Process issues in redox biocatalysis: cyclohexanone monooxygenase catalysed chiral lactone syntheses

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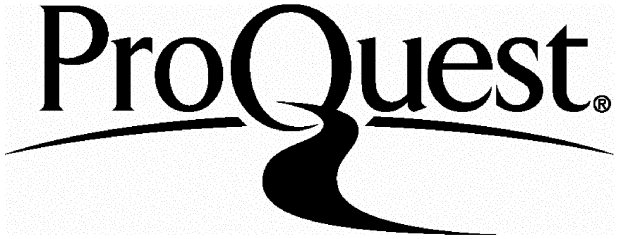

For Mum and Dad

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Abstract

This thesis investigates the Baeyer-Villiger oxidation of cyclic ketones to optically enriched lactones by the enzyme cyclohexanone monooxygenase (CHMO), cloned into *Escherichia coli* JM107 pQR210. Two model substrates were selected (2-hexyl cyclopentanone and 4-methyl cyclohexanone) to conduct investigations with. A major constraint found was that whole cell catalysis produced low reaction rates and poor enzyme stability. Isolated enzyme was stabilised effectively by using elevated levels of the cofactor NADPH.

Recycle of the expensive NADPH was investigated by detailed studies of thermostable glucose and alcohol dehydrogenases. These were characterised by marked product inhibition. Alcohol dehydrogenase from *Thermoanaerobacter brockii* (TBADH) was chosen for the ease of removal of the acetone product from the system and the high affinity for NADPH.

The interaction between CHMO and TBADH was modeled by simultaneous numerical integration of their rate equations leading to an understanding of the effect of different enzyme ratios on system performance. This model also predicts the conditions necessary to maximise cofactor stability and re-usability. Quantification of a range of processing strategies was performed, fed-batch operation was found to be 2.5 times more productive than batch.

Multi-gram syntheses of lactones were performed at 2L scale with both free and immobilised enzymes. NADPH recycle was effective at producing over 700 reaction cycles. Immobilised CHMO was found to be significantly more stable than free enzyme under process conditions, a catalyst with retained activity of 12% and specific activity of $1.2Ug^{-1}$ was produced. TBADH produced 42% retained and $13.6Ug^{-1}$ specific activity. Co-immobilisation of both enzymes on the same support produced a catalyst with an activity of $0.6Ug^{-1}$.

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Nomenclature

ε	extinction coefficient, $mL\mu mol^{-1}cm^{-1}$
C	Final amount of cofactor after each batch, mmol
E	amount of enzyme, kg
[S]	concentration of species S, $mmolesL^{-1}$
[So]	initial concentration of species S, $mmolesL^{-1}$
Q	mass flow Rate, kgs^{-1}
X	fractional conversion, dimensionless
K_i	Inhibition constant, mM
K_La	volumetric mass transfer coefficient, s^{-1}
K_i	inhibition constant, mM
K_m	substrate concentration that gives an activity of $V_{max}/2$
$K_{m(x)app}$	apparent Km for species x, mM
Ks	dissociation constant of the enzyme-inhibitor complex, mM
$K_{obs}(X)$	observed first order decay constant for species X, h^{-1}
STY	space time yield, $gL^{-1}day^{-1}$
$t_{1/2}$	half life, h
R	cofactor recharge required, mmol
U	Units of enzyme activity, $\mu molmin^{-1}$ product formed
V	reactor volume, m^3
V_{max}	maximum enzyme reaction rate, $mmols^{-1}$
Y_n	yield of product in organic phase, mmol

Abbreviations

2-HCP	2-hexyl cyclopentanone
3-CPBA	3-chloroperoxybenzoic acid
4-MCH	4-methyl cyclohexanone
5-MOP	(S)-5-methyl oxepane-2-one
6-HTPO	(S)-6-hexyl tetrahydropyran-2-one
ADH	alcohol dehydrogenase
BSA	bovine serum albumin
BSTR	batch stirred tank reactor
bpt	boiling point
CHMO	cyclohexanone monooxygenase
CLEC	cross linked enzyme crystal
CPFR	continuous plug flow reactor
CPMP	Council for Proprietary Medicinal Products
CSTR	continuous stirred tank reactor
DSP	downstream processing
ee	enantiomeric excess
FAD	flavin adenine dinucleotide
FDA	Food and drug administration
FDH	formate dehydrogenase
FID	flame ionisation detector
FMN	flavin mononucleotide
FBSTR	fed batch stirred tank reactor
GC	gas chromatography
GDH	glucose dehydrogenase
G6PDH	glucose-6-phosphate dehydrogenase
HPLC	high performance liquid chromatography
I	inhibitor
IPTG	isopropyl β-D-thiogalacto pyranoside

ISPR	*In-situ* product removal
mpt	melting point
MW	molecular weight
n	batch number
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
OD	optical density
PEG	polyethylene glycol
RO	reverse osmosis
rpm	revolutions per minute
TAGDH	glucose dehydrogenase from *Thermoplasma acidophilum*
TBADH	alcohol dehydrogenase from *Thermoanaerobacter brockii*
TEPP	tetraethyl pyrophosphate
TLPBSTR	two liquid phase batch stirred tank reactor
TLC	thin layer chromatography
Tris-HCL	Tris-(hydroxymethyl) aminomethane
TTN	total turnover number
TTN_n	total turnover number after batch n
UV	ultraviolet
vvm	volume.volume^{-1}minute^{-1}

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1.0 Introduction

1.1 Chirality

1.1.1 Definition

Chirality is a term used to describe a property of molecules that exists when an atom (usually carbon) has attached to it four different functionalities. The consequence of this is to create two distinct forms of the same molecule that differ only in their spatial arrangement, forming mirror images that are not superimposable upon one another. A molecule with this property is termed *chiral*. **Figure 1.1** below illustrates how these two forms of the same compound can exist, and are in fact mirror images.

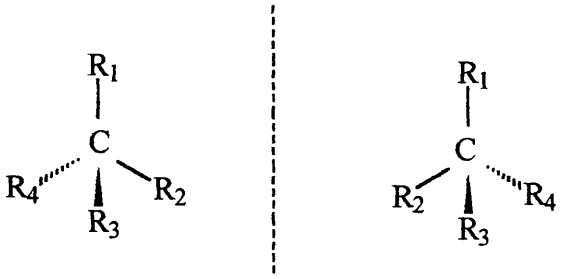


Figure 1.1 Optical isomers

The two mirror image forms of a particular compound are called *enantiomers* and can be distinguished by the fact that they rotate plane polarised light in opposite directions when one form is present in excess over the other, hence the traditional use of the phrase *optical isomers*. A mixture of equal proportions of both enantiomers is known as a *racemic* mixture.

Enantiomeric excess (ee) is a term widely used to define the proportion of the two enantiomers of a compound within a mixture and is calculated using equation (1) where the two enantiomers are termed A and B.

$$ee = \left(\frac{[A]-[B]}{[B]+[A]}\right) \text{ x } 100 \qquad (1)$$

Thus a racemic mixture will have an ee of 0%, and a ratio of enantiomer A to B of 300 will have an ee of 99%.

In almost any environment the two enantiomeric forms of any compound exhibit identical chemical behaviour. The exception to this is when interactions occur with another chiral molecule, this is extremely common within biological systems and so becomes a serious issue in the pharmaceutical industry when evaluating the potency and toxicity of potential chiral drug candidates.

1.1.2 Advantages of enantiomeric purity over racemic mixtures

Two enantiomeric forms of the same compound often have different pharmacological effects when administered as a therapeutic drug or agrochemical (Collins *et al*, 1997). The most infamous example of this is the different effect each enantiomer of thalidomide has on the body. One enantiomer produces the desired antiemetic effect, whilst the other is a powerful teratogen causing limb deformity in developing foetuses. The drug was administered as a racemic mixture, thus generating the side effects in patients caused solely by the undesired enantiomer.

Other possibilities with administering racemic mixtures are that one enantiomer may be completely inactive thus the dose given to a patient is twice that theoretically

necessary generating the possibility of side effects due entirely to the large dosage. It is also possible that both enantiomers have the same effect, but not with the same potency. An example of this is the anti-inflammatory Ibuprofen (Cannarsa, 1996). **Table 1.1** below summarises the potential advantages of enantiopure pharmaceuticals over racemates.

Properties of racemate	**Potential benefits of enantiomer**
One enantiomer is exclusively active	Reduced dose and load on metabolism
The other enantiomer is toxic	Increased latitude in dose and broader use of the drug
Enantiomers have different pharmokinetics	Better control of kinetics and dose
Enantiomers metabolised at different rates in the same person	Wider latitude in setting the dose; reduction in variability of patients' response
Enantiomers metabolised at different rates in the population	Reduction in variability of patients' responses; greater confidence in setting a single dose
One enantiomer prone to interaction with key detoxification pathways	Reduced interactions with other common drugs
One enantiomer is agonist, the other is antagonist	Enhanced activity and reduction of dose
Enantiomers vary in spectra of pharmacological action and tissue specificity	Increased specificity and reduced side effects for one enantiomer; use of other enantiomer for different indication

Table 1.1. Potential therapeutic benefits of enantiomerically pure drugs.

1.1.3 Industrial significance of chiral molecules

The global market for optically pure pharmaceuticals is extremely large, in 1995 approximately $50 bn worth of sales of optically pure pharmaceuticals were achieved, amounting to one fifth of the total pharmaceutical market, with sales in recent years achieving 20% growth per annum (Cannarsa, 1996). Sales rose to $71.1 in 1997 with half the top 100 selling drugs being single enantiomers (Stinson, 1998). It has been estimated that 75% of synthetic pharmaceuticals and 20% of agrochemicals will be single enantiomers by the year 2000 (Collins *et al,* 1997). **Table 1.2.** lists the top fifteen single enantiomer drugs in terms of sales.

Drug Name	Company	1995 sales/ $M
Epogen	Amgen	3060
Vasotec	Merck	2460
Premarin	Various	2080
Amoxil	Various	2080
Pravachol	BMS	2020
Humulin	Various	1970
Zocor	Merck	1825
Cardizem	Zeneca	1790
Capoten	BMS	1670
Zestril	Merck	1350
Mevacor	Merck	1260
Sandimmune	Sandoz	1250
Neupogen	Amgen	1230
Ceclor	Eli Lilly	1050
Zoloft	Pfizer	990

Table 1.2. The top single enantiomer drugs by sales (Cannarsa, 1996)

There is a growing trend to producing compounds with multiple chiral centres, such as newer protease inhibitors designed to combat the spread of HIV e.g. Abbott's Norvir and Merck's Crixivan (Davies and Reider, 1996). A recent review (Stinson, 1998) covers the current developments in new single enantiomer drug candidates.

Regulatory issues are also increasingly important. The FDA stipulates that both enantiomers of a potential new drug must be compared in terms of biological activity. Only if there is no appreciable difference may a racemate be used. This is usually not the case in that nearly all drugs have isomeric forms with different pharmacological effects. The European CPMP has a similar, if weaker, policy at present that says a racemic mixture can be used providing no data is available that illustrates improved safety of the single isomeric form over the mixture. Several drugs have recently switched from racemic to single isomer forms, i.e. desfenfluramine (anoretic), levofloxacin (antibiotic), ibuprofen (anti-inflammatory) and dilevalol (beta-blocker) (Juaristi, 1997). It seems almost certain that this trend will continue and regulations will become more restrictive with time, becoming the chief driving force in the development of improved methods of single enantiomer synthesis.

1.1.4 Methods for enantioselective synthesis

The methods of introducing enantioselectivity in syntheses are diverse. Many current targets are reactions termed *racemic switches*, single enantiomer forms of drugs that are currently on sale as racemates, such as Astra's stomach ulcer treatment Prilosec which has been recently patented as a single enantiomer variant (Stinson, 1998).

There are two general classes of reactions that generate enantiomerically enriched products, depending on the properties of the starting material. The first class of reaction is known as *kinetic resolution* and occurs when the starting material is a racemic mixture. Formation of product occurs from only one enantiomer of the

starting material, the other being essentially unreactive. Formation of product is thus limited to a 50% yield. An example of this is the esterification of a racemic carboxylic acid shown in **Figure 1.2** (Chen *et al*, 1987). One enantiomer forms the ester, the other is unreacted.

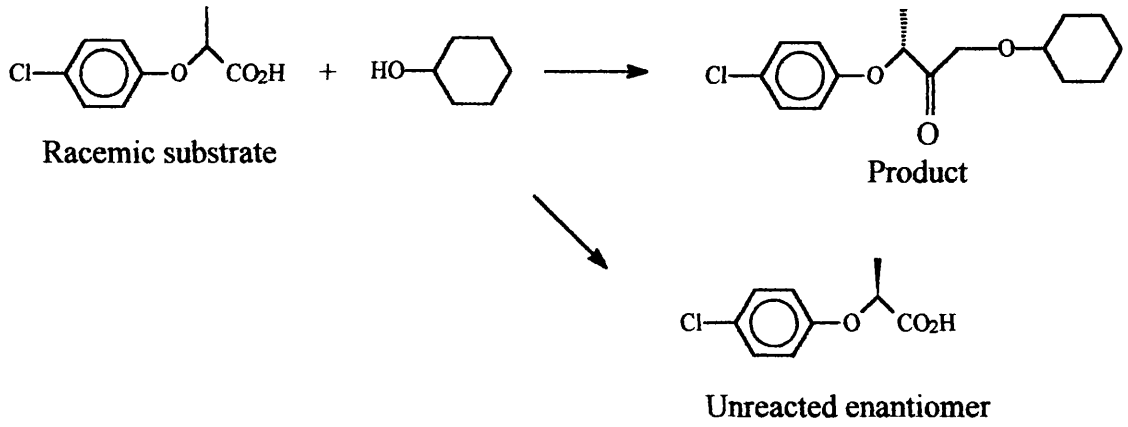
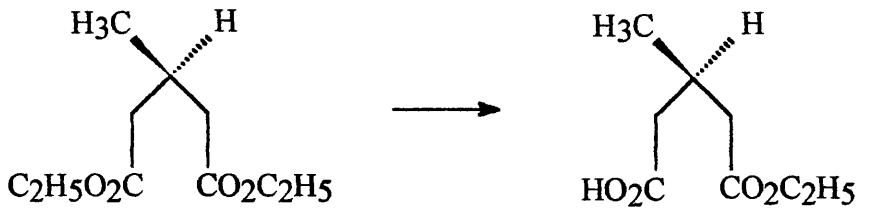


Figure 1.2 Kinetic resolution of a racemic carboxylic acid

This reaction class is not ideal due to the low yield and the required separation of the unreactive enantiomer post reaction. More emphasis has been placed on the second class of reaction termed *asymmetric synthesis*. In this case the starting material does not contain a chiral centre, the reaction introduces chirality and forms only one enantiomeric form of product, such as the hydrolysis reaction shown in **Figure 1.3** (Chen *et al*, 1982).



Non chiral substrate Single enantiomer product

Figure 1.3. Asymmetric synthesis from a prochiral substrate

The reaction is theoretically able to reach 100% yield and as such would require less purification. Asymmetric syntheses are now generally the favoured technology in developing routes to enantiopure pharmaceuticals (Cannarsa, 1996).

One area of intense interest is the use of chiral catalysts based on transition metal chemistry (Cannarsa, 1996) such as palladium, silver and platinum, often it is possible to prepare chiral forms of existing and established catalysts to convert racemic syntheses to enatiospecific reactions, making racemic switching a viable technology.

The main area of focus in developing enantioselective synthesis is currently the use of enzymes either in an isolated form or within a microorganism to perform reactions (Stinson, 1998). Enzymes by their very nature tend to be selective in processing one enantiomer of a compound over the other due to the biological origin of the catalyst. As new drug candidates become larger and more complicated with many different functionalities it is often necessary with chemical catalysis to introduce extra synthetic steps in their preparation in order to protect some of these functionalities from undesired side reactions (Davies and Reider, 1996). Enzymatic catalysts have a very significant advantage as generally the selectivity of their reaction is so complete and the reaction conditions are mild such that no protection (and subsequent deprotection) is required, allowing the number of synthetic steps to be reduced. The

use of an enzyme (in any form) to perform any specified chemical reaction, whether enantioselective or not is termed *biocatalysis*.

1.2 Biocatalysis

1.2.1 Industrial examples of biocatalytic processes

Biocatalysis is used widely in the process industries, from pharmaceutical manufacture, where the enzymatic reaction may be a single step in a multi step chemical synthesis, to waste treatment (Roberts *et al*, 1995; Faber, 1992). Biocatalysts were originally entirely from microbial sources, (Wang *et al*, 1979) however advances in genetic engineering have expanded the range available (Dordick *et al*, 1991; Bliem *et al*, 1991). The first commercially important application was the hydroxylation of progesterone in 1952 (Peterson *et al*, 1952). Enzymes show particular promise in their ability to catalyse enantioselectively, generating chirally pure products often in applications where there is no equivalent chemical technique available.

Biocatalysis can also offer advantages over traditional chemical syntheses in terms of energy usage, waste generation and purity. The biosynthesis of indigo is a process that can dispense with toxic chemical reagents and does not generate toxic waste material (Tramper, 1996). Biocatalysis is not yet able to compete on cost effectiveness with existing chemical methods for bulk chemical manufacture, although polyacrylamide is currently produced in Japan at a rate of 30000 tonnes/annum (Ashina and Masuro, 1993) in a process that has significantly "greener" credentials than the established chemical route. Biocatalysis also can offer potential routes to new compounds, which cannot be made via traditional chemical syntheses.

Several large scale biocatalytic processes are currently in late phase development or have just gone into production (Stinson, 1998). A recently developed process is that for the synthesis of the potent potassium ion channel blocker MK-499 (Merck) that uses an aminotransferase enzyme to introduce the correct enantioselectivity into an intermediate molecule (Davies and Reider, 1996). NSC operates a multi-ton process producing single enantiomer forms of the amino acids phenylalanine and tyrosine using an aromatic transaminase enzyme, operating in 180000L batches. BASF plan to have a 1000 tonnes/annum single isomer amine plant operational by the middle of the year 2000 based on lipase catalysed acylation, enantiomerically pure alcohols are also targets of this technology. Glaxo-Wellcome operate an enantiospecific biocatalytic step to produce a potent anti-HIV agent on a multi-ton scale (Mahmoudian *et al,* 1993).

Currently most commercially operating biocatalytic processes involve hydrolytic enzymes, particularly lipase, esterase and protease (Faber and Franssen, 1993). This is mainly due to the availability, high stability in aqueous and organic solvents and lack of cofactor requirement. These processes are well established and the fundamental chemistry, biology and engineering have been well characterised.

1.2.2 Future directions for biocatalysis

Biocatalysis can offer advantages in the preparation of enantiopure compounds over chemical methods. The environmental impact of a biocatalytic process may be significantly lower than the equivalent chemical process. Additionally the increasing complexity of target compounds would result in necessary protection steps if produced chemically. These three factors will inevitably make the use of biocatalysis more widespread in the future, however there are other issues that arise when evaluating the potential for these technologies. Redox biocatalysis (particularly cell free systems) has generally yet to establish itself, due in part to the limited

commercial availability of redox enzymes and the technical difficulties in regenerating the unstable and expensive cofactors required for enzyme function. Carbon-carbon bond syntheses are another important target that have yet to emerge as viable technologies.

A reluctance to use biocatalysis is in part due to several widely held views that the technology is not robust enough to operate as a process, for example catalyst stability is perceived to be low; thermophillic biocatalysts have been shown to be stable above 70°C (Lamed and Zeikus, 1981) and immobilisation of enzymes to solid supports confers superior stability to many diverse enzymes (Gerhartz, 1990). It is also believed that the only solvent that can be employed is water; the use of co-solvents, second organic phases and pure organic solvents has often been demonstrated (Klibanov, 1990; Woodley, 1990; Woodley and Lilly, 1992).

Other problems such as low reaction rates and yield due to interference from the reaction components can be overcome or their effect limited by the use of different reactor modes or processing strategies (Freeman *et al*, 1993; Woodley and Lilly, 1994). This requires a thorough understanding of the system under investigation in order to rationally select a process design. Future progress in this technical area is crucial in increasing acceptance of biocatalysis, as is application of recombinant DNA techniques.

1.3 Biocatalytic process selection

1.3.1 Selection of catalyst

Biocatalysts are generally in two forms, whole cells or isolated enzyme preparations. (Lilly, 1977; Gerhartz, 1990; Dordick *et al,* 1991 and Wingard *et al,* 1976).

Whole cell biocatalysts are usually non-reproducing and may even be non-viable, as long as sufficient stable enzyme activity exists within the cells. Problems can arise due to the organism being difficult to grow, substrate and product toxicity, mass transfer problems into the cell, side reactions (such as further enzymes in a pathway degrading a desired product) and cell lysis. Whole cells are historically the only method available for example in a multi-enzyme conversion or when expensive cofactors are necessary (redox biocatalysis), it is also possible that the enzyme cannot be isolated with retention of activity. Some of these problems can be overcome in some cases with the advent of recombinant DNA technology, by engineering the relevant genes into a more flexible host, such as *Saccharomyces cerevisiae* or *Escherichia coli.*

Purified enzymes can overcome some of these constraints, but have their own characteristic problems, for example re-use of enzyme, product contamination, enzyme stability and the additional cost of purification. Purified enzymes are generally only used if whole cells are not suitable. The purity of the enzyme preparation need not be high, as it is only necessary to remove from the catalyst other enzymes that may interfere with the target reaction and compounds that may lower the activity of the desired enzyme. The issues of stability, re-use and product contamination can be addressed by attaching the biocatalyst to a solid support in a procedure known as immobilisation.

