralone is shown in Appendix B 1.2

B1.4 Whole Cell Bioconversion without Recovery

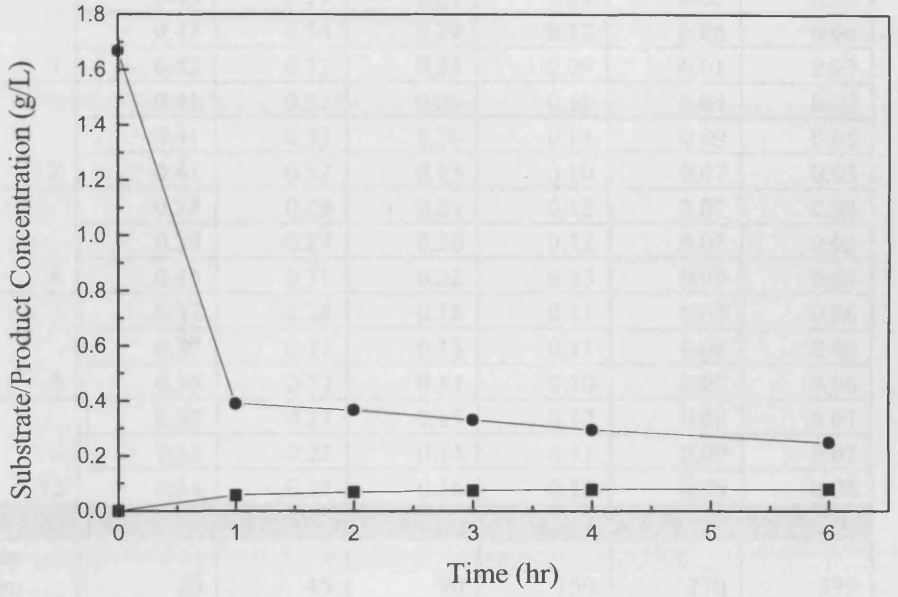

Figure B1.4 Whole Cell Bioconversion at Substrate Concentration of 0.9g/L
A whole cell bioconversion of 6-bromo-β-tetralone (●) to 6-bromo-β-tetralol (■). Initial substrate concentration is 1.7g/L with a solvent concentration 17% (v/v). The graph illustrates how the substrate is rapidly removed from the aqueous media but a concurrent increase in product is not observed.

B1.5 Raw Data Whole Cell Bioconversion Change in Solvent Concentration (see Figure 5.7)

Raw Data Whole Cell Bioconversion						
Substrate Concentration (g/L)						
Time (min)	20	45	90	150	270	390
	0.43	0.34	0.27	0.09	0.05	0.04
	0.42	0.34	0.29	0.12	0.06	0.06
1	0.42	0.32	0.25	0.09	0.01	0.03
	0.41	0.31	0.23	0.10	0.04	0.02
	0.41	0.33	0.26	0.11	0.09	0.05
2	0.41	0.32	0.25	0.10	0.07	0.03
	0.38	0.29	0.21	0.12	0.07	0.05
	0.39	0.27	0.20	0.12	0.07	0.05
4	0.41	0.31	0.22	0.13	0.09	0.05
	0.37	0.26	0.18	0.11	0.07	0.06
	0.37	0.27	0.18	0.11	0.06	0.06
8	0.36	0.27	0.17	0.10	0.07	0.06
	0.33	0.23	0.15	0.12	0.09	0.07
	0.33	0.22	0.14	0.11	0.09	0.07
12	0.34	0.24	0.16	0.12	0.08	0.08
Product Concentration (g/L)						
Time (min)	20	45	90	150	270	390
	0.07	0.16	0.23	0.40	0.45	0.46
	0.08	0.17	0.23	0.40	0.47	0.47
1	0.08	0.17	0.23	0.39	0.46	0.47
	0.09	0.18	0.25	0.40	0.44	0.48
	0.09	0.18	0.26	0.39	0.43	0.48
2	0.09	0.18	0.25	0.40	0.43	0.47
	0.11	0.21	0.29	0.37	0.42	0.45
	0.11	0.22	0.30	0.38	0.43	0.46
4	0.10	0.20	0.28	0.37	0.42	0.45
	0.13	0.24	0.33	0.40	0.44	0.44
	0.13	0.23	0.31	0.39	0.43	0.44
8	0.13	0.24	0.33	0.40	0.43	0.44
	0.17	0.27	0.35	0.38	0.41	0.43
	0.18	0.28	0.36	0.38	0.41	0.43
12	0.18	0.28	0.35	0.40	0.42	0.43