The immobilisation of biocatalysts to a solid support was first reported by Grubhofer and Schleith in 1953 (Gerhartz, 1990) who immobilised a variety of digestive enzymes including trypsin and pepsin. The first practical application was the immobilisation of fungal aminoacylase by Chibata and coworkers in 1969 to enantioselectively produce L-amino acids. The rationale behind immobilisation is that it enables a relatively expensive biocatalyst such as an intracellular enzyme to be removed from solution into a more recoverable insoluble form, which can then be re-used to lower costs and can significantly simplify downstream processing of the product, as the enzyme is retained within the reactor. Immobilisation of cells is also practiced, and can give extra mechanical support to fragile organisms.

However immobilisation is not without drawbacks, and can lead to diffusional problems of both substrate and product with the potential creation of undesirable micro-environments around the biocatalyst. Also the activity of an immobilised enzyme can be reduced due to steric interference of the support near the active site, activity will also be lost due the immobilisation procedure itself.

By either entrapping the catalyst within a matrix i.e. a porous gel or encapsulating within a bead an immobilised catalyst is created (Wingard *et al,* 1976). This could help alleviate substrate inhibition, by limiting the local substrate concentration, but cause diffusional problems. In practice this is not easy to achieve. Cell layering by viable cell growth within a bead can further add to this problem, making the commercial use of immobilised cells rare.

Another range of immobilisation techniques are catagorised as binding to a solid surface. This could be simple adsorption to the surface of a support, or covalent linkage to either a support, or bridging between individual enzyme molecules. A recent technique pioneered by Altus Biologics (Cambridge, USA) is the preparation of cross linked enzyme crystals (CLECS) by reaction with a bi-functional reagent forming extremely stable insoluble enzyme crystals that are active in a range of solvents (Margolin, 1994). Diffusional limitations are likely to be less severe than entrapment. The binding process can add to enzyme stability with respect to temperature and pH due to steric resistance to unfolding, allowing higher

temperatures to be used to increase reaction rates and decrease the possibility of microbial contamination. Covalent linkage of enzyme molecules is the most widely used in practice due to the relative ease of the procedure and the lack of enzyme loss into the medium due to the covalent nature of the bonding (Gerhartz, 1990).

1.3.2 Reactor modes

The choice of reactor for a biocatalytic process is largely based on the characteristics of the reaction itself and the form of catalyst employed. The design should be flexible to allow for process changes and it is established wisdom that operating at the limits of the reaction constraints often yields the best performance (Wang *et al*, 1979). Assuming "ideal" conditions a kinetic analysis can be undertaken to allow an analysis of the suitability of different reactor configurations of which there are considered three "ideal" types (Jenkins *et al*, 1992).

1) Batch Stirred Tank (BSTR)
2) Continuous Stirred Tank (CSTR)
3) Continuous Plug Flow (CPFR)

The reactors are considered ideal in that the stirred tank reactors are perfectly mixed (including a uniform dispersion of biocatalyst) and all the material contained within a CSTR will have a single residence time. Also a CPFR should have no axial mixing, with radial homogeneity. In all cases the reactors are considered isothermal and no mass transfer limitations (both internal and external) exist (Wingard *et al*, 1976).

A mass balance can be written and combined with the Michaelis-Menton equation to yield design equations for the BSTR, CPFR and CSTR (Lilly and Dunill, 1976).

Batch stirred tank $$X.So + K_m.\ln\left(\frac{1}{1-X}\right) = k.\frac{E.t}{V} \quad (2)$$

Continuous stirred tank $$X.So + K_m.\left(\frac{X}{1-X}\right) = k.\frac{E}{Q} \quad (3)$$

Continuous plug flow $$X.So + K_m.\ln\left(\frac{1}{1-X}\right) = k.\frac{E}{Q} \quad (4)$$

If the initial substrate concentration is much greater than K_m, then the left hand term of all the equations dominates, and all the reactors perform equally well, the batch mode in this case is penalised due to downtime between runs. If the initial substrate concentration is below the value of K_m, then at high conversions (i.e. 99%) the amount of enzyme needed in a CSTR compared to that in a BSTR is roughly 20 times greater (depending on the particular enzyme) This is due to the low concentration of substrate in a CSTR which makes inefficient use of the enzyme. This mode of operation is inefficient in it's use of catalyst and rarely used in practice for just this reason. The advent of recombinant DNA technology and over expression has considerably reduced the cost of biocatalyst in some cases, and so a CSTR may be operable now, compared to a similar process conducted ten years ago. This is by no means universally so.

A packed bed plug flow reactor allows high concentrations of enzyme to be available (up to 65% by volume) however the flow pattern is not well mixed and so there will be regions of very high substrate and product concentrations which can be toxic to the cells or cause inhibition of the enzyme. A similar problem is encountered if there is a pH shift due to the reaction and acid/alkali has to be added; since there is no mixing, areas of extreme pH will exist. This can be partially addressed with the use of a recycle loop, which draws off the process stream to a small stirred tank where pH control takes place before the material is fed back to the beginning of the reactor. If a gaseous component is required by the reaction a CPFR reactor is not suitable as

it is very difficult to disperse the gas bubbles effectively, when compared to a well mixed system. A CPFR system will generate few mechanical forces of consequence on the catalyst (mainly pumping) but it can cause problems with biocatalysts with short operational lifespans as replacing the packing can be very time consuming.

The BSTR suffers in that due to agitation (which generates good mixing) the maximum concentration of biocatalyst that can be accommodated in the reactor is limited to roughly 10% by volume due to increased viscosity and grinding (particularly with immobilised beads) and so if the kinetics require a large amount of catalyst to achieve the reaction in a realistic time-scale this could preclude this mode of operation. Also the degree of mixing could be lowered to such an extent due to the viscosity increase that the vessel could no longer be considered well mixed. A variant of this design is when substrate is supplied continuously or intermittently over a production run, the so-called fed batch stirred tank reactor (FBSTR) which can overcome substrate toxicity by feeding in substrate regularly so the concentration in the vessel never exceeds a predetermined value. The limit of this design is the maximum feed concentration which is defined by the aqueous solubility of the substrate. **Table 1.3**. summarises the abilities of the three general reactor modes to cope with a number of common features of biocatalytic systems. It must be noted that in this analysis presented it is considered that the cost of the biocatalyst is significant in the overall process economics and as such is not pertinent to all systems.

Characteristic	BSTR	CPFR	CSTR
Liquid flow pattern	well mixed	plug flow	well mixed
pH control	possible	difficult	possible
Temperature control	possible	difficult	possible
Gaseous reactants/ products	possible	unsuitable	possible
Presence of second liquid phase (solvent/reactant/product)	possible	unsuitable	possible
Control of substrate inhibition	poor (better in FBSTR)	poor	good
Control of product inhibition	better	better	poor
solid feed solution	possible	unsuitable	possible
Mechanical catalyst damage	possible	unlikely	possible
Concentration limit of catalyst	low	high	low

Table 1.3. General characteristics of three types of reactor for biocatalytic reactions (Woodley and Lilly, 1994)

1.3.3 Structured biocatalytic design methodology

1.3.3.1 Introduction

A biocatalytic process can, depending on several different factors be designed toward a number of different and often conflicting criteria such as high final product concentration where it is perceived that downstream processing may be difficult and/or expensive, volumetric productivity such as in conversion cost intensive processes or toward catalyst re-use where the enzyme is the dominant cost. It is thus often the case that issues common to biocatalysis are not considered and design is very much on an individual case basis. This approach has several shortcomings, for

instance the engineer may conduct experiments that are not pertinent to the particular study and the final design may be very inflexible if and when the process is subject to change by the use of a new substrate, improved biocatalyst or change in final product formulation. Thus design time may be unnecessarily long and re-engineering and revalidation is a real possibility in the future.

Several attempts have been made to generate a more systematic approach encompassing all the issues raised above. Dervakos and coworkers (Dervakos *et al*, 1995) proposed a knowledge based system. However such as system can be problematic to set up in the first place, and requires data on a large number of diverse bioconversions in order to be confident in its use which in the scope of this thesis is impractical. Another approach has been proposed (Woodley and Lilly, 1994, 1996) that illustrates the benefit of examining only the constraints imposed on a particular conversion through the collection of a defined data set on the fundamental properties of the substrates, products, catalyst and possible interactions between the three in order to highlight the process designs that are impractical and those that should be highlighted for further evaluation via the use of heuristics. This approach, through the identification of process constraints also should allow the designer to focus in areas of potential process improvement such as targets for protein engineering. The key steps within this design philosophy are shown in **Figure 1.4**. The methodology described has been successfully applied to design of the transketolase process (Hobbs *et al*, 1993; Mitra, 1996) and the fluorocatechol synthetic process (Lynch, 1994). The proposed data set is shown in **Table 1.4.**

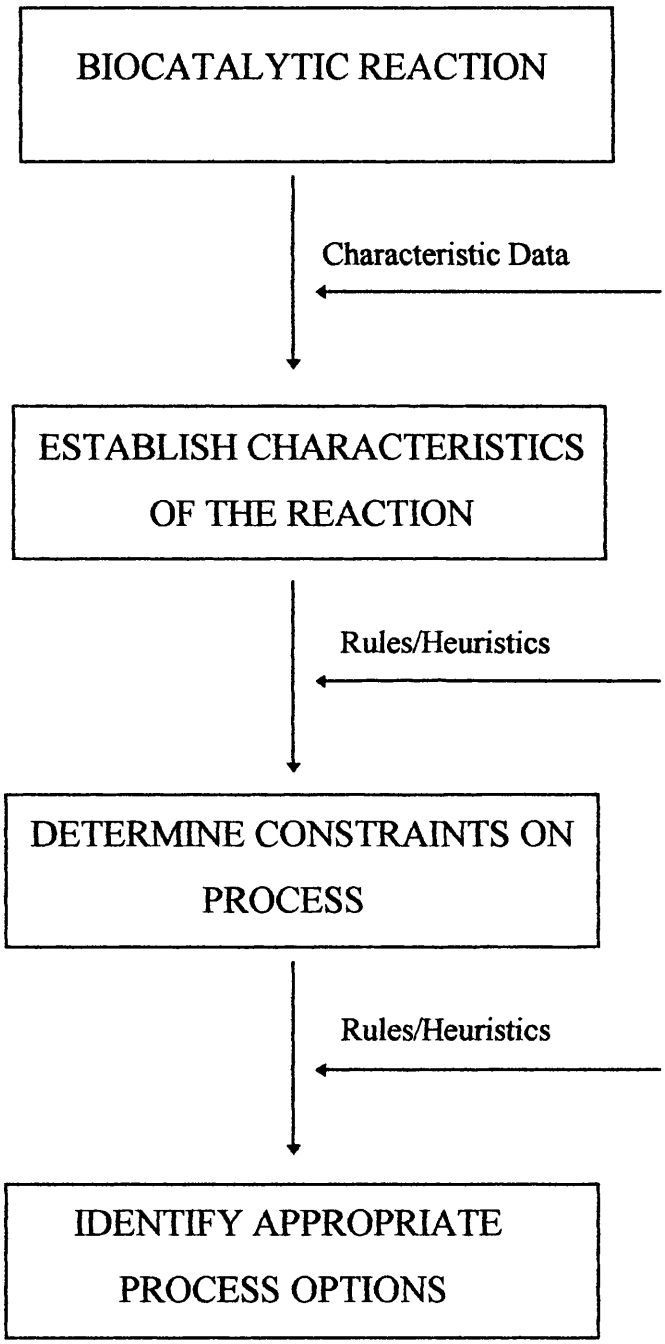


Figure 1.4. Decomposition of biocatalytic process design methodology (Dervakos *et al*, 1995)

SUBSTRATE/PRODUCT PROPERTIES
Melting/boiling points
Water solubility
Temp/pH/time Stability
Volatility,
REACTION PROPERTIES
Equilibrium -pH/temp dependence
Michaelis-Menton Kinetics
Enantioselectivity
Acid/base production
Water consumption/production
Gas consumption/production
Heat evolution
BIOCATALYST PROPERTIES
Single/multienzyme
Cofactors
Coenzymes
Enzyme location
Location of enzyme activity
Requirement for water
Stability
INTERACTIONS BETWEEN BIOCATALYST/PRODUCT/SUBSTRATE
Substrate inhibition
Product inhibition
Activity
Substrate toxicity
Product toxicity

Table 1.4. The proposed key data set to determine process selection (Woodley and Lilly, 1994)

1.3.3.2 Characterisation of the system with a model reaction

In general a potential biocatalyst will be able to react a wide range of related substrates and so if a process is to be designed to be efficient and flexible in substrate range the choice of a particular substrate for characterisation studies must accommodate this. It would be impractical and time consuming to characterise all the potential conversions and so it becomes necessary to select a model substrate to conduct studies with.

It has previously been shown that by using a simple, representative and readily available substrate valuable design data can be collected that is applicable to more complex targets (Mitra, 1996), requiring minimal further experimental studies.

1.3.3.3 Reaction properties

The data collected regarding the reaction will be in general independent of the model substrate, such as the pH/enzyme activity profile and the relation of temperature to reaction rate. These data will narrow down the conditions which studies in the other areas need to focus on, such as the range of pH for product stability studies.

Requirement or generation of a gaseous phase within the reactor will have a constraining effect on reactor performance due to supply limitation of for example oxygen which is only sparingly soluble in water (Stanbury and Whittaker, 1995). De-bottlenecking can be achieved through number of methods including membrane oxygenation (Wang *et al,* 1988; Henzler and Cauling, 1993; Weiss *et al* 1996; Drury *et al,*1988), use of additives, such as perflourocarbons as a dispersed second phase (Mattiasson and Aldercreutz, 1983, King *et al,* 1989; Rols and Goma, 1989; Mano *et al,* 1990; Martin *et al,* 1995; Elibol and Mavituna, 1996; Cho and Wang, 1988) and

in-situ oxygen generation from hydrogen peroxide (Ibrahim and Schlegel, 1980[1,2]). Generation of carbon dioxide as a by-product would cause a pH shift within the reaction due to the formation of carbonate ions, requiring the reactor configuration to be well mixed.

1.3.3.4 Biocatalyst properties

The fundamental properties of the enzyme are critical in evaluating process options. Multi-enzyme pathways and membrane bound catalysts would make the selection of an isolated catalyst form unlikely. Similarly the requirement for cofactors requires careful consideration, as cell free regeneration systems are difficult to operate economically. Stability toward shear and gas/liquid interfaces also may merit investigation and could severely limit the range of process options.

1.3.3.5. Substrate and product properties

The properties of the reaction components will influence all aspects of the biocatalytic process. Issues regarding the stability of the compounds within the reaction environment need to be addressed, data generated from the characteristics of the reaction will for example illustrate the range of pH to investigate stability over.

Low water solubility is a common feature of many target compounds, which can be enhanced by addition of a water miscible co-solvent, i.e. ethanol in small amounts (typically an increase of 10% is seen); or a second immiscible organic phase which can act as a reservoir for the substrate (Duarte, 1983, Woodley, 1990[1,2], Woodley and Lilly, 1992, Collins *et al,* 1995). The substrate itself can act as the second phase in some cases. Substrate solubility becomes more important with kinetic resolution

reactions as the unwanted enantiomer is effectively lowering the solubility of the desired enantiomer, potentially causing yields to drop to well below the theoretical 50% value.

Volatilisation can also be a problem with low boiling point components, particularly if gaseous substrates or products are present. Passing gas through a medium can quite easily strip out a significant proportion of substrate or product, which is then lost in the exit gas.

Product instability or interference with the reaction may necessitate the removal of the product from the reaction environment. Any separation process cannot be adequately considered without knowledge of the physical parameters that will govern it's behaviour. Physical and chemical differences between the product and the rest of the system (substrate, catalyst, water) can be exploited to determine the viability of a number of techniques which have attracted much interest, collectively called *In-situ* Product Removal (ISPR) (Freeman *et al,* 1993; Meyer *et al,* 1997; Chauhan, 1996) whereby ideally the product is removed from the viscinity of the reaction and from the medium as soon as it is produced giving it no time to interfere with the reaction and/or lessen the impact of degradation effects. ISPR has been successful in generating step change increases in product titres in a variety of biocatalytic processes. There are five general techniques by which *in-situ* recovery can be accomplished:

1) Evaporation (vacuum or stripping)
2) Extraction into another phase (e.g. organic solvent)
3) Size selective processes based on dialysis
4) Complex formation (e.g. biorecognition)
5) Product immobilisation via binding/adsorption

The choice depends on the characteristics of the product, for example evaporation would be more applicable for low molecular weight products such as ethanol. The

use of a second organic phase to partition the product into could be of particular interest if the same process was used to increase the concentration of a poorly water soluble substrate in the reactor. If the substrate and product are reasonably similar in hydrophobicity then it is quite possible to combine ISPR with enhanced substrate feeding

Generally the removal of product has rarely been scaled up when the extraction takes place internally within the main reactor itself; usually a recycle loop is added to reactor; the extraction takes place in a separate unit with media continually circulating. Ideally an internal system would allow faster removal and a less complicated setup but problems of e.g. limited solids concentrations and mechanical damage to membranes can be too problematic to implement.

These considerations emphasize the need for physical data on all the substrates, without which the various process alternatives cannot be properly evaluated.

1.3.3.6 Interactive Effects

The substrate and product may affect the activity and stability of the catalyst within a process. This can occur as an irreversible deactivation of the catalyst (toxicity) or a reversible change in reaction rate (inhibition). Product inhibition is a natural consequence of the biological origin of enzymes, many have their activity regulated in their natural environment by the product they produce in order to control cellular metabolism (Zubay *et al*, 1995). This can be overcome or reduced by the use of ISPR or substrate feeding.

The advent of recombinant DNA technology has allowed these effects to be removed in some cases by altering the amino acid sequence of proteins to delete or replace residues vulnerable to damage from the reaction environment (Wells and Estell, 1988). Immobilisation can confer improved stability by restraining the three

dimensional structure of the enzyme, making unfolding of the polypeptide chain(s) more difficult.

1.4 Baeyer-Villiger as an important future target

1.4.1 Reaction

The Baeyer-Villiger reaction is the conversion of a ketone to an ester, or a cyclic ketone to a lactone. (Baeyer and Villiger, 1899; Solomons, 1992), summarised in **Figure 1.5.** The reaction can be achieved via established chemical methods and also by a variety of bacterial and fungal enzymes (Holland, 1992). The biocatalytic route to commercially important lactones will be considered in this thesis in the context of rational reactor selection and design.

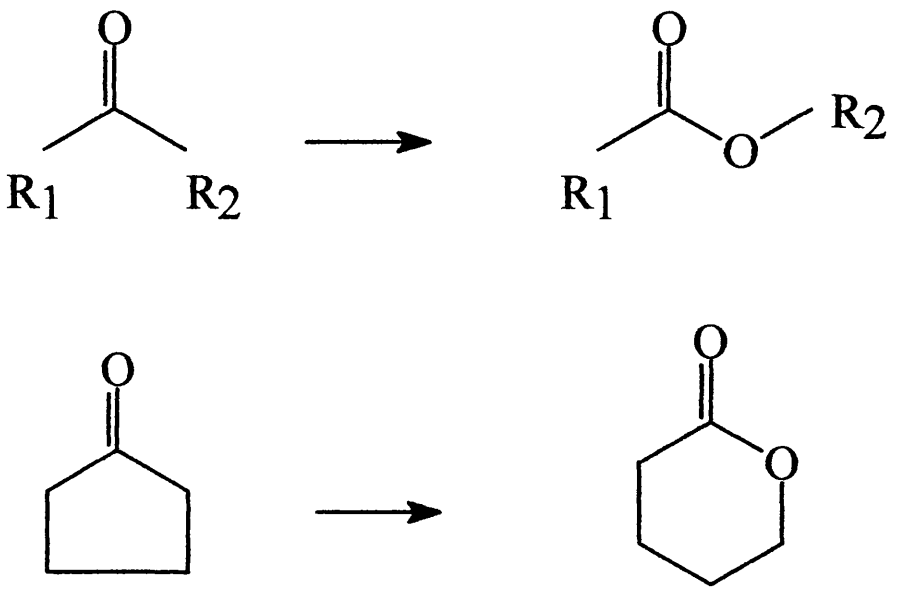


Figure 1.5 The generalised Baeyer-Villiger reaction

1.4.2 Applications of the Baeyer-Villiger reaction

The Baeyer-Villiger oxidation has been used to produce a variety of industrially useful chemicals. α-substituted δ-lactones with an aliphatic side chain of between 5 and 7 carbon atoms are useful constituents of some fruit flavours (Tressl *et al*, 1978), particularly peach and mango. Deng and Overmann (Deng and Overmann, 1994) demonstrated the synthesis of preussin, an effective anti-fungal agent proceeded by several steps including a Baeyer-Villiger oxidation.

Substituted, optically active δ-lactones are commonly found as insect hormones which are potentially high value biopesticides. For example undecanyl valerolactone and dimethyl valerolactone are pheromones of the oriental hornet and the carpenter bee respectively (Gerth and Giese, 1986). The insect antifeedant clerodin also has lactone functionalities (Petit and Furstoss, 1992; Willetts, 1997). The major banana weevil pheromone sordidin has been produced (Beauhaire *et al*, 1995) in a total synthesis involving Baeyer-Villiger oxygen insertion into a 2,6-disubstituted cyclohexanone.