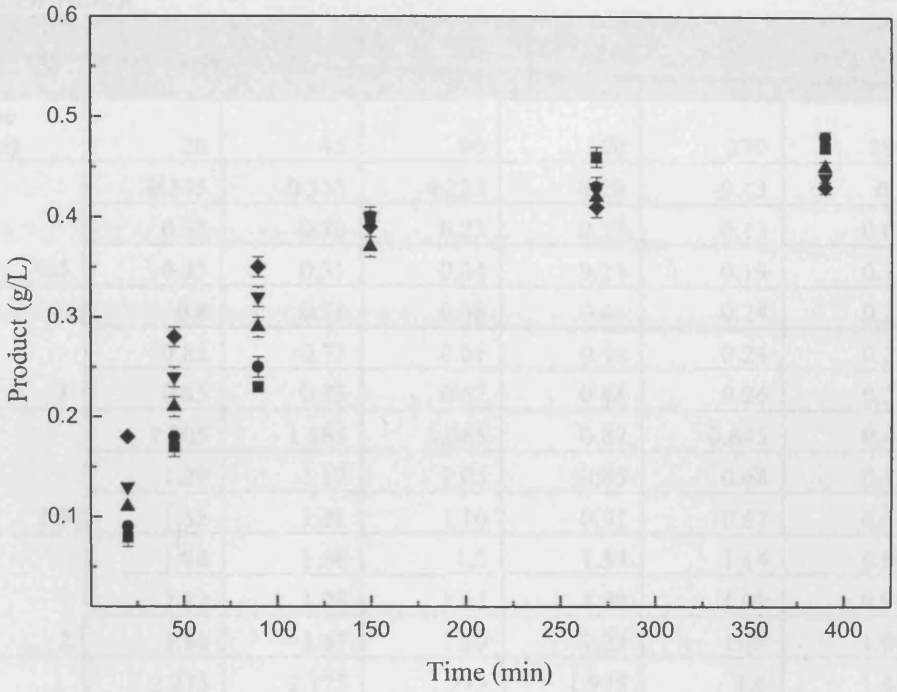

Figure B1.5 Whole Cell Biocatalyst – Reaction Profile
Whole cell bioconversion reaction profile with solvent concentration as the experiment variable. The solvent concentrations used were as follows: ■ (1%) ● (2%) ▲ (4%) ▼ (8%) ◆ (12%) - See Figure 5.7 for more detail.

B1.6 Raw Data Whole Cell Bioconversion - Change in Substrate Concentration

Raw Data Whole Cell Bioconversion							
Substrate (g/L)							
Time (min)	20	45	90	150	270	390	450
	0.335	0.315	0.225	0.18	0.13	0.1	0.105
	0.33	0.30	0.23	0.19	0.13	0.09	0.09
0.5	0.33	0.31	0.24	0.21	0.19	0.13	0.13
	0.8	0.71	0.58	0.43	0.24	0.21	0.21
	0.82	0.73	0.61	0.44	0.24	0.21	0.20
1	0.85	0.73	0.62	0.48	0.26	0.27	0.26
	1.305	1.185	1.065	0.87	0.645	0.45	0.42
	1.29	1.17	1.05	0.85	0.63	0.43	0.40
1.5	1.32	1.21	1.10	0.91	0.67	0.49	0.47
	1.78	1.66	1.5	1.34	1.14	0.96	0.94
	1.82	1.73	1.53	1.49	1.09	0.97	0.97
2	1.80	1.67	1.50	1.35	1.19	1.00	1.00
	2.275	2.175	1.975	1.975	1.6	1.45	1.475
	2.28	2.19	1.99	1.95	1.55	1.43	1.43
2.5	2.28	2.19	1.98	1.96	1.56	1.46	1.46
	2.82	2.64	2.49	2.25	2.13	1.91	1.9
	2.86	2.68	2.53	2.29	2.17	1.95	1.94
3	2.80	2.62	2.40	2.23	2.11	1.92	1.91
Product (g/L)							
	0.17	0.19	0.28	0.32	0.37	0.40	0.40
	0.16	0.19	0.26	0.30	0.36	0.40	0.40
0.5	0.17	0.19	0.26	0.29	0.31	0.38	0.38
	0.20	0.29	0.42	0.57	0.76	0.79	0.79
	0.19	0.28	0.40	0.57	0.77	0.80	0.81
1	0.16	0.28	0.40	0.54	0.75	0.74	0.75
	0.20	0.32	0.44	0.63	0.86	1.05	1.08
	0.19	0.30	0.41	0.59	0.81	0.99	1.03
1.5	0.19	0.30	0.41	0.60	0.84	1.02	1.04
	0.22	0.34	0.50	0.66	0.86	1.04	1.06
	0.22	0.32	0.48	0.63	0.84	1.00	1.02
2	0.21	0.34	0.51	0.66	0.82	1.01	1.01
	0.23	0.33	0.53	0.53	0.90	1.05	1.03
	0.21	0.30	0.50	0.54	0.94	1.06	1.06
2.5	0.23	0.32	0.53	0.55	0.95	1.05	1.05
	0.18	0.36	0.51	0.75	0.87	1.14	1.10
	0.16	0.33	0.51	0.72	0.80	1.07	1.09
3	0.19	0.37	0.59	0.76	0.88	1.16	1.08

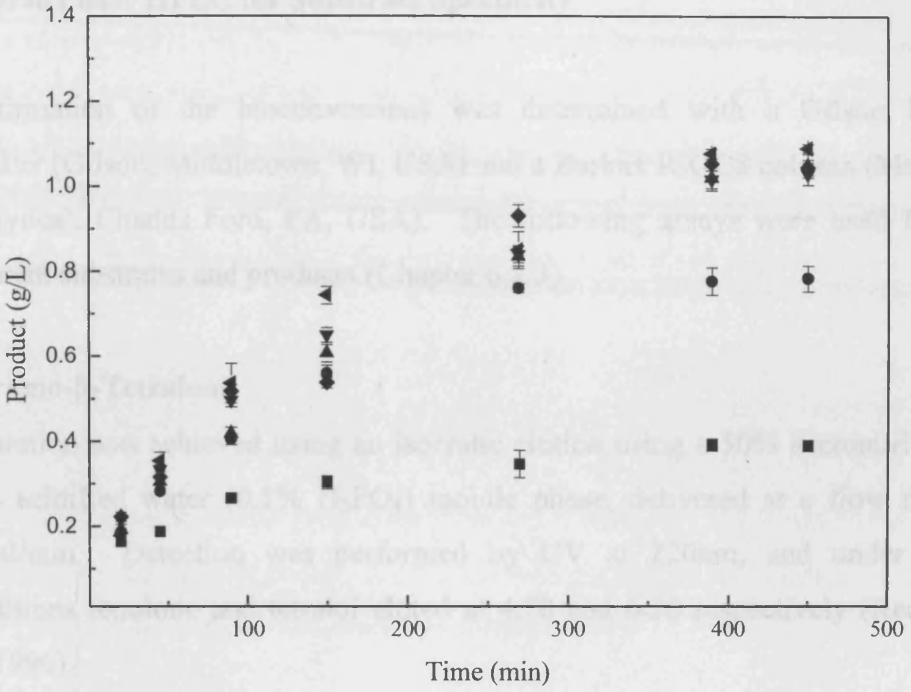

Figure B1.6 Whole Cell Biocatalyst Reaction Profile
Change in Substrate Concentration. Initial substrate concentrations are as follows■ (0.5g/L) ● (1g/L) ▲ (1.5g/L) ▼(2g/L) ◆ (2.5g/L) ◀ (3g/L) - See Figure 5.9 for more detail.