The synthesis of 6'-fluorocarboxylic nucleosides (anti-viral agents) includes a Baeyer-Villiger step (Levitt *et al* 1990). Likewise the Baeyer-Villiger oxidation's importance in the synthesis of a variety of hydroxylated alkaloids has also been demonstrated (Cook *et al*, 1994) as well as the synthesis of R-bacolofen, a potent inhibitor of neurotransmition (Mazzini *et al*, 1997).

1.4.3 Chemical methods

The Baeyer-Villiger oxidation is one of only a few methods capable of breaking a C-C bond under mild conditions and is most commonly achieved by using a peroxy acid reagent such as 3-chloroperoxybenzoic acid (3-CPBA), trifluoroperoxyacetic

acid and also hydrogen peroxide (Roberts and Wan, 1998). Major problems occur due to the instability of these compounds and their oxidative potential, making their large scale use difficult although a 160kg synthesis of a substituted norbornan-2-one has been demonstrated (Coleman *et al*, 1997) using the cheaper and safer peracetic acid.

Within the last five years this reaction has also been catalysed using transition metal complexes and molecular oxygen as the oxidant (Roberts and Wan, 1998; Strukul, 1998) particularly nickel (II) and platinum (II) based catalysts.

The chief drawback in using chemical syntheses to prepare lactones is that almost exclusively the reaction proceeds with no enantioselectivity (if indeed there is the potential for enantiomeric excess) such that any lactone produced will be racemic and have an ee of 0. Platinum (II) derived catalysts can introduce enantioselectivity in some cases although the yields and ee are both poor, a recent oxidation of a range of α-substituted cyclohexanones produced no greater than 9% yield and 44% ee (Strukul, 1998). For reasons already outlined this is unfavourable particularly when viewed in the context of current and future legislation driving toward enantiopure pharmaceuticals and agrochemicals.

1.4.4 Biological methods

A diverse range of micro-organisms have been found to perform Baeyer-Villiger oxidations usually as part of catabolic pathways allowing growth on unusual carbon sources (Roberts and Wan, 1998). The biological Baeyer-Villiger oxidation, unlike many of the chemical methods does not use a peracid, but molecular oxygen (Fang *et al*, 1995; Roberts *et al*, 1992). The commonest type of enzyme catalysing this reaction is known as a monooxygenase, or mixed function oxidase due to the fact that only one atom of oxygen is incorporated in the product molecule with the other forming water. The advantage of using a biocatalyst is that a racemic lactone is

generally not produced, instead the reaction is enantioselective, often with ee's approaching 100% (Holland 1992; Roberts, 1993)

In the 1950's it was found that the formation of testolactone by *Penicillium* species and *Aspergillus flavus* proceeded by successive Baeyer-Villiger oxidations (Holland 1992). This system has since been found to be common in steroid synthesis and as such is of potential importance in biocatalysis. Other fungi, including *Fusarium* and *Septomyxa* species have since been found capable of similar reactions. Similar results have been shown with *Cylindrocarpon radiciola*, the monooxygenase showing a strong affinity to large ketosteroids and progesterone (Itagaki 1986) and bicyclic ketones (Konigsberger *et al*, 1990). *Curvularia lunata* has been demonstrated to show high enantioselectivity in the reaction of (R,S)-2,2,5,5-tetramethyl-4-hydroxy-cyclohexanone, forming the S-lactone (Ouazzani-Chahdi *et al*, 1987).

Pseudomonas multivorans, *Pseudomonas aeruginosa*, *Pseudomonas flava* and *Pseudomonas cepacia* are all capable of Baeyer-Villiger reactions, via flavoproteins. *Pseudomonas putida* has been studied extensively (Roberts and Willetts, 1993, Trudgill 1990, Griffin and Trudgill 1972) and contains a pair of two enzyme complexes used in the degradation of camphor; 2,5,-Diketocamphane 1,2-monooxygenase consisting of a flavin mononucleotide *iso*-enzyme and an NADH oxidase. The other complex is identical, except that the monooxygenase is 3,6,-Diketocamphane 1,2-monooxygenase. Each enzyme processes a different enantiomer of camphor. The same organism also contains another Baeyer-Villiger oxidase, 2-oxo-Δ^3-4,5,5,-trimethylcyclopentenyl acetyl-CoA monooxygenase (known as MO2) (Ougham *et al*, 1983). This organism has been shown to be effective at carrying out bioconversions, such as the enantioselective oxidation of bicyclo[2.2.1]heptan-2-ones to (1S,5S,6R) lactones in ee's over 95% (Gagnon *et al*, 1995[2]). Purified MO2 has shown excellent enantioselectivity with 2-alkyl and 2-phenyl cyclohexanones (Alphand *et al*, 1996).

Candida antarctica contains a lipase capable of performing enantioselective Baeyer-Villiger oxidations (Lemoult *et al,* 1995) but the mechanism is more akin to the common chemical methods, the lipase forms a peracid which performs the oxidation.

Lasiodiplodia theobromae and *Acremonium roseum* have both been found to produce enantiopure products, the former is able to utilise cyclic alkenes as a carbon source, the Baeyer-Villiger step being part of this metabolism. (Holland 1992).

The most investigated of all microbial Baeyer-Villiger oxidases are the cyclohexanone 1,2-monooxygenases, allowing growth on cyclohexanols or cyclohexanones. They were first isolated from *Nocardia globerula, Xanthobacter* sp. and *Acinetobacter* sp (Willetts, 1997). A related cyclopentanone monooxygenase from *Pseudomonas* sp NCIMB 9872 has also been reported. These enzymes are NADPH dependent, single chain flavoproteins showing wide substrate specificity.

Only a handful of the bacterial monooxygenases discussed have been purified and no crystal structures have been reported to date. The ten most characterised monooxygenases are summarised in **Table 1.5** (Willetts, 1997).

Enzyme and source micro-organism	Cofactor	Protein composition	Coenzyme	pH optimum
Cyclohexanone monooxygenase *Acinetobacter calcoaceticus* NCIMB 9871	NADPH	Monomer	FAD	9.0
Cyclohexanone monooxygenase *Nocardia globerula* CL1	NADPH	Monomer	FAD	8.4
Cyclohexanone monooxygenase *Xanthobacter* sp	NADPH	Monomer	FMN	8.8
Cyclopentanone monooxygenase *Pseudomonas* sp NCIMB 9872	NADPH	Homotetramer	FAD	7.7
Tridecan-2-one monooxygenase *Pseudomonas cepacia*	NADPH	Homodimer	FAD	7.9
2,5-diketocamphane monooxygenase *Pseudomonas putida* NCIMB 10007	NADH	Heterodimer	FMN	9.0
3,6-diketocamphane monooxygenase *Pseudomonas putida* NCIMB 10007	NADH	Heterodimer	FMN	9.0
2-oxo-Δ^3-4,5,5,-trimethyl cyclopentenyl-acetyl-CoA monooxygenase *Pseudomonas putida* NCIMB 10007	NADPH	Homodimer	FAD	9.0
Steroid monooxygenase *Cylindrocarpon radiciola* ATCC 11011	NADPH	Homodimer	FAD	7.8
Oxocineole monooxygenase *Pseudomonas flava* UQM 1742	NADH	Heterodimer	FMN	8.5-9.0

Table 1.5. Common characterised monooxygenases (Willetts, 1997).

1.4.5 Cyclohexanone monooxygenase from *Acinetobacter calcoaceticus*

The enzyme chosen for study is cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus*. This was chosen for two main reasons. Firstly CHMO is the most studied monooxygenase and is the most promising candidate to date of all the characterised monooxygenases for producing enantiopure commercially

important lactones due to the wide substrate range shown and high product ee's (Stewart, 1998). Secondly the enzyme is a single protein chain and is thus easier to clone and over express into a host such as *E.coli* than a multi-subunit enzyme such as the pair of diketocamphane monooxygenases from *Pseudomonas putida.*

CHMO is a single polypeptide chain enzyme of 542 amino acids with a molecular weight of 59000 (Chen *et al,* 1988), and a tightly bound flavin adenine dinucleotide (FAD) molecule near the N-terminus, acting as a co-enzyme (Walsh and Chen 1988). The enzymatic conversion of cyclohexanone also requires one molecule of NADPH and one of oxygen; which undergoes a four electron reduction, to form water and the lactone product. Several groups (Ryerson *et al,* 1982; Donaghue *et al,* 1976 and Trudgill *et al,* 1990) have attempted to elucidate the mechanism by which the enzyme catalyses the reaction between cyclohexanone, oxygen and NADPH. It is believed that NADPH binds to the enzyme first, generating reduced flavin coenzyme. Oxygen binds to form a flavin hydroperoxide, which is the oxygen transfer agent; nucleophillically attacking the carbonyl group of the ketone. The product ε-caprolactone is further metabolised by *A.calcoaceticus* by a lactonase enzyme which opens the ring and successive steps generate adipate from which eventually acetyl-CoA is generated (Donaghue and Trudgill, 1975). **Figure 1.6** shows the metabolic pathway of *Acinetobacter* using cyclohexanol as a carbon source.

(Ottolina *et al,* 1996; Kelly *et al,* 1995 and Wright *et al,* 1994) have produced evidence that the enzyme contains only one active site and that enantioselectivity of the reaction observed with a wide range of substrates is due to the ability of the enzyme to stabilise only one hydroperoxide configuration. A similar model is also proposed (Alphand and Furstoss, 1992) in which selectivity is partly controlled by a "forbidden" zone within the enzyme molecule which disallows certain configurations from binding and reacting.

A.calcoaceticus is a rod shaped gram negative bacterium (Lautrop, 1974) and appears very similar to *Moraxella* species but is distinguished by the fact that *Acinetobacter* is penicillin resistant due to a differing cell wall structure. *A.calcoaceticus* can be grown to cell densities of 7-8gL^{-1} and enzyme activities of

$20Ug^{-1}$, using simple salts, e.g. succinate and glutamate as a carbon source and inducing CHMO production using cyclohexanol, upon which rapid growth can also be achieved (Donaghue *et al,* 1976).

An *E.coli* clone (*E.coli* JM107 pQR210) is available in-house and will be used to produce the catalyst, the CHMO gene being expressed on a *tac* promoter, with ampicillin resistance. A previous *E.coli* clone has been reported (Chen *et al,* 1988) with an enzyme titre comparable to that found in *A.calcoaceticus.* Recently the gene has also been successfully cloned into *Saccharomyces cerevisiae* (Stewart *et al,* 1996[1,2]) with active enzyme expression although no data on enzyme titre is presented. The use of a recombinant *E.coli* has three main advantages over the original organism as a source of CHMO.

1) The lactonase enzyme in *A.calcoaceticus* has an activity approximately 27 times greater than CHMO (measured with the 'natural' substrates) and so accumulation of lactone is not seen with some substrates. Bioconversions can be undertaken by inhibiting the lactonase enzyme. One such inhibitor found to be effective is tetraethypyrophosphate (TEPP) (Alphand *et al,* 1990[1]). A major disadvantage is that TEPP and other related compounds are extremely toxic mammalian cholinesterase inhibitors and would be impractical and expensive on a process scale. This lactonase is not present in *E.coli,* as only the CHMO gene has been cloned thus making this issue irrelevant.

2) *E.coli.* JM107 pQR210 can produce higher levels of enzyme activity than the native organism, in a shorter fermentation (Barclay *et al,* 1997)

3) *E.coli* is classified as a class 1 organism, whereas *A.calcoaceticus* is a class 2 pathogen requiring specialist containment to grow and handle on a process scale.

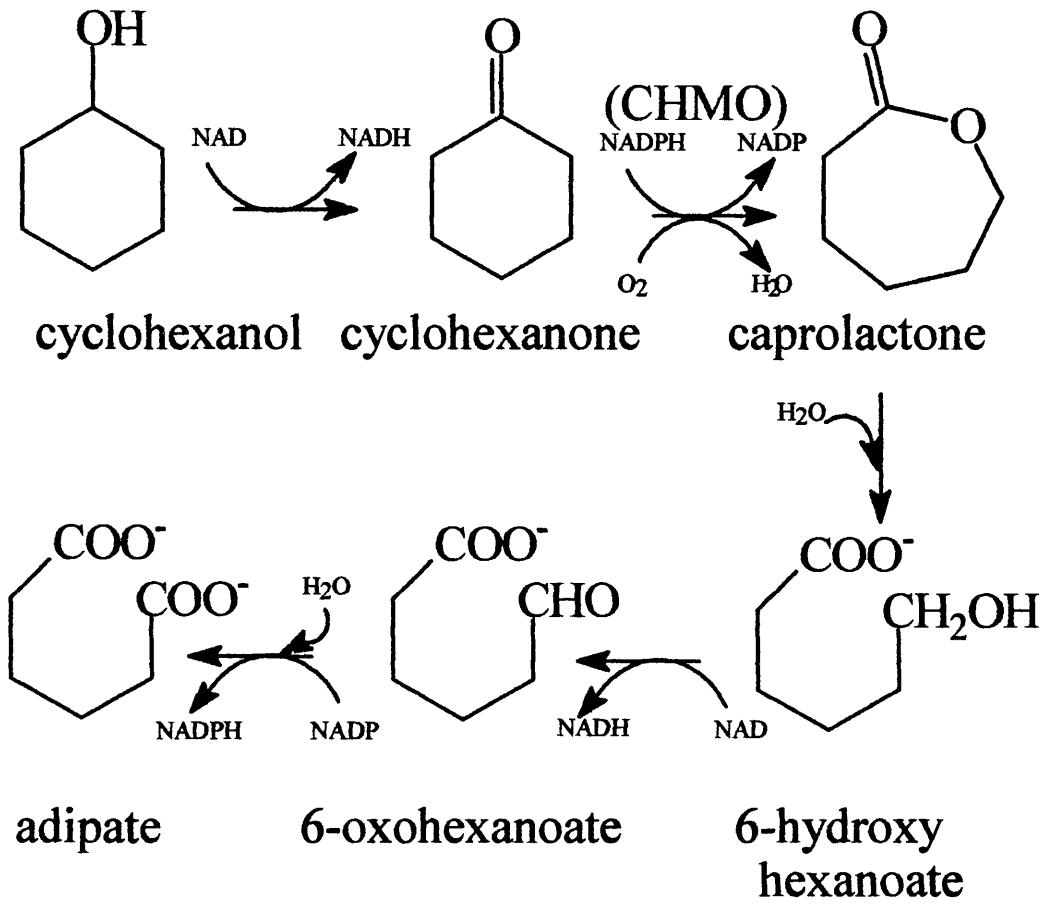


Figure 1.6. Reaction sequence for the oxidation of cyclohexanol to adipate (after Donoghue and Trudgill, 1975)

1.4.6 Previously reported syntheses

(Trudgill *et al*, 1990) demonstrated that the enzyme has a high substrate specificity for a variety of ketones, from cyclobutanone to complex cyclic molecules such as (+)-camphor and dihydrocarvonone.

(Schwab *et al*, 1983) showed that even with small substitutions on the ring structure such as is found on 2-methyl cyclohexanone enantioselectivity was shown by the enzyme. After 50% conversion the ee dropped due to the other enantiomer of the ketone being processed. Examples do exist however when CHMO performs a Baeyer-Villiger reaction with no selectivity (Gagnon *et al*, 1994), where the reaction using a racemic norbanone produced the lactone with no enantiomeric excess, in a similar fashion to that which can be achieved using a peracid in the classical chemical Baeyer-Villiger reaction.

(Abril *et al*, 1989) examined a range of substrates derived from cyclohexanone, including 4-phenylcyclohexanone and 4-*tert*-butylcyclohexanone as well as more complicated molecules such as 7-benzyloxymethyl-2-norban-5-one using immobilised CHMO. It was found that substrate specificity was broad but CHMO did not accept α,β-unsaturated ketones or 1,3-diketones. High yields were obtained with most ketones, although no data on enantioselectivity was reported.

The focus of much work in recent years has been on various bicyclic ketones; (Sandey and Willetts 1992; Alphand *et al*, 1989; Shipstone *et al*, 1992; Lenn and Knowles 1994; Alphand and Furstoss, 1992, and Grogan *et al*, 1992). All have studied the oxidation of bicyclo[3.2.0]hept-2-en-6-one and found that each isomer undergoes oxygenation at a different position, forming in high yield and enantiomeric purity (>90% ee) two regioisomers. It was also found that *Acinetobacter* TD63 and *Pseudomonas putida* can also catalyse this reaction. Lenn and Knowles also found that substitutions at the 7-*endo* position can increase selectivity; whereas substitutions at the 7-*exo* position has the opposite effect.

(Carnell *et al,* 1991) also studied derivatives of bicyclo[3.2.0]hept-2-en-6-one, and found that regioselectivity and enantioselectivity varied considerably, possibly due to the enzyme's preferred method of approach to the substrate being via the *exo* face, which is more open. (Sandey *et al,* 1992) also describe this reaction, additionally reporting that 1% of the products formed are as a result of an alternative, reductive pathway. Bicyclo[2.2.1]hepten-7-ones have also been investigated, with the usual excellent enantioselectivity (Taschner and Peddada, 1992; Konigsberger *et al,* 1991).

(Taschner and Chen, 1991) successfully used *A.calcoaceticus* to generate the correct stereochemistry in an intermediate of the antibiotic ionomycin, a key step to developing a competitive synthetic route to the antibiotic.

Investigations using substituted cyclohexanones have been reported, (e.g. Alphand and Furstoss, 1992[2]; Tascher *et al,* 1993). The latter produced (S)-lactones in high ee (>98%) using 4-substituted cyclohexanones when the substituted group was ethyl, propyl or *tert*-butyl; but n-butyl generated only 52% ee with the (R)-configuration. This suggests this could be a point of changeover in enantioselectivity, but no high alkyl groups were used to verify this. (Alphand and Furstoss, 1990) worked with disubstituted cyclohexanones, menthone and dihydrocarvone. High ee's were reported for both reactions, the only unusual result being the formation of 3R,6S-lactone from 2-methyl, 6-propenyl cyclohexanone with the oxygen inserted between the carbonyl group and the least substituted carbon atom; normally the insertion is between the carbonyl group and the most substituted. This was explained by steric interactions in the active site only allowing the more unusual configuration to be produced.

(Gagnon *et al,* 1995[1]) reported CHMO transformations of various 3-substituted cyclobutanones from 3-butyl to 3-phenylethylacetyl cyclobutanone producing (S)-lactones exclusively, with ee's between 50 and 95%.

Work has also been carried out with various 2-alkyl substituted (C_5-C_{11}) cyclopentanones presented to *A.calcoaceticus* (Alphand *et al,* 1990), it was found that both yield and enantiomeric excess depended on the length of substitution.

Yields increased with increasing chain length; explained by the possibility of better positioning in the active site, or the ease of passage across the cell membrane due to increased hydrophobicity. Also possible is the existence of a second metabolic pathway which acts only on the shorter chain ketones or their lactone derivatives. This is supported by the fact that adding 1,2-cyclohexanediol, a proposed intermediate in this pathway, increases yields of short chain lactones. Enantiomeric excess seems to act in the opposite way; increasing as chain length decreases. 97% ee was reported for C_5 down to 73% with C_{11}. EE drops after 50% conversion as the unfavoured enantiomer of the ketone is reacted. Interestingly it seems that the lactones produced are all (S)-configuration, the same result as has been found previously with 3-substituted cyclobutanones and 4-substituted cyclohexanones. 2- and 3- substituted cyclopentanones have also been studied using whole cell *S.cerevisiae*, producing S-lactones in high ee (>95%) for 2- substituted and poorer ee (30-40%) using 3-substituted cyclopentanones (Kayser *et al*, 1998).

It has also been shown that along with nucleophillic attack on carbonyl species producing the Baeyer-Villiger reaction, unusual substrates containing species such as iodine, boron, selenium and sulphur allow the oxygen to act as an electrophile to a nucleophillic electron pair on the substrate (Branchaud and Walsh 1985). This makes CHMO unique among flavin monooxygenases in it's ability to act as both a nucleophile and an electrophile.

Numerous oxygenation reactions of sulphides and sulphoxides have been reported, often producing products with ee's close to 100% (Donaghue *et al*,, 1976; Carrea *et al*, 1992; Secundo *et al*,, 1993; Pasta *et al*,, 1995, Kelly *et al*, 1996, Colonna *et al*, 1997). This reaction has also been reported in *Helminthosporium* sp. (Holland *et al*, 1997) and rabbit monooxygenase (Fisher and Rettie, 1997). Recently the range of sulphur containing substrates has been increased, such as the synthesis of thioacetal and thioketal sulphoxides (Alphand *et al*, 1997) and the conversion of organic sulphites to sulphates (Colonna *et al*, 1998). Molecules with a chiral centre in proximity to a S=0 bond are useful pharmaceutical intermediates (Davies and Reider, 1996; Collins *et al*, 1997).

1.4.7 Categories of CHMO lactone syntheses

CHMO is capable of three general categories of ketone to lactone conversions, as shown in **Figure 1.7** below. Non chiral synthesis results when neither the product nor substrate have a chiral centre. Kinetic resolution occurs when only one enantiomer of the ketone forms product leaving the other enantiomer of the ketone unreacted. Finally asymmetric syntheses are possible with prochiral substrates, all of which can potentially form a single enantiomer product. Chiral centres are shown where appropriate.

The drive toward asymmetric synthetic technologies and the commercial importance of the lactones produced makes the study of this enzyme and it's process development important in the context of trends in future pharmaceutical production.