APPENDIX C: Materials and Methods

Reverse Phase HPLC for Substrate Specificity

Confirmation of the bioconversions was determined with a Gilson Liquid Handler (Gilson, Middletown, WI, USA) and a Zorbax RX-C8 column (MacMod Analytical, Chadds Ford, PA, USA). The following assays were used for the different substrates and products (Chapter 6.2.3).

6-Bromo-β-Tetralone

Separation was achieved using an isocratic elution using a 50% acetonitrile and 50% acidified water (0.1% H_3PO_4) mobile phase, delivered at a flow rate of 1.5ml/min. Detection was performed by UV at 220nm, and under these conditions tetralone and tetralol eluted at 4.70 and 6.20 respectively (Reddy *et al.*, 1996).

Benzyl Acetoacetate

Separation was achieved using a gradient of acetonitrile and acidified water (0.1% H_3PO_4) at a flow rate of 1ml/min. The gradient was 20/80 (v/v) acetonitrile/water to 80/20 over 20min, held for 5min and the re-equilibrated for 5min before the next injection. Detection was performed at 220nm, the alcohol and ketone eluted at 10.7min and 12.8min respectively.

Cyclohexylphenyl Ketone

Separation was achieved using an isocratic elution of a 50% acetonitrile and 50% acidified water (0.1% H_3PO_4) mobile phase, delivered at a flow rate of 1.5ml/min. Detection was performed by UV at 210nm, and under these conditions the ketone eluted at 11.2min, no peak was observed for the alcohol (Chartrain *et al.*, 1997).

β-Ketoester

Separation was achieved using a gradient of acidified acetonitrile (0.1% H_3PO_4) and acidified water (0.1% H_3PO_4) at a flow rate of 1.5ml/min. The gradient was 20/80 (v/v) acetonitrile/water to 70/30 over 10min, held for 20min. The system the re-equilibrated to 20/80 (v/v) for 7min before the next injection. Detection was performed at 200nm, and under these conditions the ketone eluted at 14.7min, no peak was observed for the alcohol (Chartrain *et al.*, 1995).

Ketone

Separation was achieved using a gradient of acetonitrile and acidified water (0.1% H_3PO_4) at a flow rate of 1ml/min. The gradient was 20/80 (v/v) acetonitrile/water to 80/20 over 20min, held for 5min and the re-equilibrated for 5min before the next injection. Detection was performed at 220nm, the ketone eluted at 8.2min, no peak was observed for the alcohol

Separation was achieved using a gradient of acidified acetonitrile (0.1% H_3PO_4) and acidified water (0.1% H_3PO_4) at a flow rate of 1.5ml/min. The gradient was 10/90 (v/v) acetonitrile/water to 90/10 over 25min then re-equilibrated for 5min before the next injection. Detection was performed at 254nm. (Roberge *et al*, 1996)

1,2-Indanedione

Separation was achieved using a gradient of acetonitrile and acidified water (0.1% H_3PO_4) at a flow rate of 1ml/min. The gradient was 10/90 (v/v) acetonitrile/water to 90/10 over 20min, held for 3min then re-equilibrated for 2min before the next injection. Detection was performed at 220nm, the ketone eluted at 9.8min, no peak was observed for the alcohol (Stahl *et al.*, 1999).

Bisaryl Ketone

Separation was achieved using an isocratic elution using a 50% acetonitrile and 50% acidified water (0.1% H_3PO_4) mobile phase, delivered at a flow rate of 1ml/min. Detection was performed by UV at 210nm. (Chartrain *et al.*, 2000).