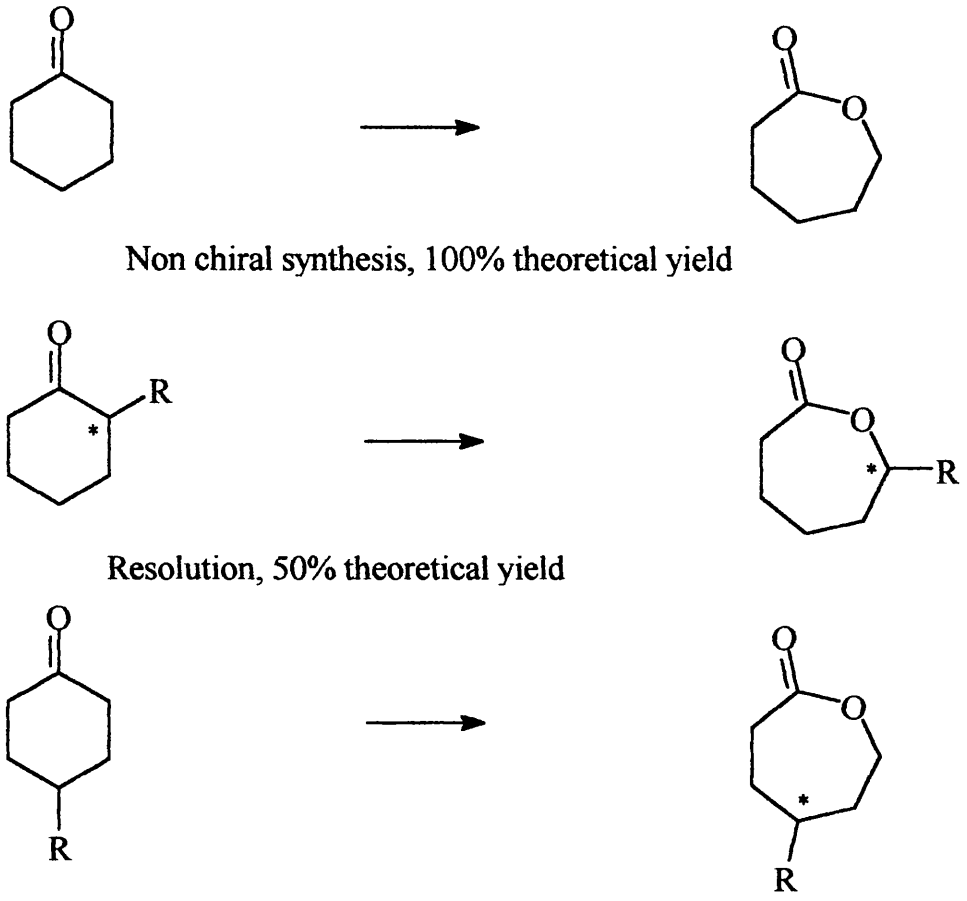


Figure 1.7. Synthetic potential of CHMO, showing chiral centres(*).

1.5 Thesis aims

This thesis aims to investigate the applicability of a structured design approach to the selection and design of a reactor system to perform CHMO catalysed lactone syntheses on a preparative scale, and is part of a larger research program to develop a whole process approach to the biocatalytic Baeyer-Villiger reaction from catalyst production to final product formulation.

This approach has been investigated previously (Lynch, 1994). Lynch considered the whole cell conversion of fluorobenzene to fluorocatchecol, concluding that the structured approach identified substrate volatility and product toxicity as constraints. This work demonstrated a step change increase in productivity when these constraints were identified and removed by changes to the process. Later work (Mitra, 1996) used the same approach to investigate the carbon-carbon bond synthetic Transketolase reaction. It was shown that a simple model substrate can be used to gather characterisation data that identified key process constraints. Additionally these constraints applied equally to more complicated substrates such that the process could rapidly be modified to operate effectively with a new substrate.

It is clear from these two studies that this approach is predominately useful as a guiding tool, providing qualitative information only on the choice of reactors.

This thesis aims to achieve the following goals:

1) To examine the degree of usefulness that the structured design approach offers to redox biocatalysis using the commercially significant Baeyer-Villiger reaction as an example through the use of an example of both a kinetic resolution and an asymmetric reaction.
2) To extend this approach to the more specific issues arising from redox biocatalysts, principally cofactor recycle.

3) Investigate the potential for coupling quantitative data to the qualitative design information that should arise from studies in (1) through numerical techniques, in order to predict the performance of a range of reactor modes, allowing the verification of conclusions from (1).
4) Using the data gained through studies (1)-(3) perform preparative scale bioconversions of the model ketones to enantiomerically pure lactones.

2.0 Characterisation of the model system

2.1 Introduction

The aim of this chapter is to apply a structured design approach to the biocatalytic Baeyer-Villiger reaction in order to investigate the suitability of this approach to redox biocatalysis and highlight the shortcomings of this technique (if any). The biocatalyst of choice is cyclohexanone monooxygenase produced from recombinant *E.coli*. The analysis of this set of experiments should lead to a number of possible process options for both kinetic resolution and asymmetric synthetic reactions. The data should provide the grounding upon which to construct a process design to perform these reactions on a preparative scale which will be explored in later chapters.

In order to carry out these studies it is first necessary to select appropriate ketone substrates to act as model systems, which must be representative of the commercially important reactions this technology could be called upon to perform. The screening of a range of candidate molecules will therefore form the initial stages of this chapter.

2.2 Materials and Methods

2.2.1 Commercially sourced solvents and chemicals

Unless otherwise stated otherwise all chemicals were of the highest purity available and supplied by Sigma-Aldrich (see Appendix 1). Water used was produced by reverse osmosis (RO).

2.2.2 Chemical syntheses

2.2.2.1 2-hydroxymethyl cyclopentanone

Cyclopentanone (500g) was added to a stirred solution of potassium carbonate (82.15g) in water (4L). Aqueous formaldehyde (40%) was added in 100mL volumes and the mixture cooled to offset the exotherm. The mixture was stirred at room temperature for three hours and followed by TLC (Boros *et al*, 1994). The mixture was washed with cyclohexane (1.5L) and the aqueous phase separated. The aqueous phase was then saturated with sodium chloride and extracted with dichloromethane (3 × 500mL). The combined organic extracts were dried over magnesium sulphate and the solvent evaporated under reduced pressure producing the crude product which was purified by vacuum distillation (0.5 mm Hg, vapour temperature 80°C) producing 2-hydroxymethyl cyclopentanone (14% yield, 70g).

2.2.2.2 Preparation of racemic lactones by chemical oxidation

Under a nitrogen atmosphere 5g of the ketone under investigation was dissolved in 100mL dichloromethane, added to which was 10g 3-chloroperoxybenzoic acid (3-CPBA) and 3.25g sodium bicarbonate and left to stir for 24 hours at 0°C (Krow, 1993). After completion (confirmed by TLC) the mixture was filtered and washed sequentially with a saturated sodium sulphite solution (3 x 150mL), distilled water (150mL) and saturated sodium chloride solution (150mL). The organic phase was dried over magnesium sulphate and the solvent removed under reduced pressure. The crude lactone product was stored at -18°C to allow impurities to crystalise out of solution. Liquid lactone was then decanted from the solid crystals. Yields were in the range 50-70%.

2.2.3 Chromatography

2.2.3.1 Thin layer chromatography

Thin layer chromatography was carried out using silica on aluminium plates using ethyl acetate as solvent. Visualisation was performed by staining with aqueous potassium permanganate followed by heating. Reaction components were revealed as yellow spots.

2.2.3.2 Gas Chromatography

Separation and quantification of ketones and lactones was performed using a Perkin-Elmer autosystem XL-2 gas chromatograph, with an autosampler linked to a FID detector. Data capture was achieved using PE Nelson Turbochrom software running on a PC. **Table 2.1.** lists the operating condition used.

Column	Alltech series AT-1701
Film Thickness	1μm
Dimensions	0.53mm x 30m
Oven Temperature Program	100°C for 5mins, 100-200°C at 10°C/min
Carrier gas split	100:1
Injection volume	1μL

Table 2.1. Operational parameters for GC separation of ketones and lactones

Aqueous samples (0.5mL) were mixed with 1mL of chilled 2-propanol. Precipitate (if any) was removed by centrifugation for 2 minutes at 15000rpm. The sample was

loaded into a vial and used in this form. All samples were run in triplicate. In the case of whole cell bioconversions the cells were initially removed by centrifugation prior to addition of 2-propanol.

Standard curves of concentration against FID response for the compounds under investigation and sample chromatograms can be found in Appendix 2.

2.2.4 Spectrophotometric methods

2.2.4.1 Cell growth analysis

Growth of *E.coli* JM107 pQR210 was monitored by measuring the optical density (OD) of suspended cells at a wavelength of 670nm in a Kontron Uvikon 922 spectrophotometer. Dry cell weight could be calculated from the equation below:

$$\text{dry cell weight, } gL^{-1} = OD \times 0.51 \qquad (5)$$

2.2.4.2 Cyclohexanone monooxygenase rate assay

Cyclohexanone monooxygenase rate assays were carried out on a Kontron Uvikon 922 variable wavelength spectrophotometer equipped with a variable temperature cell carrier, utilising circulating water. The assay depends on following the time course of the enzymatic consumption of NADPH, which adsorbs strongly at 340nm (ε = 6.22mLμmol^{-1}cm^{-1}) whereas $NADP^+$ does not. Consumption of NADPH is stoichiometrically linked to product formation and so is a direct measurement of enzyme activity (Donoghue *et al*, 1976). Enzyme activity is expressed in Units of

activity (1 Unit = 1μmolmin^{-1} of product formed). This activity is calculated over the linear region of the $\Delta A / \Delta t$ profile in order to be considered accurate.

Activity of a sample can be calculated from the equation:-

$$\text{Number of Units in the assay} = \frac{\Delta A(340nm)/\Delta t}{6.22} \quad (6)$$

$$\text{Total sample activity} = \text{Assay activity x sample dilution in assay} \quad (7)$$

In a final volume of 1mL the following reagents, expressed in their final concentrations were added to a 1.5mL cuvette: Tris-HCL buffer (50 mM, pH 9.0), bovine serum albumin, 5 mgmL^{-1}; NADPH, 0.161 mM; CHMO source, 0.02-0.15 UmL^{-1}. A background rate of NADPH oxidation was measured over two minutes before CHMO activity was measured following the addition of cyclohexanone to a final concentration of 2 mM. The background rate was subtracted from the measured CHMO activity.

Crude solutions of CHMO were prepared by harvesting cells (0.5-2mL) by centrifugation at 15000rpm for 2 minutes (Heraeus Megafuge 1.0R) at 8°C, resuspending in phosphate buffer (50mM, pH7). Cellular disruption was achieved by sonication (Soniprep, 5x10 seconds, with 10 seconds between cycles) in an ice bath for cooling. Clarification was achieved by centrifugation for 5 minutes as described previously.

2.2.4.3 CHMO rate assay using other ketones

For activity measurements using substrates other than cyclohexanone the procedure was identical to the method described in section 2.2.4.2 except cyclohexanone was substituted by other ketones at concentrations from 2-250mM depending on the observed solubility of the ketone under investigation.

2.2.4.4 Protein assay

Soluble protein levels were determined by the colorimetric Bradford protein assay. Bovine serum albumin (fraction V powder, Sigma) in the range 0-1mgmL^{-1} was used to generate a standard curve of concentration against absorbance (see Appendix 2) Samples were diluted in RO water into the range 0.1-0.9 mgmL^{-1} for absorbance measurement at 595nm, 25°C in a Uvikon 922 spectrophotometer.

2.2.5 Cyclohexanone monooxygenase from *E.coli*

E.coli JM107 PQR210 was a kind gift from Dr John Ward, Department of Biochemistry and Molecular Biology, University College London. The organism was maintained at -74°C in 1mL volumes in 50:50 glycerol: phosphate buffer (50mM, pH7). The cells were grown by batch fermentation in a 2L LH series 210 bioreactor on a media described in **Table 2.2**, sterilised at 121°C for 20 minutes prior to use. Integral ampicillin resistance was present and was used to select for those cells carrying the CHMO gene. Cellular production of CHMO was induced during late logarithmic growth (6-7 gL^{-1} dry cell weight) by the addition of 2mM isopropyl β-thiogalactopyranoside (IPTG). CHMO titre reached a maximum approximately one hour post induction (Barclay *et al*, 1997). CHMO activity was typically 0.5-0.7 UmL^{-}

^{1} and 0.2 Umg^{-1} (total cellular protein) in sonicated and clarified lysates. Lysate could be stored for up to 24 hours at 4°C with no loss of activity.

Media component	**Concentration, gL^{-1}**
Glycerol	10
Yeast extract	8
Tryptone	8
Sodium chloride	7.5
Ammonium sulphate	5
Ampicillin (filter sterilised)	0.05
PEG	0.2

Table 2.2. Media components for growth of *E.coli* JM107 pQR210 (Barclay *et al*, 1997)

2.2.6 Substrate and product stability studies

2mL solutions of ketone or lactone were prepared in Tris-HCL buffer (50mM, pH7-10) and placed in glass chromatography vials. The liquid level was kept as high as possible in order to reduce the possibility of loss by evaporation into the vial headspace. The vials were placed in an oven kept at 30^0C and periodically removed for analysis by GC. Concentrations used were 50mM (4-MCH/5-MOP) and 2mM (2-HCP/6-HTPO).

2.2.7 Substrate and product solubility studies

To 10mL of Tris-HCL buffer (50mM, pH8, 30°C) stirring in a waterbath was added ketone or lactone dropwise until a second phase was seen to form. The solution was left stirring for one hour and the aqueous layer separated and analysed by GC as described previously.

2.2.8 Enzyme stability studies

2.2.8.1 CHMO stability studies in whole cells

Cells of *E.coli* expressing CHMO were re-suspended in 50mM Tris-HCL buffer, pH8 and incubated in chromatography vials in an oven at 30°C. Samples (0.2mL) were periodically removed and the cells disrupted as described previously. Active CHMO was quantified using the spectrophotometric rate assay described in section 2.2.4.2.

2.2.8.2 Interfacial damage experiments

Clarified *E.coli* lysate (10mL in Tris-HCL buffer, 50mM, pH 8) containing 0.8mM $NADP^+$ was incubated in a 25mL glass beaker at 30°C in a water bath. The beaker was equipped with a sealable top with a gas sparger connected to either air or nitrogen through a pressure regulator to supply gas at 2vvm. A hypodermic syringe was used to periodically remove 0.2mL samples of lysate to quantify CHMO levels by the spectrophotometric rate assay described in section 2.2.4.2.

2.2.8.3 Other time dependent studies

Clarified *E.coli* lysate (2mL Tris-HCL buffer, 50mM, pH 8 containing 0-0.8mM NADP(H)) was placed in glass chromatography vials containing ketone and lactone to a final concentration from 0-150mM. The vials were placed in an oven kept at 30^0C and 0.2mL samples periodically removed for analysis by the spectrophotometric CHMO rate assay. In samples where there was ketone present, the assay was initiated with addition of NADPH, no background rate being measured.

2.2.9 Whole cell bioconversions

2.2.9.1 Equipment

Whole cell reactions (100mL) were conducted in 1L conical shake flasks kept at 30°C shaken at 200rpm in an orbital shaker (New Brunswick Scientific, Eddison, USA).

2.2.9.2 Cell preparation

E.coli was grown, CHMO activity induced and cells harvested as described previously. Cells were resuspended in 50mM Tris-HCL buffer, pH 8 to an OD of 10 and used immediately. Toluene (0-1mL/L) and ethanol (0-5% v/v) were added followed by ketone and glucose to a final concentration of 2.5-9mM to initiate the reaction.

2.2.9.3 Monitoring the reaction

Samples (1mL) were periodically removed and the cells collected by centrifugation (2 minutes, 15000rpm). Supernatant was used for GC analysis. Cells were washed twice in the same buffer and resuspended to the same volume. Intracellular CHMO levels were assayed as described previously following cell lysis and clarification.

2.3 Criteria required for a model system

To assist in the selection of model substrates it was decided to assemble a list of criteria that a model substrate would have to fulfill in order to be suitable for extensive investigation. The list of criteria developed are shown below.

- Commercial availability
- Cost
- High specific activity with CHMO
- High product enantiopurity

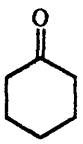
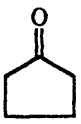
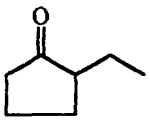
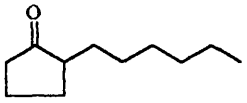
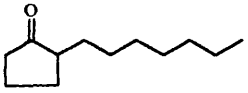
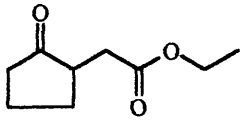
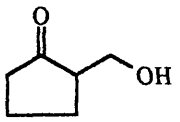
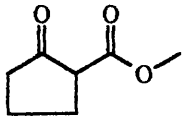
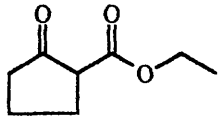
For reasons of time a commercially available chemical makes sense as one of the main benefits proposed for the structured design approach is that the design procedure is rapid. However it does mean that it cannot be prohibitively expensive. If possible the substrate should be processed to a lactone at a high specific rate in comparison to other available substrates.

A high ee is desirable in order to demonstrate fully the usefulness of CHMO as a method of producing enantiopure lactones. A product ee of 95% was set as an arbitrary cut off point for minimum purity. In many cases this data was not available and so this data is incomplete. However in many cases a side chain length adjacent to the carbonyl group greater than 3 carbon atoms (or equivalent) provides the required enantioselectivity (Kayser *et al*, 1998). Molecules with this chain length were assumed to have the required selectivity.

2.4 Screening of potential model substrates

2.4.1 Substrates considered

The substrates evaluated were restricted to those with simple pentanone or hexanone rings as it would be likely that high activity substrates would be those being of a similar structure to cyclohexanone (the natural substrate). A preliminary selection of eleven candidate substrates was made based initially on their commercial availability and included three that produced lactones with no chiral centre and one that had to be synthesised. These were included on the grounds of assessing the relative specific activities of as large a range of ketones as possible with CHMO. These are listed below in **Table 2.3.**

Compound Name	Molecular Structure	Reaction Type	Cost, £/g
Cyclohexanone		Non-Chiral	0.006
Cyclopentanone		Non-Chiral	0.02
Cyclobutanone		Non-Chiral	10.2
4-methyl cyclohexanone		Asymmetric synthesis	0.43
2-ethyl cyclopentanone*		Kinetic resolution	2.2
2-hexyl cyclopentanone♥		Kinetic resolution	2.0
2-heptyl cyclopentanone♣		Kinetic resolution	0.38
Ethyl-2-oxo cylopentyl acetate♠		Kinetic resolution	25.2
2-hydroxymethyl cyclopentanone		Kinetic resolution	N/A
Methyl 2-oxo cyclopentan carboxylate		Kinetic resolution	0.36
Ethyl 2-oxo cyclopentancarboxylate		Kinetic resolution	0.29

*Fluorochem Ltd; ♥Avocado Ltd; ♣Lancaster synthesis; ♠Acros Organics. (See Appendix 1)

Table 2.3 The substrates chosen for investigation

2.4.2 Specific activity measurements

The eleven candidate ketones were assayed for specific CHMO activity as described in section 2.2.4.3 up to the concentration where the ketone was seen to form a second phase, but is not strictly quantitative in terms of solubility. **Table 2.4** summarises the activities produced and enzyme inhibition data.

Compound Name	Maximum CHMO activity observed, Umg^{-1} total protein	K_i, mM	Product ee, %
Cyclohexanone	0.20	40	N/A
Cyclopentanone	0.11	160	N/A
Cyclobutanone	0.13	30	N/A
4-methyl cyclohexanone	0.21	50	98 (Taschner and Black, 1988)
2-ethyl cyclopentanone	0.16	55	46 (Kayser *et al*, 1998)
2-hexyl cyclopentanone	0.17	-	>98 (Kayser *et al*, 1998)
2-heptyl cyclopentanone	0.07	-	>98 (Kayser *et al*, 1998)
Ethyl-2-oxo cyclopentyl acetate	0.15	-	Not available
2-hydroxymethyl cyclopentanone	0.01	-	Not available
Methyl 2-oxo cyclopentan carboxylate	0.02	100	Not available
Ethyl 2-oxo cyclopentancarboxylate	0.03	100	Not available

Table 2.4 Screening data for candidate model substrates.

2.4.3 Selection of model substrate

Using the data on activity, cost and enantioselectivity it was possible to select appropriate model substrates. The non-chiral activities were not considered further due to the lack of enantioselectivity shown in these reaction and are shown only for completeness and to investigate the range of specific activities shown by CHMO toward the candidate ketones. The two esters (ethyl 2-oxo cyclopentan carboxylate and methyl 2-oxo cyclopentan carboxylate) as well as 2-hydroxymethyl cyclopentanone were also discarded due to the low activities seen with CHMO and the lack of a commercial source of the latter.

In terms of enzyme activity, all the remaining candidates produced an inhibitory effect on CHMO activity at concentrations above 2mM except for ethyl-2-oxo cyclopentyl acetate which had an activity of 0.15 Umg^{-1} over the concentration range 2-100mM. This compound however is the most expensive, at £25/g. This is an order of magnitude more than any of the others and for this reason it was not considered further. Of the three 2-alkyl cyclopentanones 2-ethyl cyclopentanone has previously been shown to produce lactones in only modest enantiopurity and 2-heptyl cyclopentanone has the lowest activity. However 2-hexyl cyclopentanone has both good activity and enantioselectivity. An added bonus with this molecule is that the product lactone is itself a high value food additive; a potent peach flavour compound (Tressl *et al*, 1978). This compound is thus both a good model reaction whilst being commercially significant. 4-methyl cyclohexanone was the only asymmetric reaction considered due to a lack of commercially available substrates. It does however fit the criteria required in being both inexpensive, of high specific activity and producing a lactone in a highly enantioselective manner.

The two substrates, 2-hexyl cyclopentanone (2-HCP) and 4-methyl cyclohexanone (4-MCH) were therefore selected to be investigated as model substrates in parallel, 4-MCH as an example of asymmetric synthesis and 2-HCP as an example of kinetic resolution. CHMO has been shown to oxidise 4-MCH to (S)-5-methyl exepane-2-one

(5-MOP) with an ee of 98% (Taschner and Black, 1988). 2-HCP is oxidised by CHMO to (S)-6-hexyl tetrahydropyran-2-one (6-HTPO) with an ee of >98%, the unreacted R-enantiomer is left with a similar enantiopurity (Kayser *et al*, 1998). The two model reactions are summarised below in **Figure 2.1**, firstly the oxidation of 2-HCP and secondly the oxidation of 4-MCH.

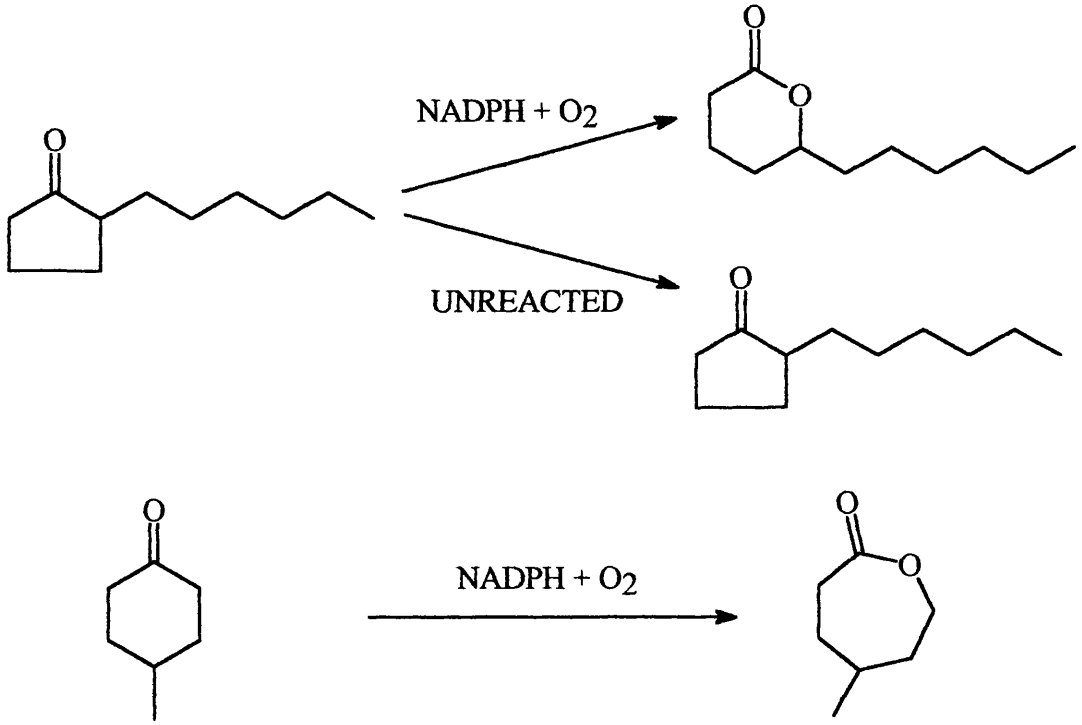


Figure 2.1 The model reactions under investigation

2.5 Characterisation of the reaction

2.5.1 General

CHMO has been studied extensively and has been shown to catalyse the synthesis of lactones in an essentially irreversible fashion (Donoghue *et al*, 1976) and so equilibrium effects are not relevant in this case. The reaction does not produce or consume acid or base and so no pH change will be seen. There is a stoichiometric requirement for oxygen and reduced cofactor (NADPH), the reaction mechanism is believed to proceed through a sequential ordered binding mechanism, with the order of addition being oxygen, cofactor and finally ketone (Ryerson and Walsh, 1982). The oxidised form of cofactor, $NADP^+$, can also bind to the enzyme, producing a competitive inhibition effect. Michaelis-Menton parameters have previously been described (Ryerson and Walsh, 1982), K_m (NADPH) = 0.023mM; K_m (4-MCH) = 0.016mM; K_i ($NADP^+$) = 0.038mM. However while no studies have been conducted using 2-HCP it has been shown that many monocyclic ketones have K_m values in the micromolar range and so it was assumed to be the case here also.

2.5.2 Effect of pH on initial CHMO activity

The optimum pH for enzyme activity has been reported to be pH 9 (Trudgill, 1990) but there is no information as to the relative activity at other values of pH, so this was determined, as shown in **Figure 2.2**. It is evident that the data presented agrees with literature, showing an optimum pH of 9, with activity dropping to 50% at pH 8 and 25% at pH 7.

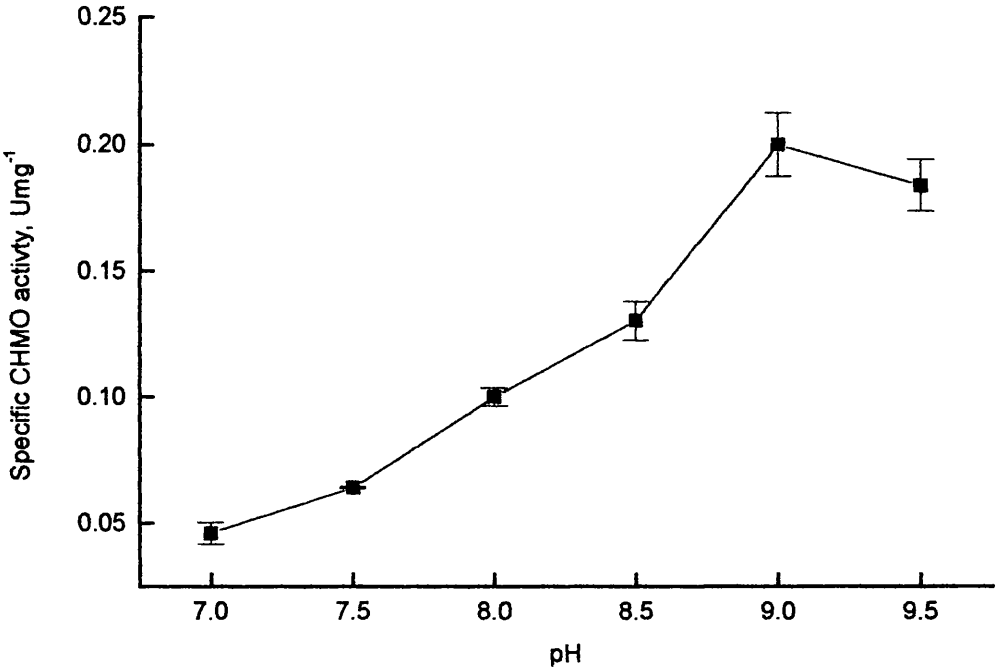


Figure 2.2. The initial specific activity of CHMO (■) as a function of pH

2.6 Characterisation of substrates and products

2.6.1 Volatility

As the reaction has been described as requiring oxygen the issue of substrate/product volatility assumes a greater importance as the supply of oxygen through sparging air has the potential to remove volatile components by a gas stripping mechanism. This problem has been documented previously (Lynch, 1994). Of the four components under consideration (two substrates and two products) 4-MCH would be appear on inspection be the most volatile due to it having the lowest molecular weight. The vapour pressure at 30°C is quoted as 10mmHg (Marsden, 1963) which would classify this as non-volatile. No data was available for the other compounds. The boiling points of 4-MCH and 2-HCP are 169°C and 270°C (Marsden, 1963). No values were obtained for 5-MOP and 6-HTPO, however from the boiling points of the corresponding ketones they would appear to be in excess of 250°C. All four ketones are thus liquids under typical reaction temperatures.

2.6.2 Solubility of substrates and products

Table 2.5 lists the measured aqueous solubility of the four components at 30°C.

Compound	Molecular weight	Solubility, mM	Solubility, gL^{-1}
4-MCH	112	196	21.9
5-MOP	128	280	35.8
2-HCP	168	2.5	0.4
6-HTPO	184	5	0.9

Table 2.5 Aqueous solubility of the reaction components in 50mM Tris buffer.

The solubility's of 4-MCH/5-MOP are an order of magnitude greater than the 2-HCP/6-HTPO system, which is unsurprising due to the large number of hydrophobic residues present in 2-HCP/6-HTPO compared to hydrophilic functions, in 2-HCP there is only one carbonyl; in 6-HTPO one carbonyl and one ester function.

2.6.3 Aqueous stability studies

2.6.3.1 Ketones and lactones

Studies were undertaken at 30°C, over a range of pH from 7-10, chosen because of the data gained describing the pH/activity profile of CHMO. It makes little sense to examine stability in a pH range within which there would be an unacceptably low reaction rate.

Both 4-MCH and 2-HCP were completely stable over the entire pH range for 24 hours. 5-MOP and 6-HTPO were found to be labile under aqueous conditions. The degradation occurs through a ring opening mechanism in an identical mechanism to that catalysed by the *A.calcoaceticus* lactonase shown in **Figure 1.6.** The rate of degradation appears to be accelerated at more alkaline pH, as shown in **Figure 2.3**, the residual levels of lactone after exposure for 24 hours. 6-HTPO is the most unstable, particularly above a pH of 7.5. In order to have a greater understanding of the degradation process the levels of the two lactones were monitored at a single pH (pH 8) regularly over 24 hours. This is shown in **Figure 2.4**.

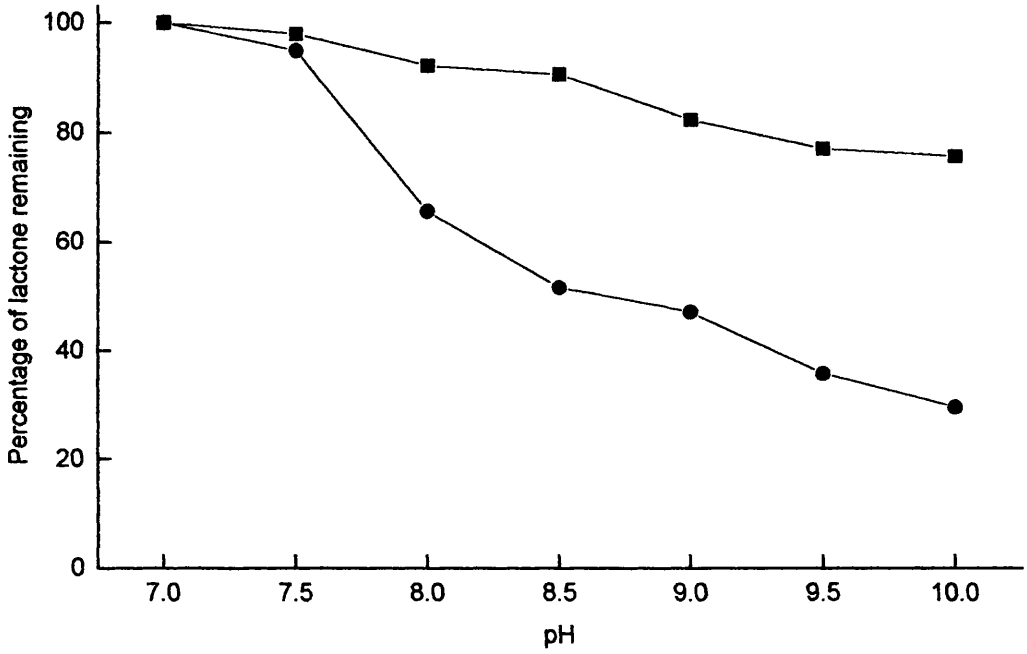


Figure 2.3 The residual levels of 5-MOP(■) and 6-HTPO(●)after 24 hours at 30°C.

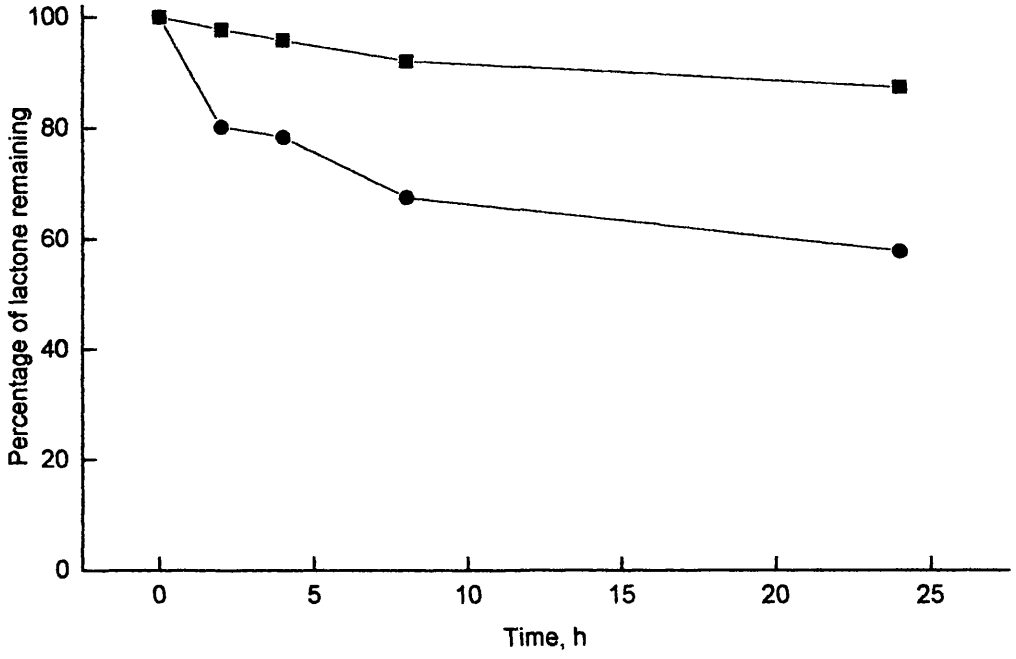


Figure 2.4 Degradation of 5-MOP(■) and 6-HTPO(●)over 24 hours at pH 8 and 30°C.

2.6.3.2 Cofactor

The stability of NADP(H) has previously been investigated by several researchers. (Lowry *et al*, 1961) were the first to examine this in detail, reporting that in general the phosphorilated forms of cofactor were less stable over a range of conditions than NAD(H). This work was given a more formal treatment (Chenault and Whitesides, 1987) in as much as the mechanism of degradation for both forms of the cofactor has been elucidated, and the kinetics described as first order decay. Acidic conditions catalyse the hydration and anomerisation of the reduced form, basic conditions catalyse the hydrolysis of the nicotinamide-ribose bond of the oxidised form (Chenault *et al*, 1988). Thus in general NADPH is stable in alkaline conditions but unstable at pH 7 and below. This is reversed for $NADP^+$. Degradation of both forms is also a function of the ionic species present; phosphate ions are able to catalyse the breakdown of both forms.

2.7 Characterisation of CHMO

2.7.1 General

Much of the fundamental characterisation of CHMO has been reported elsewhere (Chen *et al*, 1988). However no crystal structure has yet been reported. The enzyme is a single polypeptide of 542 amino acids with a molecular weight of 59,000 and a specific activity of $21Umg^{-1}$ when pure. The FAD coenzyme is tightly bound near to the N-terminus and does not dissociate easily, CHMO remaining in the *holo* form during purification (Donoghue and Trudgill, 1976). No metal ion is thought to be present although there is a catalytically important cysteine residue in the active site (Trudgill, 1990).

2.7.2 Enzyme stability

2.7.2.1 Effect of cofactor

The stability of CHMO in lysate (pH 8) at a range of cofactor concentrations is shown in **Figure 2.5**. In general a higher retained activity is seen at higher concentrations of NADPH. The form of cofactor seems to be irrelevant, as virtually identical retained activity is seen with the same concentration of either NADPH and $NADP^+$. It is possible that the cofactor is reinforcing the tertiary enzyme structure by binding to the protein, or it is protecting sensitive residues in the active site, for example the cysteine residue mentioned previously. Cysteines in proteins are often susceptible to oxidation (Wells and Estell, 1988).

The stability of the enzyme within resting *E.coli* cells is much less than when isolated in the presence of added cofactor. This could be for several reasons. The cells may be harboring active proteases that are breaking down CHMO. Also the concentration of cofactor within the cells may be extremely low and the enzyme is not being protected. It is possible that the internal pH of the cell may not be suitable for CHMO, and denaturation is occurring. Equally the cause could be a combination of several or all of these factors. It was determined that cell lysis was not occurring as no CHMO was detected in the suspending buffer.

2.7.2.2 Effect of gas/liquid interfaces

The effect of exposure of CHMO to aeration with air and nitrogen is shown in **Figure 2.6**. compared to a non-aerated sample. The lowest stability is seen when the enzyme is exposed to air bubbles. This would saturate the lysate with oxygen, and if the hypothesis concerning oxidative damage to the enzyme is correct, then this

would explain the drop in activity compared to the non-aerated sample. However a second mechanism of deactivation must be considered. It is understood that gas/liquid interfaces are damaging to proteins, allowing hydrophobic residues normally internalised in the molecule to be exposed, disrupting the tertiary structure irreversibly. Aeration with nitrogen would purge all the oxygen from the sample, and any activity loss must be entirely due to physical damage. It is clear from the data that this is occurring, although aeration with nitrogen causes less activity to be lost than when oxygen is present.

2.7.2.3 Effect of pH

Enzyme stability over 24 hours with lysate at pH from 7-10 is shown in **Figure 2.7**. Within this band of pH CHMO has the highest activity and so is the region of most interest (assuming CHMO is the most expensive part of the process). Over 80% of activity is retained between pH 8-10, with stability decreasing at neutral pH. It would seem that the enzyme has significantly higher stability in the region of highest specific activity, possibly due to the molecule being more distorted below pH 8, lowering both activity and the activation energy for denaturation.

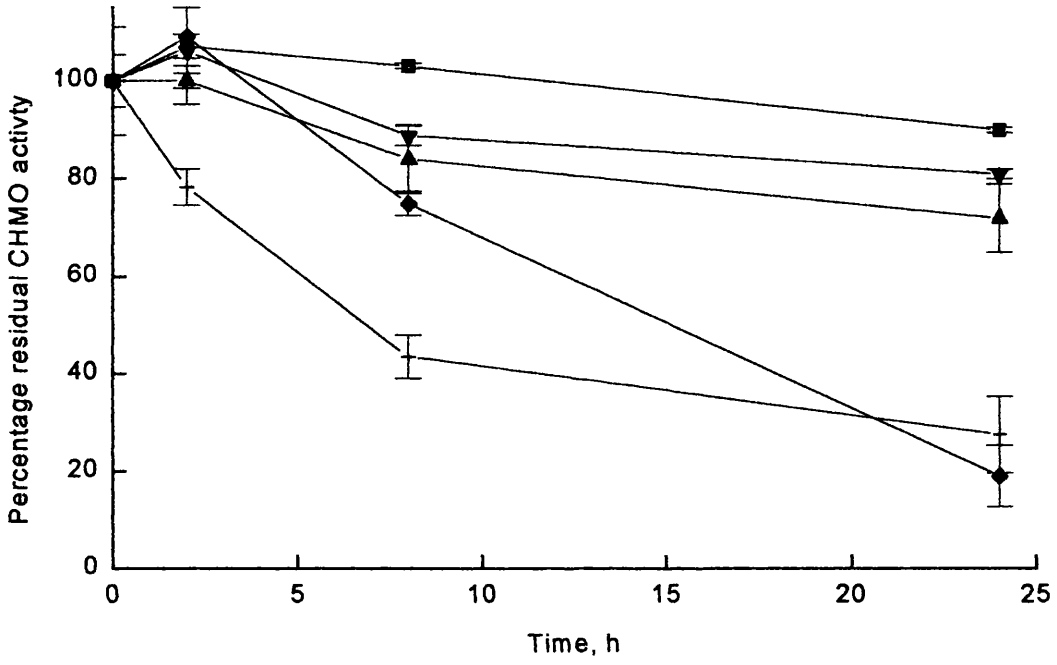


Figure 2.5. The loss of CHMO activity over time for whole cell (+), lysate (◆),lysate with 2mM NADPH (■),lysate with 0.2mM NADPH (▲) and lysate with 0.2mM $NADP^+$ (▼).