Ketosulfone

Separation was achieved using a gradient of acetonitrile and acidified water (0.1% H_3PO_4) at a flow rate of 1ml/min. The gradient was 20/80 (v/v) acetonitrile/water to 90/10 over 20min, held for 5min then re-equilibrated for 5min before the next injection. Detection was performed at 220nm, the ketone eluted at 13.5min, no peak was observed for the alcohol, no peak was observed for the alcohol.

Chloroketone

Separation was achieved using a gradient of acetonitrile and water at a flow rate of 1ml/min. The gradient was 40/60 (v/v) acetonitrile/water to 90/10 over 15min, held for 10min then re-equilibrated for 5min before the next injection. Detection was performed at 220nm, alcohol and ketone eluted at 5.9min and 8.2min respectively.

Ketoester

Separation was achieved using a gradient of acetonitrile and water at a flow rate of 1ml/min. The gradient was 40/60 (v/v) acetonitrile/water to 90/10 over 15min, held for 10min then re-equilibrated for 5min before the next injection. Detection was performed at 220nm, the

1-indanone

Separation was achieved using a gradient of acetonitrile and acidified water (0.1% H_3PO_4) at a flow rate of 1ml/min. The gradient was 20/80 (v/v) acetonitrile/water to 80/20 over 20min, held for 5min and the re-equilibrated for 5min before the next injection. Detection was performed at 220nm, the ketone eluted at 10.9min no peak was visible for the alcohol

2-Indanone

Separation was achieved using a gradient of acetonitrile and acidified water (0.1% H_3PO_4) at a flow rate of 1ml/min. The gradient was 20/80 (v/v) acetonitrile/water to 80/20 over 20min, held for 5min and the re-equilibrated for 5min before the next injection. Detection was performed at 220nm, ketone eluted at 11.5min.

β-Tetralone

Separation was achieved using a gradient of acetonitrile and acidified water (0.1% H_3PO_4) at a flow rate of 1ml/min. The gradient was 20/80 (v/v) acetonitrile/water to 80/20 over 20min, held for 5min and the re-equilibrated for 5min before the next injection. Detection was performed at 220nm, the alcohol eluted at 10.3min.

α-Tetralone

Separation was achieved using a gradient of acetonitrile and acidified water (0.1% H_3PO_4) at a flow rate of 1ml/min. The gradient was 20/80 (v/v) acetonitrile/water to 80/20 over 20min, held for 5min and the re-equilibrated for 5min before the next injection. Detection was performed at 220nm, the ketone eluted at 31.2min, no peak was visible for the alcohol.

APPENDIX D: List of Suppliers

Amersham Pharmacia Biotech
UK Ltd.,
Amersham Place
Little Chalfont
Bucks
HP7 9NA

Amersham Pharmacia Biotech Inc.,
800 Centennial Ave
PO Box 1327
Piscataway
NJ
08855
USA

APV Eura
23 Gatwick Road
Crawley
W Sussex
RH10 2JB
UK

Bio-Rad Laboratories Ltd
1000 Alfred Nobel Drive
Hercules
CA 94547
USA

Beckman Coulter, Inc
4300 N. Harbour Boulevard
PO Box 3100
Fullerton
CA 92634-3100
USA

Roche Diagnostics
F.Hoffmann – La Roche Ltd
Dianostics Division
Grenzacherstrasses
CH – 4070 Basel
Switzerland

Bio-Rad Laboratories Ltd
Bio-Rad House
Maylands Ave
Hemel Hempstead
Herts
HP2 7TD

Dionex
4 Albany Court
Camberley
Surrey
GU16 7QL
UK

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

Mac-Mod Analytical, Inc.
127 Commons Court
Chadds Ford
PA 19317
USA

Merck and 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

Phenomenex
2320 North 205th Street
Torrance
CA 90501-1456
USA

Röhm GmbH
Chemische fabrik
Kirschenalle
64293 Darmstadt
Germany

Sigma-Aldrich Company Ltd
Fancy Road
Poole
Dorset
BH12 4QH
UK

USA Scientific Inc.
PO Box 3565
Ocala
FL 24478-3565
USA

Whatman Plc
Whatman House
St Leonard Street
Maidstone
Kent
UK