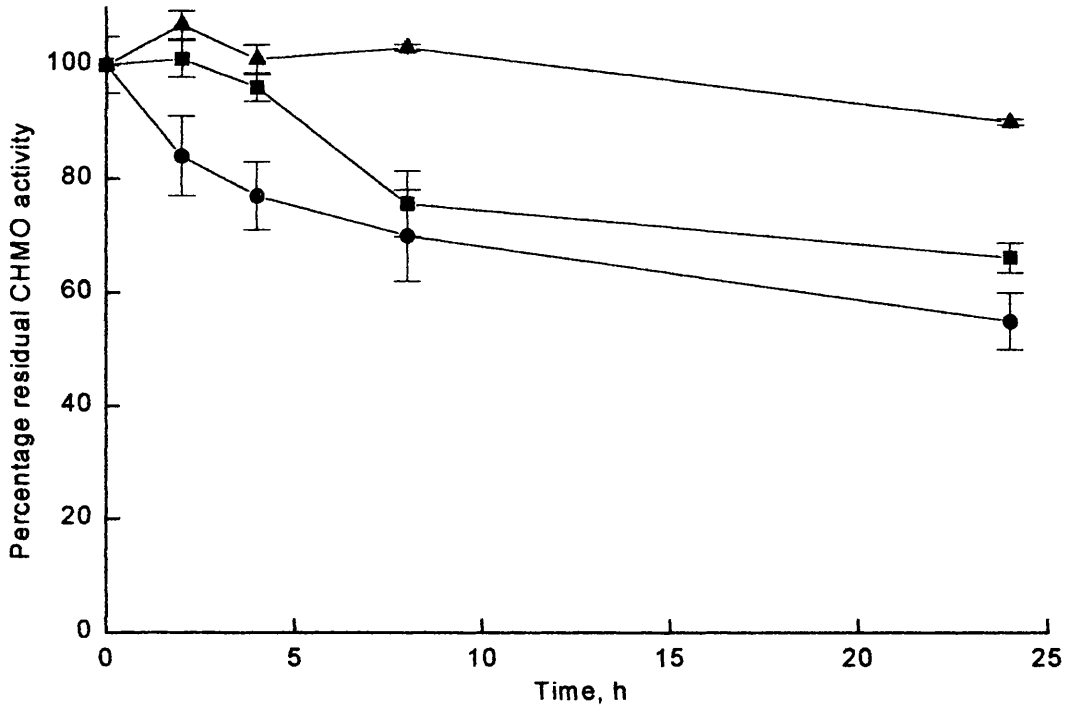


Figure 2.6. The loss of CHMO activity over time for lysate with 2mM NADPH (▲), lysate with 2mM NADPH aerated with 2vvm nitrogen (■) and lysate with 2mM NADPH aerated with 2vvm air(●).

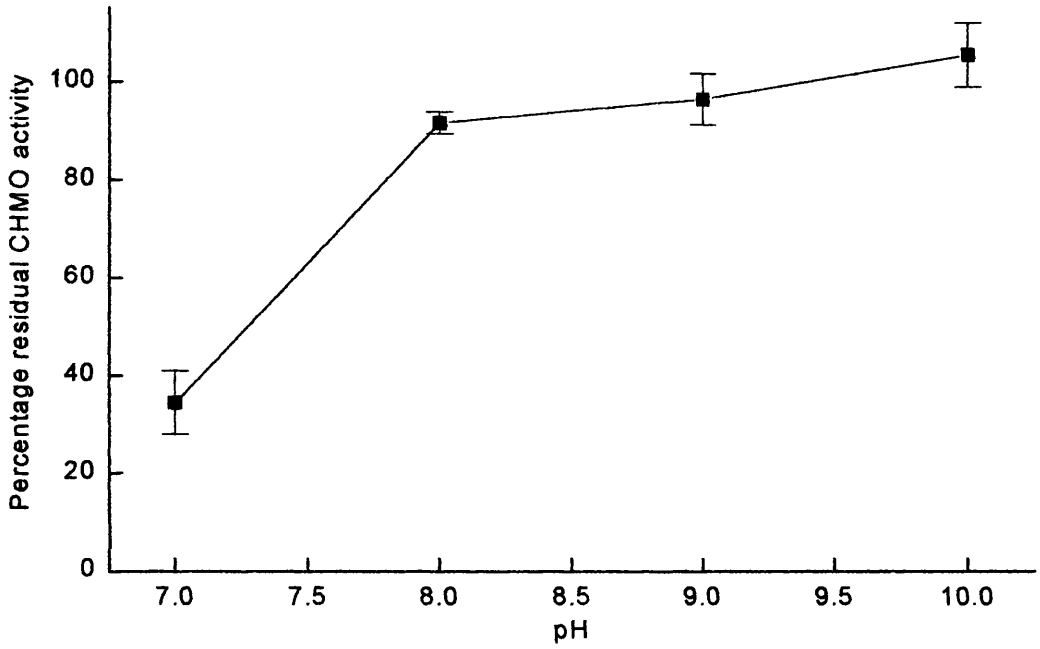


Figure 2.7 The residual activity of CHMO in lysate (■) after incubation for 24 hours at 30°C as a function of pH.

2.8 Characterisation of interactive effects

2.8.1 Substrate inhibition

As has previously been described in section 2.4.2, 4-MCH shows substrate inhibition against CHMO above 2mM, with a K_i of 50mM. 2-HCP does not show an inhibitory effect up to the aqueous solubility limit.

2.8.2 Product inhibition

Enzyme rate determinations at elevated levels of 6-HTPO showed no product inhibition up to the aqueous solubility limit. 5-MOP produced an inhibitory effect that was pronounced at a concentration above 2mM, shown in **Figure 2.8**. The source of 5-MOP was via chemical synthesis so that it was in fact a racemate. It is possible that the two enantiomers would have different inhibitory potential against CHMO, the S-enantiomer which is the form predominantly produced by CHMO may have easier access to the active site and so be the more effective inhibitor. However in the context of this investigation the discovery of product inhibition is of more relevance to the structured approach than absolute quantification.

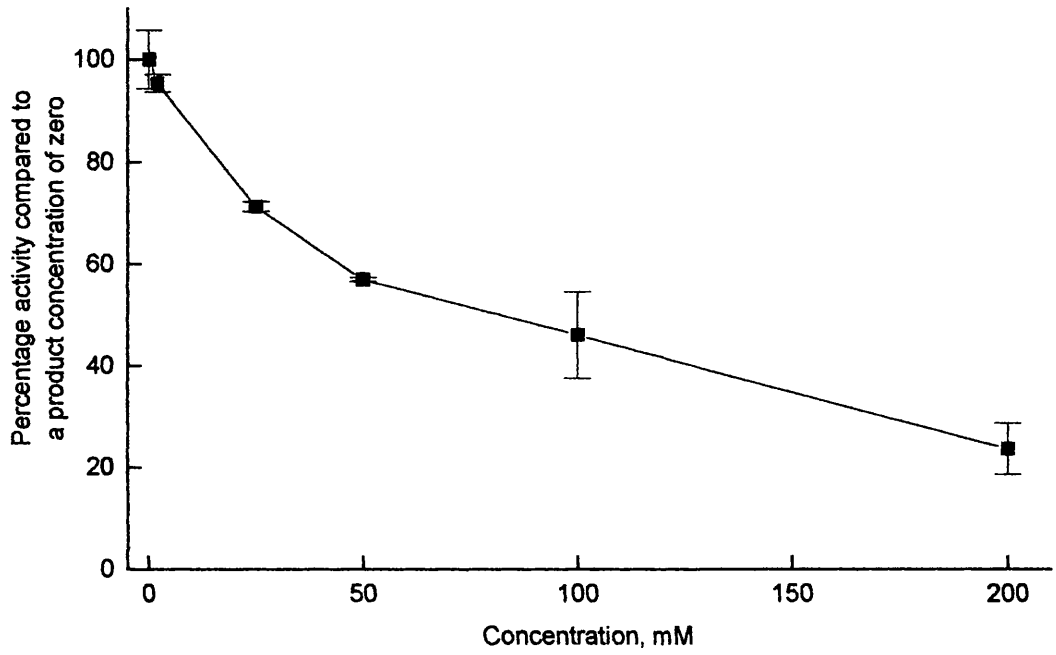


Figure 2.8. The activity of CHMO as a function of 5-MOP concentration(■) as a percentage of the activity with initially zero product.

2.8.3 Substrate toxicity

Incubation of lysate with 2-HCP up to the solubility limit did not irreversibly lower CHMO activity compared to samples containing no ketone.

4-MCH toxicity has previously been addressed (Rissom *et al*, 1997) and was found to rapidly denature CHMO at and above a concentration of 50mM, but had no effect below this value. Studies conducted here were in agreement. After 24 hours the residual activity of a sample containing 50mM 4-MCH was 61% +/- 5.6% (compared to 70% retained with zero product) indicating that the ketone showed a significant toxic effect at this level.

2.8.4 Product toxicity

Racemic 6-HTPO caused no permanent damage to CHMO after incubation for 24 hours up to the solubility limit. Racemic 5-MOP could be dissolved to much higher concentrations and the result of incubation studies is shown in **Figure 2.9**. After five hours there seems to be no loss of activity up to 80mM and above this there is a rapid loss of activity. This is mirrored by studies at 24 hours where no activity remains at 100mM. Irreversible loss is seen at this time at 40mM and above. Lower concentrations (25mM) would appear to have a protective effect on the enzyme, possibly shielding the active site, as a higher retained activity is seen compared to the sample containing no 5-MOP.

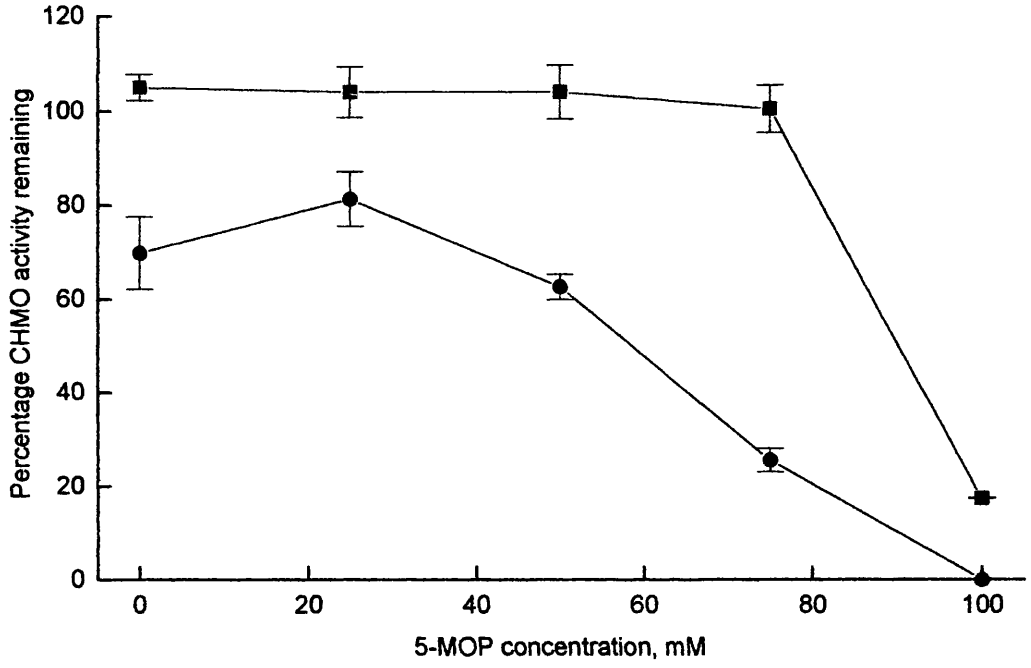


Figure 2.9. Residual CHMO activity after exposure to 5-MOP for five hours(■) and 24 hours(●).

2.8.5 Whole cell bioconversions

2.8.5.1 Oxidation of 4-MCH and 2-HCP

Profiles for whole cell conversions can be seen in **Figure 2.10** and **Figure 2.11** for 4-MCH and 2-HCP respectively. Also shown is the intracellular amount of enzyme. This is not the reaction rate observed but a quantification of the maximum possible if the enzyme was working at 100% efficiency (i.e. the enzyme is under standard rate assay conditions).

It can be seen that the intracellular enzyme activity dropped off rapidly from 32U to zero in only 4 hours with 4-MCH. This rate of decrease was much more rapid than in resting cells or crude enzyme preparations. This trend was mirrored when the cells were presented with 2-HCP.

By integrating under the intracellular activity plot on **Figure 2.10.** a total theoretical conversion of 3.3mol of substrate was calculated. The data shows that only 0.0505mol was actually produced. Expressing this as a percentage efficiency of enzyme use this is only 1.5% of the enzyme's potential. A similar analysis of **Figure 2.11** indicates that this occurs when 2-HCP is a substrate, the usage being 1.4%.

The mass balance for both reactions was good suggesting that no product was being stored in the cells, which was confirmed from GC analysis of lysed cells post-reaction showing no trace of either component.

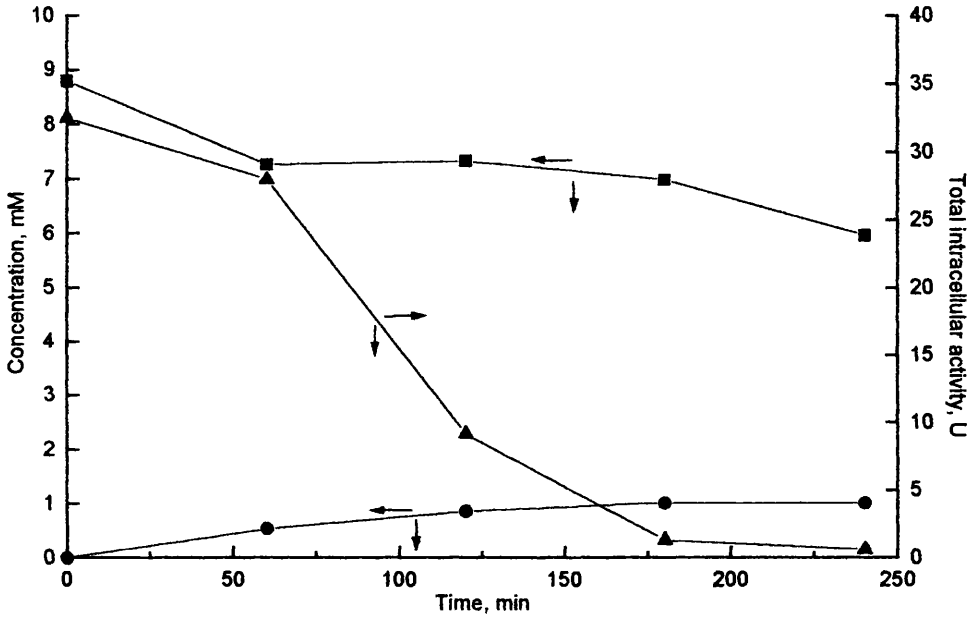


Figure 2.10 Whole cell *E.coli* bioconversion showing 4-MCH(■), 5-MOP(●) and intracellular enzyme activity(▲).

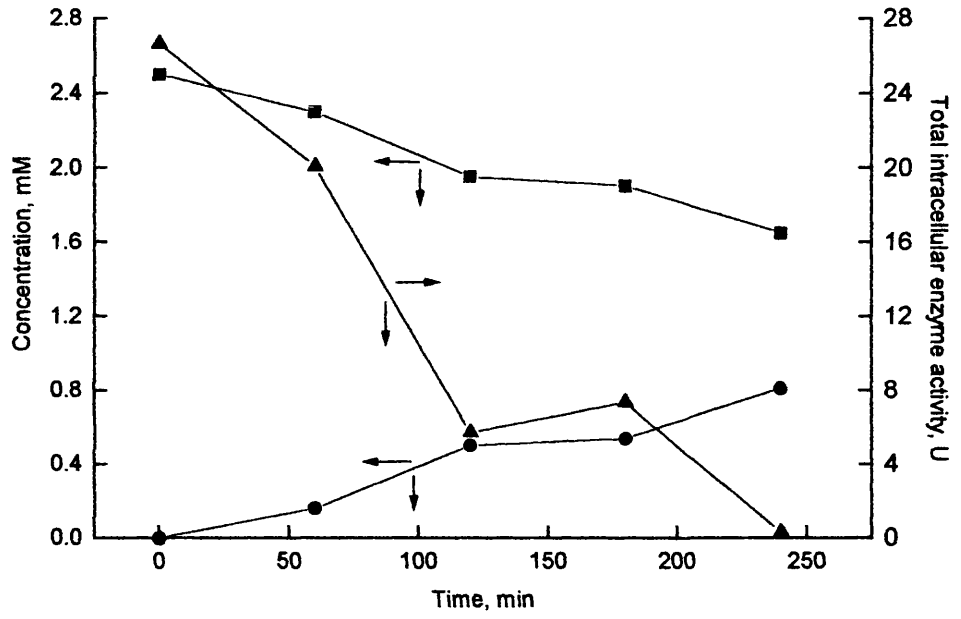


Figure 2.11 Whole cell *E.coli* bioconversion showing 2-HCP(■), 6-HTPO(●) and intracellular enzyme activity(▲).

2.8.5.2 Reactions with added toluene and ethanol

Repeating the whole cell experiments in the presence of toluene showed a similar pattern to previous experiments, in so much as the enzyme activity was lost over a period of 4-5 hours and substrate conversion was limited. The overall efficiencies produced were 1.1% for 4-MCH and 1.4% for 2-HCP. Substituting 5% ethanol for the toluene produced similar results; efficiencies for 4-MCH and 2-HCP were 1.4% and 0.9% respectively.

It is clear that addition of toluene and ethanol, which was designed to permeablise the cells to see if mass transfer across the cell membrane was restricting reaction rate were unsuccessful in increasing efficiency. This would seem to suggest that transport across the cellular membrane was not the limiting factor. This appears to be the case as it would be expected that 2-HCP would pass more easily through the cell membrane due to it's greater hydrophobicity. The results show that the substrate choice is not affecting reaction rate. Another more likely cause is that the intracellular turnover of NADPH is limiting the reaction rate. CHMO is not native to this organism and it's metabolism may not be able to cope with the demand even in the presence of glucose which provides a carbon source for $NADP^+$ to NADPH conversion. Kinetic considerations may also apply; the turnover of NADPH may be fast enough but the absolute concentration could be significantly below the K_m value. A combination of these two factors could quite easily account for the extremely low rate observed.

2.9 Discussion of model systems

2.9.1 Process constraints and implications

The relationship of specific CHMO activity to pH shows a maximum activity at pH 9, with good retention of activity found from pH 8-10. These data are valuable since they highlight the pH range within which both enzyme and reaction component stability studies need to be performed. In this way the structured approach is helpful in leading the design process to more focused experimentation.

The solubility of the asymmetric reaction components was an order of magnitude greater than the kinetic resolution reaction components. This immediately identifies probable divergent process designs for the two systems even from this limited data, highlighting the importance of the characteristics of the components. The 2-HCP system has very low solubility ($<1 gL^{-1}$) and so realistically could not be operated as a batch reaction unlike the 4-MCH system which theoretically could produce product titres in excess of $30gL^{-1}$. There was no constraint found due to volatilisation of the substrates and products or by the stability of the ketones. Both lactones were found to be alkali labile, probably as a consequence of base catalysed hydrolysis (Marsden, 1963). Significant losses over 24 hours were shown for both ketones at pH 7.5 and above. This constraint conflicts with the region of high enzyme activity, high reaction rates are seen at pH 8 and above. Degradation was particularly marked with 6-HTPO. This would suggest that product removal techniques need particular attention with this reaction.

The choice of reaction pH will have to be a compromise. The compromise must maximise product yield, the compromise being between product formation rate and product degradation rate. The point of highest difference between the two rates is the ideal condition, although this is true only if productivity over time is not a consideration. Upon inspection pH 7.5-8 seems to be the most satisfactory to allow

this. The stability of the catalyst is also of importance, and follows a similar pattern to the region of high activity, suggesting the process should not be operated below pH 8 (if the enzyme is considered to be a significant cost).

The choice of reaction pH can be illustrated diagramatically by constructing a window of operation (Woodley and Titchener-Hooker, 1996) which plots key variables to show the feasible limits of operation in the system under investigation. By assuming a basis (arbitrarily) of no greater than 10% 5-MOP lost due to degradation and either a CHMO activity of 30% or 50% of it's maximum (which occurs at a product concentration of zero and pH9) it is possible to plot out a series of windows, as shown in **Figures 2.12.** and **2.13.** It can be seen that the window is larger if the constraint of CHMO activity is relaxed. It is possible to plot other important variables such as substrate concentration or CHMO degradation rate, however pH and 5-MOP concentration are likely to be the most important variables and as such illustrate this technique.

Enzyme stability was shown to be a strong function of cofactor concentration. After 24 hours the residual activity of CHMO in a crude extract and whole cells was roughly equal at 25-30%. Deactivation in whole cells is more rapid than in homogenate, possibly due to active proteolysis. The addition of cofactor to the isolated enzyme increased stability markedly, with the addition of 2mM NADPH nearly all inactivation was prevented. It has been suggested that there is a catalytically important cysteine residue at the active site (Donoghue and Trudgill, 1976) and these residues are known to be susceptible to oxidation (Wells and Estell, 1988). It is possible that the cofactor is able to bind to the active site and prevent access by oxygen to the cysteine. The protective effect appears to be a function of concentration, but not a function of the cofactor form as $NADP^+$ offers equivalent protection. This could offer another explanation as to instability of the enzyme within whole cells, as the intracellular concentration of cofactor may be very low and therefore not prevent oxidation. The role of oxygen in enzyme deactivation is reinforced by the lower rate of inactivation found when free enzyme is exposed to nitrogen compared to air. The effect of increased interfacial contact by bubbling gasses through a solution of the enzyme is to accelerate inactivation, however a

significantly greater amount of activity remains in the oxygen free state (using nitrogen) than the use of air. CHMO deactivation would thus seem to be a combination of oxidation and physical deactivation due to interfacial effects. This has important implications for the choice of catalyst form, whole cells would protect the enzyme from interfacial damage but will be inherently unstable Free enzyme would be protected by cofactors but aeration damage will dominate and require artificial regeneration of NADPH. One solution may be to use immobilised CHMO that should be generally more stable to these effects.

With the 4-MCH system significant substrate and product inhibition was observed, as was enzyme degradation due to 4-MCH and 5-MOP. The type of inhibition was not elucidated. However due to the irreversible kinetics of the reaction it is not an equilibrium effect. The nature of the compounds involved may affect the monooxygenase by distorting the tertiary protein structure and disrupting intramolecular hydrogen bonds. No such effects were noted for the 2-HCP system. The probable reason for the difference is simply the maximum achievable aqueous concentration of the compounds. 2-HCP and 6-HTPO cannot exist in a high enough concentration within the enzymes environment to have a detrimental effect.

Performing whole cell reactions with both systems showed that the observed reaction rate was unexpectedly low, compared to the potential reaction rate. The potential reaction rate was easily determined by quantifying the activity of CHMO within the cells after cell disruption and clarification using the spectrophotometric rate assay. With both the 2-HCP and 4-MCH systems the reaction rate observed was less than 2% of the potential velocity. This may be due to several factors; the internal pH of *E.coli* may not suit CHMO activity; the cell's metabolism may not be able the recycle cofactor quickly enough; 4-MCH/2-HCP may be extremely toxic to cells or there is a diffusional limitation, bottlenecking the transmission of ketone across the cell membrane. Repeating the reactions in the presence of toluene or ethanol in an attempt to permeablise the cell membrane showed no improvement in reaction rate, suggesting that diffusion was not limiting. This is supported by the fact that the two ketones used differ greatly in hydrophobicity but showed no significant difference in reaction rate. The reaction rate with 2-HCP would be higher if diffusion limited

because the more hydrophobic the molecule the easier the passage across the membrane should in theory be.

Intracellular CHMO activity seemed to be much more unstable than in resting cells, complete loss of active CHMO occurred in under five hours. This is possibly due to the cells degrading CHMO in an attempt to generate energy for NADPH turnover to drive the reaction, although it would be expected that supplying glucose would have limited this effect. As such no satisfactory answer to this phenomenon could be produced.

It would appear that a whole cell catalyst is not appropriate and so even with the disadvantages associated with an isolated enzyme (principally the recycle of NADPH) the increase in catalyst stability and reaction rates achievable with an isolated enzyme make this the catalyst form of choice.

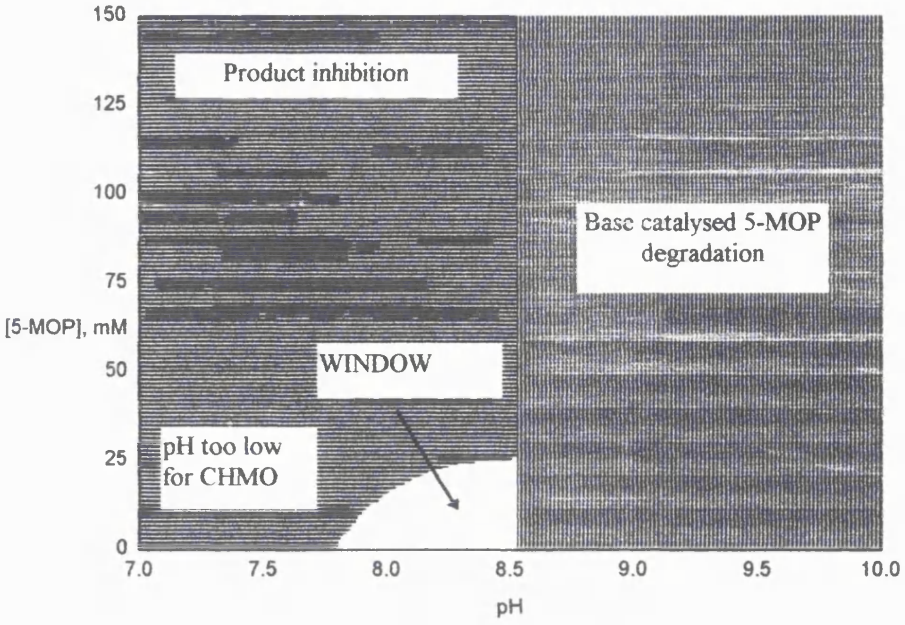
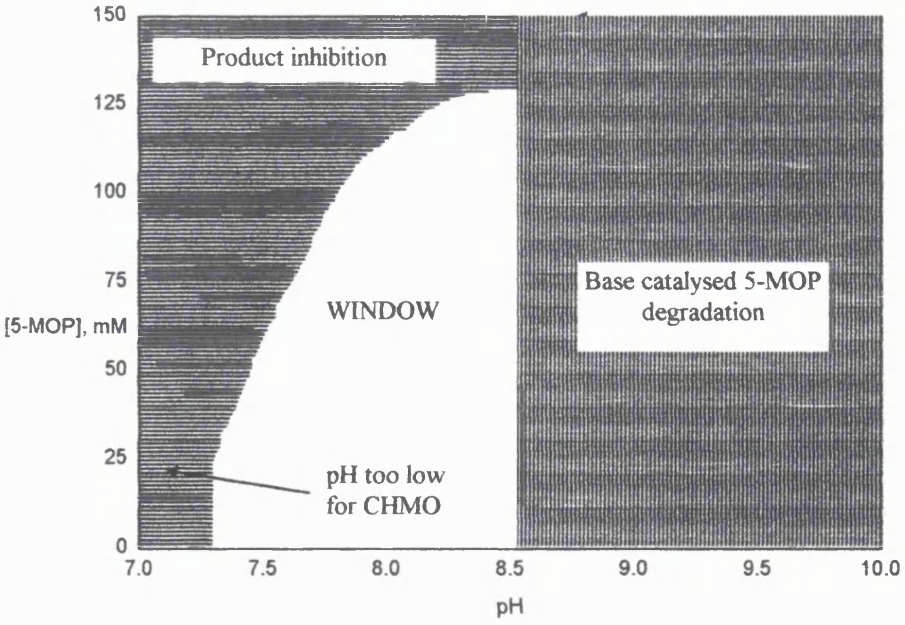


Figure 2.12 Operating window for 90% residual 5-MOP over 24 hours and a CHMO efficiency of >50%.

Figure 2.13 Operating window for 90% residual 5-MOP over 24 hours and a CHMO efficiency of >30%.



2.9.2 Reactor options

The constraints identified in section 2.9.1. help to define the reactor options by showing the limits to stable operation that the physical environment in a reactor will have on the process for both model reactions. The alkaline lability of the lactones would present a problem if there was a pH shift as good mixing and pH control would be needed to avoid yield loss. As this is not the case then a well mixed plug flow system could not be ruled out. The need to supply oxygen and the lack of volatility seen with the substrates and products allow the use of aeration by sparging, which is simple and effective but demands a well mixed system precluding the use of plug flow or packed beds (due to channeling and poor interfacial contact). It is clear that the use of whole cells as catalyst is not practical and free enzyme must be used, the choice largely weighted towards immobilisation as opposed to soluble due to the damage aeration would cause to soluble protein.

The effect of substrate inhibition and toxicity seen with the 4-MCH system would be alleviated by substrate feeding, needing either a FBSTR or CSTR. Immobilised enzyme would allow continuous operation, however product inhibition and toxicity would tend to limit the use of continuous modes. It is here that the divergence occurs with the constraints on the two model systems. The 2-HCP system does not produce inhibition or toxicity and is only sparingly soluble in aqueous solution. Using a 2-phase reactor, with the substrate forming a second phase would present the ketone to the enzyme at a concentration suitable for efficient synthesis. Product removal would also be achieved, as it would partition into the organic phase. This would not be possible with the 4-MCH system as 5-MOP is not sufficiently hydrophobic to partition satisfactorily into a solvent phase and so a different method of removal is needed with this more water soluble component, such as adsorption to a solid carrier.

Continuous processing with soluble cofactors is not practical unless they are retained in some way such as the use of ultrafiltration membranes and covalent attachment of

the cofactor to water soluble polymers such as PEG (Ottolina *et al*, 1990) and recycled. This constitutes a novel reactor scheme and is beyond the scope of this thesis. A similar benefit (in that cofactor is retained) of a 2-phase reactor would be the potential re-use of cofactor by simply reusing the aqueous phase after separation from the organic layer, lowering the overall cost.

It is interesting to note that the main influence on the difference between the two model system reactor modes is the reactant and product solubility, and not the asymmetric/resolution nature of the reaction that may have been expected. It would seem that if indeed these reactions are representative then the toxic and inhibitory effects are only a function of solubility and thus the reactor design is largely a function of reactant/product solubility also. It was anticipated that the nature of the two reactions, particularly the build-up of the unwanted enantiomer in resolution would have a major influence on the reactor design. Simply it seems that highly water soluble reactions will need to be FBSTR possibly with product removal, whereas poorly water soluble reactions will require a TLPBSTR, the second phase acting as a method of both product removal and reservoir for the unconverted ketone enantiomer in the case of resolution reactions. It must be noted that in general oxidation reactions produce products with increased water solubility compared to the substrate and so product removal into the organic phase may loose some selectivity. The pro's and con's of the various reactor modes are summarised in **Table 2.6** (high solubility reactions) and **Table 2.7** (low solubility reactions).

There does remain the case of a system with 'intermediate' solubility that will form a second phase but with an aqueous concentration that is too high, causing inhibition and/or toxicity. It is likely that a inert solvent could be used as the second phase, preferentially partitioning the ketone and lactone away from the aqueous phase.

	Mode					
Characteristic	**FBSTR**	**BSTR**	**CPFR**	**CSTR**	**TLPBSTR**	**Comment**
Solubility of substrate/product	❍	❍	-	-	●	2-phase not suitable
pH lability of lactone	-	-	-	-	-	Operate at pH 8 to compromise with enzyme activity
oxygen needed	❍	❍	●	❍	❍	Sparge air, immobilise enzyme for protection
substrate inhibition	❍	●	-	❍	-	Substrate feeding
substrate toxicity	❍	●	-	❍	-	Substrate feeding
product inhibition	-	-	●	●	-	Batch modes have high product levels only toward the end of the reaction compared to continuous reactors, possible use of ISPR
product toxicity	-	-	●	●	-	Batch modes have high product levels only toward the end of the reaction compared to continuous reactors, possible use of ISPR
NADPH requirement	❍	❍	●	●	❍	Recycle cofactor, batch processing to prevent cofactor 'wash-out'
SUITABILITY	❍	●	●	●	●	**FBSTR with immobilised CHMO and cofactor recycle, sparge with air.**

Table 2.6. The possible reactor modes for high aqueous solubility reactions (e.g. 4-MCH/5-MOP), highly unsuitable(●); no strong effect(-); highly suitable(❍).

Characteristic	Mode					Comment
	FBSTR	BSTR	CPFR	CSTR	TLPBSTR	
Solubility of substrate/product	●	●	●	●	❍	Substrate as second phase
pH lability of lactone	-	-	-	-	❍	Operate at pH 8 to compromise with enzyme activity, product will largely partition into the organic phase
oxygen needed	❍	❍	●	❍	❍	Sparge air, immobilise enzyme for protection
NADPH requirement	❍	❍	●	●	❍	Recycle cofactor, batch processing to prevent cofactor 'wash-out'
SUITABILITY	●	●	●	●	❍	**TLPBSTR with immobilised CHMO and cofactor recycle, sparge with air.**

Table 2.7. The possible reactor modes for low aqueous solubility reactions (e.g. 2-HCP/6-HTPO), highly unsuitable(●); no strong effect(-); highly suitable(❍).

2.9.3 Applicability of the structured approach

The structured design approach has been successful in highlighting the key characteristic for CHMO based syntheses in determining mode of operation, the reactant\product solubility, being the 'controlling' characteristic of the process. The possible process designs have been considered and the most appropriate identified on a qualitative basis over other designs from the constraints. It is clear however that this characterisation process allows some process options to be discussed and rejected as unsuitable, rather than positively selecting the appropriate ones.

The structured approach does not however indicate to the designer what the quantitative difference will be between the options under consideration. The ability to do this would be of great help as it would limit the number of more detailed experimental studies required. The ability to select a small number of process options on a qualitative basis, and then test their effectiveness to gain quantitative data through for example process modelling would enhance this technique as a design tool, and give greater confidence in the final design identified.

In this chapter the issue of recycling the cofactor has not been addressed. It is vital to the use of CHMO as a method of producing lactones. This issue is general to redox biocatalysis and is likely to arise in the future when considering other systems. The structured design approach needs therefore to be enlarged to encompass this issue, the investigation into which will form the following chapter.

2.10 Summary

In this chapter the structured design approach was considered to assess it's suitability to this biocatalytic reaction and to redox biocatalysis in more general terms. Two model reactions were selected, compromising a poorly water soluble kinetic resolution system and a water soluble asymmetric synthetic reaction. The rationale behind choosing two model reactions was to investigate what effect (if any) the type of enantioselective reaction would have on the process design. Characterisation of the system indicated that the key characteristic of the substrate and product was in fact solubility and not the class of reaction. The solubility defines the choice of process as either a fed batch reaction for soluble reactions and a 2-phase system for poorly soluble reactions.

The structured approach highlighted these options as the most suitable on a qualitative basis, however no numerical data can be produced from this approach to verify the choice of reactor. The design procedure would be significantly enhanced if this was possible.

The use of whole cells as the biocatalyst was found to be impracticable due to very low reaction rates and CHMO instability. The structured approach failed to identify a suitable method of supplying the biocatalyst with NADPH, the challenge that will form the basis of the next chapter.

3.0 Rational choice of NADPH regeneration method

3.1 Introduction

3.1.1 General

In the previous chapter the applicability of a structured approach to the CHMO catalysed synthesis of lactones was investigated and highlighted several process options. One serious failing in this approach was related to the choice of catalyst form. The use of whole cell *E.coli* as the catalyst was found to be unsuitable due to extremely low reaction rates with both substrates considered. Thus although the process can be defined in terms of the physical environment within which it is necessary to operate (i.e. fed batch operation) no satisfactory method for supplying the reduced cofactor, NADPH, is produced by this approach. This section aims to address this issue in a rational manner, aiming to develop an efficient process that will fit within the general design already established.

The use of isolated redox enzymes requires a method of NADPH regeneration, since it cannot be supplied stoichiometrically because of it's high cost (£57,000mol^{-1}) and limited commercial availability. NADPH is manufactured from NAD^+, itself produced as an isolate from yeast. $NADP^+$ is synthesised from this by enzymatic phosphorylation using NAD kinase and finally NADPH is produced by chemical or biochemical reduction (Chenault *et al*, 1988). Thus a necessarily smaller amount must be used and constantly regenerated from $NADP^+$ (Ikemi *et al*, 1990[1,2]). The important dimensionless parameter used in assessing cofactor regeneration is the *total turnover number*, TTN, defined as the ratio of moles of product formed to the number of moles of cofactor present. The value of TTN that must be achieved in order to produce an economically viable process is unclear, and is a function of

product value and type of cofactor (Chenault and Whitesides, 1987). Estimates for NADPH turnover have been suggested in the range from 100 to over 1000 (Kragl, 1996; Chenault *et al*, 1988). In this investigation the lactone products are expected to be of medium to high value and a target TTN of 500 will be set. Due to the aqueous instability of NADP(H) a greater than catalytic amount needs to be added to overcome losses through decomposition, lowering the potential TTN achievable. Recovery and reuse of cofactor has been demonstrated by attaching the molecule to high molecular weight water soluble polymers, i.e. PEG, and recovering them through ultrafiltration (Ottolina *et al*, 1990). The structure of NADP(H) is shown in **Figure 3.1**. The methods of NADPH regeneration vary enormously, many enzymatic techniques have been developed, also a number of chemical and electrochemical methods have been proposed.

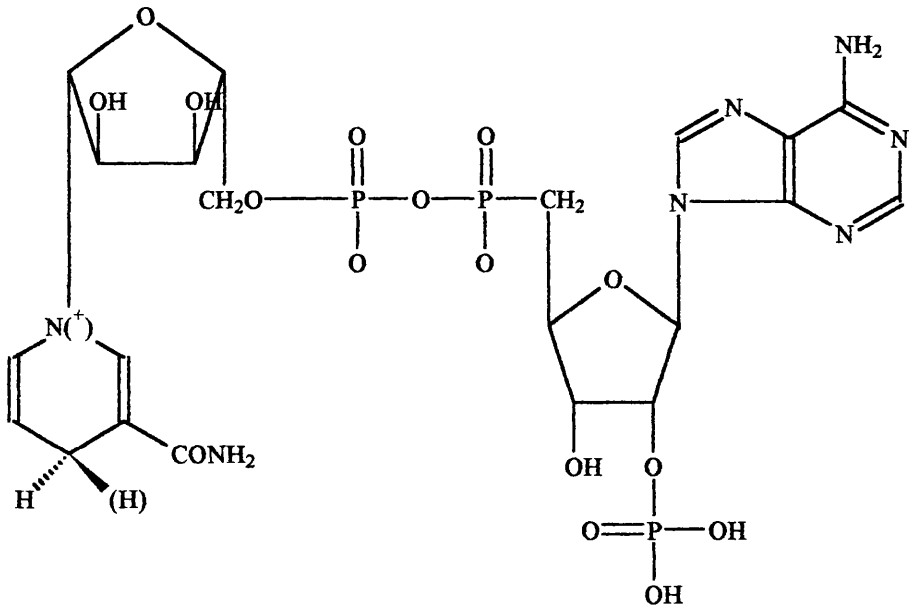


Figure 3.1. The chemical structure of NADP(H)

3.1.2 Non enzymatic methods

By far the most common non-enzymatic method is the use of electrochemistry to reduce the oxidised form of the cofactor back to the active reduced form (Devaux-Basseguy *et al*, 1997). On paper this technique appears to be the best solution to the problem as there are no byproducts produced or requirements for additional reagents. A number of problems hinder the application of this technique, for example the potential required for regeneration has to be high, so electrode fouling is common. Also direct reduction of the cofactor is not totally selective and can produce an inactive dimeric form $(NADP)_2$. Examples also exist where NADP(H) is replaced by an artificial electron carrier such as methyl viologen that is easier to electrochemically reduce. It has been shown with many examples that the kinetics of such systems are rate limiting unless high concentrations of dye are present (in some cases up to 100 times more concentrated than the equivalent "natural" cofactor) making product recovery and isolation more problematic (Lee and Whitesides, 1985).

Other quoted methods include chemical and photochemical reduction. These methods are inherently non-selective for the production of enzymatically active cofactor and can cause damage to the target enzyme (Chenault *et al*, 1988).

3.1.3 Enzymatic methods

By far the most common recycling methods published in literature consist of a coupled enzyme system that produces reduced cofactor and can be represented along with the monooxygenase reaction in **Figure 3.2**. Enzymatic methods are generally accepted as the only technique capable of producing enzymatically active cofactor over many cycles (Chenault and Whitesides, 1987). One of the most widely quoted

methods uses glucose-6-phosphate dehydrogenase producing gluconate-6-phosphate (Pasta *et al,* 1995). Other enzymes described include several glucose dehydrogenases, various alcohol dehydrogenases and a mammalian malate dehydrogenase (Wong and Whitesides, 1982; Wong *et al,* 1985). With alcohol dehydrogenases it is possible in the case of the enzymatic Baeyer-Villiger reaction to use a "closed loop" method; a two step conversion is applied to the alcohol derivative of the ketone (Gagnon *et al,* 1994), as shown in **Figure 3.3.**. The advantage of this method is that you do not require an extra, sacrificial substrate however the substrate specificity of the alcohol dehydrogenase is not always very wide (Stewart, 1998).

There are generally more NAD(H) requiring enzymes commercially available than those using NADPH, but all seem to suffer from non-competitive product inhibition (Lee and Whitesides, 1985; Chenault and Whitesides, 1988) that in many cases is the limitation in generating high TTN and STY. The enzyme of choice for NADH recycle is widely accepted to be formate dehydrogenase (FDH) that produces carbon dioxide. This can escape as a gas thereby removing product inhibition. This has been demonstrated in the production of L-*tert*-Leucine (Kragl *et al,* 1993). Although not commercially available at present a mutant FDH from *Pseudomonas sp.101* has been shown to oxidise NADPH (Seelbach *et al,* 1996) and has been used with CHMO on a 30mL scale(Rissom *et al,* 1997). Enzymes are usually specific to either phosphorilated or non-phosphorilated forms, believed to be an evolutionary strategy so that catabolism and anabolism can be kept metabolically separate (Zubay *et al,* 1995).

There are a number of technical problems that arise when applying these technologies, chiefly conflicting enzyme operating requirements make optimisation and reducing the amount of cofactor to within economically realistic limits difficult. Therefore generally NADPH requiring biocatalysts tend to be operated as whole cell reactions, even with the traditional disadvantages of this catalyst type.

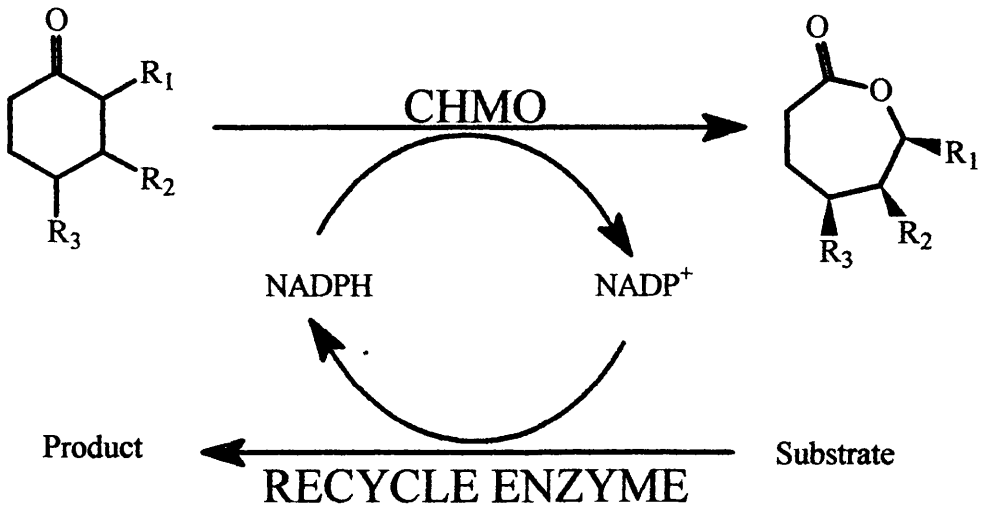


Figure 3.2. The recycling of NADPH using a linked enzyme system

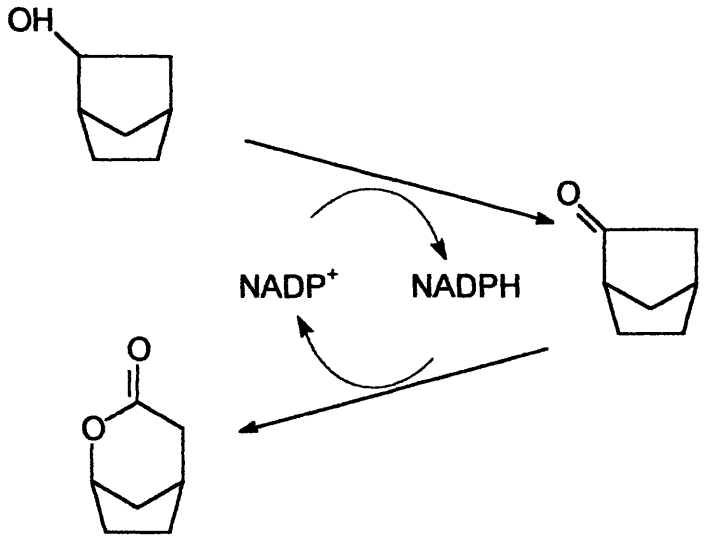


Figure 3.3. The recycling of NADPH using a two step conversion on an alcohol derivative of a cyclic ketone. (Gagnon *et al*, 1995[2])

3.2 Materials and Methods

3.2.1 Source of enzymes

Glucose dehydrogenase from *Thermoplasma acidophilum* (TAGDH) and alcohol dehydrogenase from *Thermoanaerobacter brockii* (TBADH) were purchased from Sigma (Dorset, UK) as a suspension in 3M $(NH_4)_2SO_4$ and a powder respectively. TBADH was found to have a specific activity of 1.15 Umg^{-1} solid (3.33 Umg^{-1} protein) and TAGDH 68 Umg^{-1} protein.

3.2.2 TBADH activity assay

Assays were carried out according to a literature method (Lamed and Zeikus, 1981). To a final volume of 1mL, 0.1M Tris buffer (pH7.8) containing 0.5mM $NADP^+$ and 150mM 2-propanol were pre-incubated at 30°C for five minutes, the reaction started by the addition of enzyme (0.05-0.15 UmL^{-1}). Activity was calculated as previously described in section 2.2.4.2.

3.2.3 TAGDH activity assay

The procedure used (Smith *et al,* 1989) is identical to that described for TBADH, however phosphate buffer (pH7, 50mM) was used with 0.4mM $NADP^+$ and 50mM glucose as enzyme substrates.

3.3 Economic comparison between available enzymes

Of those enzymes commercially available that can react with nicotinamide cofactors the majority react with the non-phosphorilated form, NAD(H). This severely limits the choice of enzyme for CHMO based reactions. Four commercially available enzymes have received the most attention in this type of application and for this reason were chosen for further investigation - glucose dehydrogenase from *Thermoplasma acidophilum*, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, malate dehydrogenase from chicken liver and alcohol (propanol) dehydrogenase from *Thermoanaerobacter brockii*. Initially the relative costs of using these four enzymes was compared, by assuming the use of one mole of substrate and 1000U of quoted enzyme activity. All economic data was obtained from the same source (Sigma Catalogue, 1999) and as such does not represent the cheapest price of each enzyme and/or substrate that may be obtainable from other suppliers, but for the purpose of this study it remains a useful benchmark. These data are shown in **Table 3.1**.

Enzyme	Cost of 1mol substrate, £	Cost of 1000U enzyme activity, £	Total cost, £
GDH (*T.acidophilum*)	57	99	156
G6PDH (*L.mesenteroides*)	3500	19	3519
ADH (*T.brockii*)	5	360	365
MDH (avian liver)	30	1360	1390

Table 3.1 Relative cost of four NADPH dependent dehydrogenases

It can be seen from the relative costs that both malate dehydrogenase and glucose-6-phosphate dehydrogenase would be an expensive system to operate; the former because of high enzyme cost and the latter because of the expensive phosphorilated

substrate required. The costs of the other two enzyme systems were comparable, and so were jointly used in further investigations to make a decision between them, the focus chiefly on which one of the enzymes (if either) is compatible with the conditions defined in the previous chapter. Therefore the focus for experimentation lies in the intrinsic activities of the enzymes in the region of pH, substrate concentration and cofactor concentration that CHMO requires in order to function efficiently. Also the potential of the second enzyme's substrate and product to interfere with the reaction will have to be considered.

Such a systematic approach to the selection of the most appropriate enzyme for cofactor recycling has not previously been applied to a *specific* system, although the advantages and disadvantages of many of the potential recycle enzymes has been discussed in general terms (Chenault and Whitesides, 1987). This chapter aims to address this gap in cofactor recycling processes using the CHMO reaction as a model.

3.4 Enzymes for detailed studies

3.4.1 TBADH

*Thermoanaerobacter (*formerly *Thermoanaerobium) brockii* was originally isolated from a thermal spring (Zeikus *et al*, 1979; Lamed and Zeikus 1980) and the $NADP^+$ dependent alcohol dehydrogenase first described soon after (Lamed and Zeikus, 1981). TBADH has been extensively studied, being remarkably resistant to both high temperatures and solvents. It is a homotetrameric protein with subunits of 352 amino acids with a catalytically important zinc atom per molecule (Peretz and Burstein, 1989). Increased specific activity has been demonstrated by substituting the zinc ion with either manganese or cobalt (Bogin *et al*, 1997). A crystal structure has been elucidated (Korkhin *et al*, 1998) and the gene cloned into *E.coli* producing enzyme

titres 30 fold greater than the host enzyme, accounting for 30% of the total cellular protein (Peretz *et al*, 1997).

Enzyme stability has been demonstrated to be extremely high, particularly in immobilised form, continuous operation for a month has been achieved with no loss of activity (Keinan *et al*, 1986). High stability is believed in part to be due to the extremely compact nature of the protein (Al-Kassim and Tsai, 1989). The substrate range is broad, highest activity shown with secondary alcohols i.e. 2-propanol, followed by primary alcohols and cyclic alcohols (Lamed and Zeikus, 1981). Reversibility has been demonstrated; the reduction of ketones to optically enriched alcohols can be achieved given the correct choice of reaction conditions (Peretz *et al*, 1993; Kragl, 1996).

As well as recycling the cofactor by using a sacrificial substrate, TBADH can potentially perform the recycling by oxidising the alcohol derivative of 4-MCH and 2-HTP to the ketone, allowing CHMO to oxidise this ketone through to lactone, as has been shown previously in **Figure 3.3**.

3.4.2 TAGDH

T. acidophilum is also a thermophillic bacterium. TAGDH is an enzyme involved in a modification of the glycolytic pathway found in the host organism. It has been shown to be thermally resistant and stable in the presence of a variety of organic solvents (Smith *et al*, 1989). The crystal structure is available (John *et al*, 1994) and shows structural homology to horse liver alcohol dehydrogenase.

3.5 Characterisation of the enzymes

3.5.1 pH/activity profiles

The pH/activity profile for TBADH and TAGDH using the standard assay methods was investigated with particular attention to the overlap seen with CHMO. The pH activity profiles are shown in **Figure 3.4**. The data are normalised to percentages of the maximum seen, to show more clearly the relative profiles. This is necessary as the specific activity of CHMO is a maximum of only 0.2 Umg^{-1} at pH9 compared to TAGDH which has a maximum of 68 Umg^{-1}. The maxima observed agree well with literature, observed values for TBADH and TAGDH are pH 7 and 8; literature (Lamed and Zeikus, 1981; Smith *et al*, 1989) quotes pH 7and 7.8 respectively.

The overlap between the two dehydrogenases and CHMO are very different. TBADH has a useful window of near maximal activity between pH 8 and 9.5, precisely in the region of high CHMO activity. TAGDH however has high activity at pH 7 dropping off sharply until there is virtually no activity at pH 9.

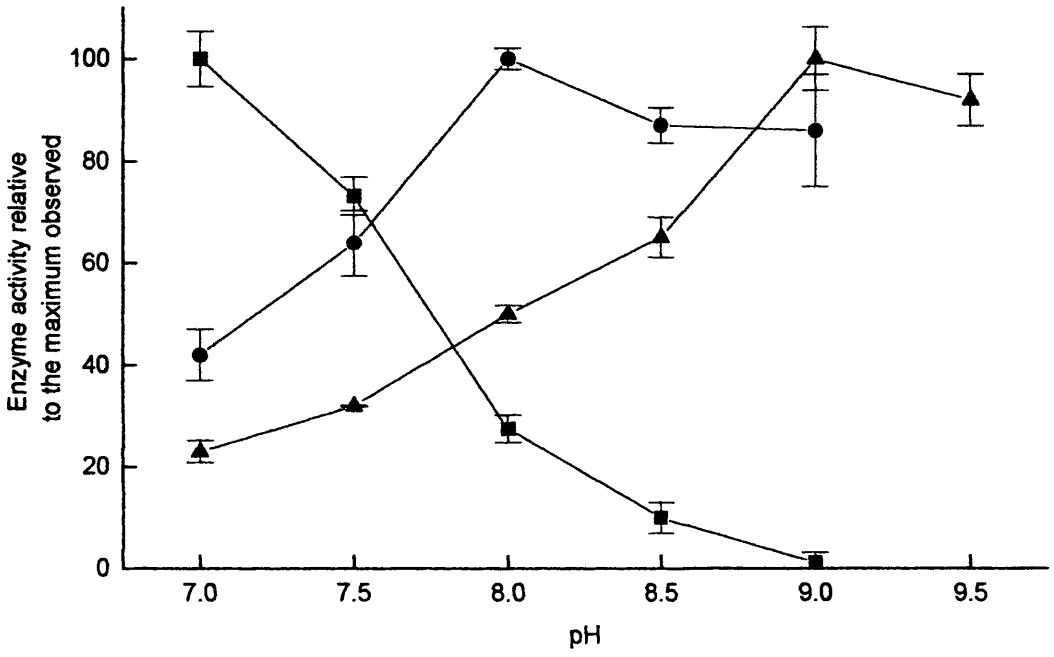


Figure 3.4. pH/activity profiles for TBADH(●), TAGDH(■), and CHMO(▲).

3.5.2 Activity of TBADH with 4-methyl cyclohexanol

The use of TBADH potentially allows the regeneration of NADPH using the closed loop method discussed in section 3.1.3 for both 4-MCH and 2-HCP systems. No commercial source of the alcohol derivative of 2-HCP (2-hexyl cyclopentanol) could be located. However the alcohol derivative of 4-MCH is commercially available (Sigma, Dorset, UK) and was used to investigate this design variant.

TBADH activity was measured by using an arbitrary concentration of enzyme ($2mgml^{-1}$) and assayed under standard conditions. The activity with 2-propanol was compared to that achieved with a range of concentrations of 4-methyl cyclohexanol from 10-150mM.

In all cases the specific activity found with 4-methyl cyclohexanol was less than 0.2% of that found using 2-propanol (<0.006 Umg^{-1} protein) and as such this technique could not be given any further consideration as the amounts of enzyme required would be large. It is probable that this would be the case if 2-hexyl cyclopentanol were employed. TBADH seems unable to process larger, substituted cyclic alcohols to any reasonable degree of specific activity (Lamed and Zeikus, 1981).

3.5.3 Substrate and product inhibition

For substrate inhibition it has been shown that TBADH is not adversely affected kinetically by 2-propanol concentrations up to 300mM (Lamed and Zeikus, 1981) and TAGDH has a low affinity for glucose; K_m = 10mM (Smith *et al,* 1989); and therefore no inhibition is likely to be seen.

It has been reported that the majority of dehydrogenase enzymes suffer from high levels of product inhibition (Chenault and Whitesides, 1988), being one reason

proposed for poor system performance in cofactor regeneration processes (Lee and Whitesides, 1985). Both enzymes were assayed for activity in the presence of varying quantities of gluconate (TAGDH) and acetone (TBADH) immediately following the product addition at the pH maxima for each enzyme. This can be seen in **Figure 3.5** The data have been normalised to the activity at zero product concentration as before in order to draw a comparison between the enzymes.

As predicted in the literature the effect of product inhibition is pronounced, for TAGDH the reaction has effectively been arrested at a concentration of only 30mM, similarly for TBADH the activity is less than 15% of that seen without any acetone present at a product concentration of 25mM.

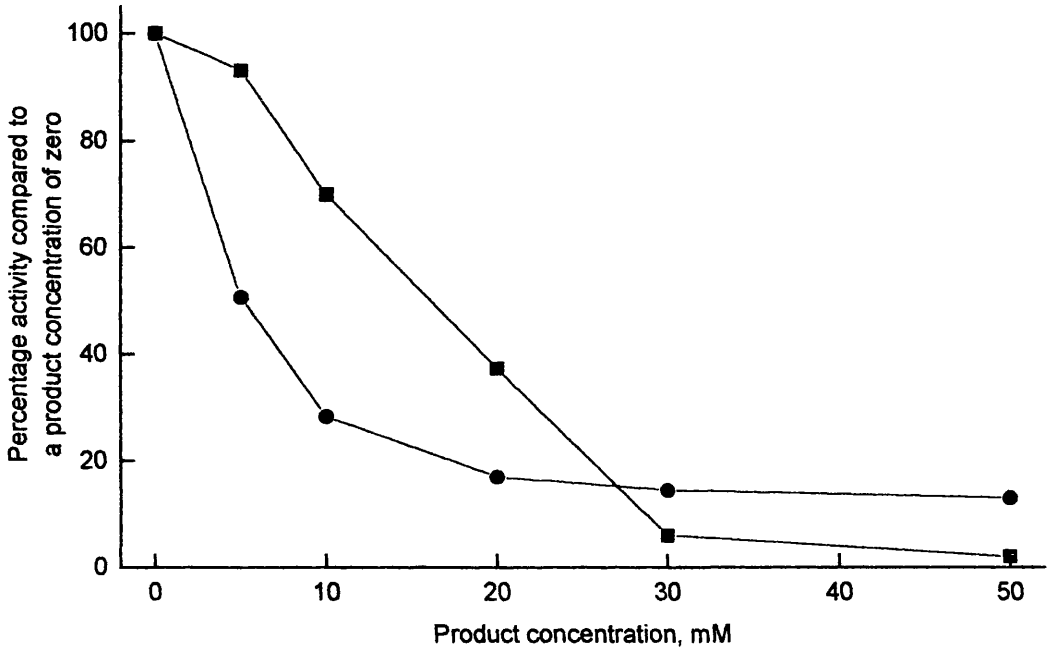


Figure 3.5. The effect of product inhibition on TBADH (●) and TAGDH (■)

3.5.4 Substrate and product toxicity

Although no substrate inhibition was observed it is possible that longer term exposure to substrates (and products) may in some way denature the enzymes similar to the effect observed with CHMO when incubated with 5-MOP at a concentration above 50mM. This effect would appear to be unlikely due to the reported stability of these catalysts but nevertheless any toxic effect could have a bearing on the eventual enzyme choice.

Specific activity measurements were taken immediately post addition of the component and at 24 hours. The residual activities (compared to a control) are summarised below, in **Table 3.2.**

Component	% remaining for TBADH	% remaining for TAGDH
glucose (300mM)	-	94.8
gluconate (25mM)	-	95
2-propanol (50mM)	99	
acetone (50mM)	102	

Table 3.2. Residual dehydrogenase activities

TBADH is unaffected by it's substrate and product even at levels exceeding those that would be likely within a process. A similar pattern emerges for TAGDH, retaining virtually all the activity initially present.

3.5.5 Effect of model reaction on TBADH and TAGDH

An investigation was undertaken to see if the presence of the model ketones and lactones would affect either of the candidate enzymes by inhibiting their catalytic action or irreversibly lowering their activity (as in section 3.5.4).

Incubation of the enzymes ($2UmL^{-1}$) for 24 hours in the presence of these compounds produced the residual activities (compared to a control) shown in **Table 3.3**. The concentrations used were representative of the maximum that the catalysts would be exposed to if used in conjunction with CHMO.

Component	% of initial enzyme activity remaining for TBADH	% of initial enzyme activity remaining for TAGDH
4-MCH (50mM)	96.4	53.7
5-MOP (200mM)	99.1	97.8
2-HCP (2.5mM)	98.0	99.4
6-HTPO (5mM)	97.9	96.5

Table 3.3. Residual dehydrogenase activities after exposure to the model reaction components

The only significant toxic effect is that of 4-MCH to TAGDH, where half the activity is seen to have been lost after 24 hours incubation. Kinetically neither the ketones or lactones had an effect on the dehydrogenase enzymes up to the concentrations listed in **Table 3.3.**

3.5.6 Effect on CHMO due to TBADH/TAGDH reactants/products

The effect of exposing CHMO to the substrates and products of TAGDH and TBADH was investigated in the region 0-100mM (2-propanol and glucose) and 0-50 mM (acetone and gluconate). Acetone, glucose and gluconate showed neither kinetic inhibition nor a toxic effect over 24 hours (compared to a control), whereas 2-propanol showed increasing inhibition as a function of concentration. This is shown in **Figure 3.6.**

Exposure of CHMO to the same concentrations of 2-propanol for 24 hours showed that the effect of 2-propanol was a form of kinetic inhibition and was not irreversibly denaturing the enzyme.

3.5.7 Affinity of TBADH and TAGDH for $NADP^+$

The cost of the cofactor often dominates the overall cost of operating such processes and therefore the intrinsic kinetics of the enzyme will have a bearing on the concentration required to produce a satisfactory reaction rate. The behaviour of both enzymes toward $NADP^+$ has been described previously. In both cases they follow Michaelis-Menton kinetics (Smith *et al*, 1989; Lamed and Zeikus, 1981). TAGDH having a K_m of 113μM and TBADH a K_m of 13μM. **Figure 3.7** illustrates the reaction rate/cofactor concentration profile for both enzymes, reconstructed from these published data.

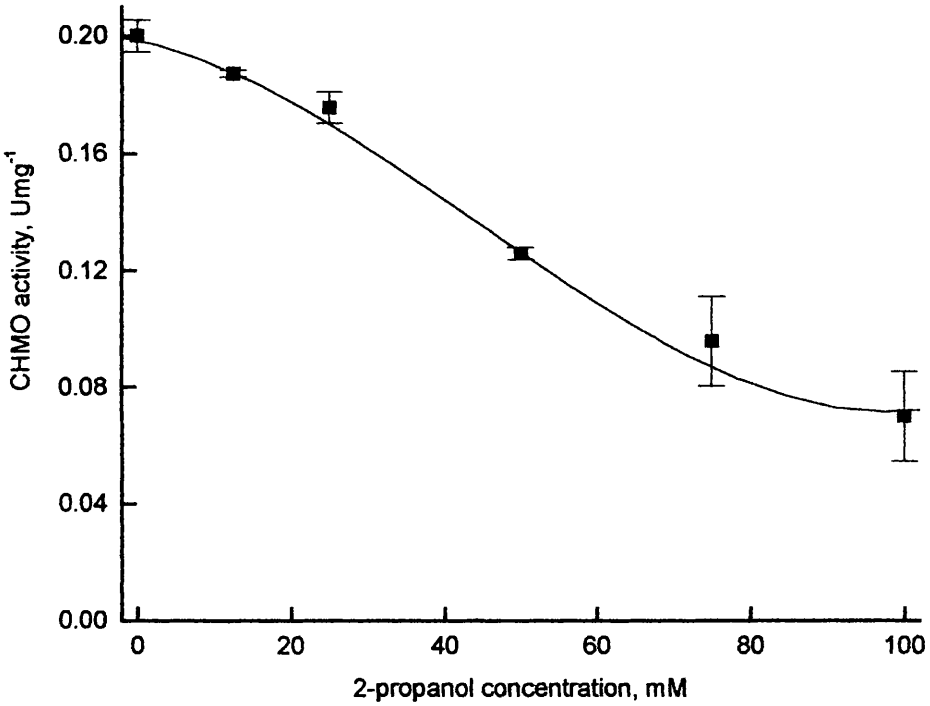


Figure 3.6. The effect of 2-propanol on CHMO activity(■)

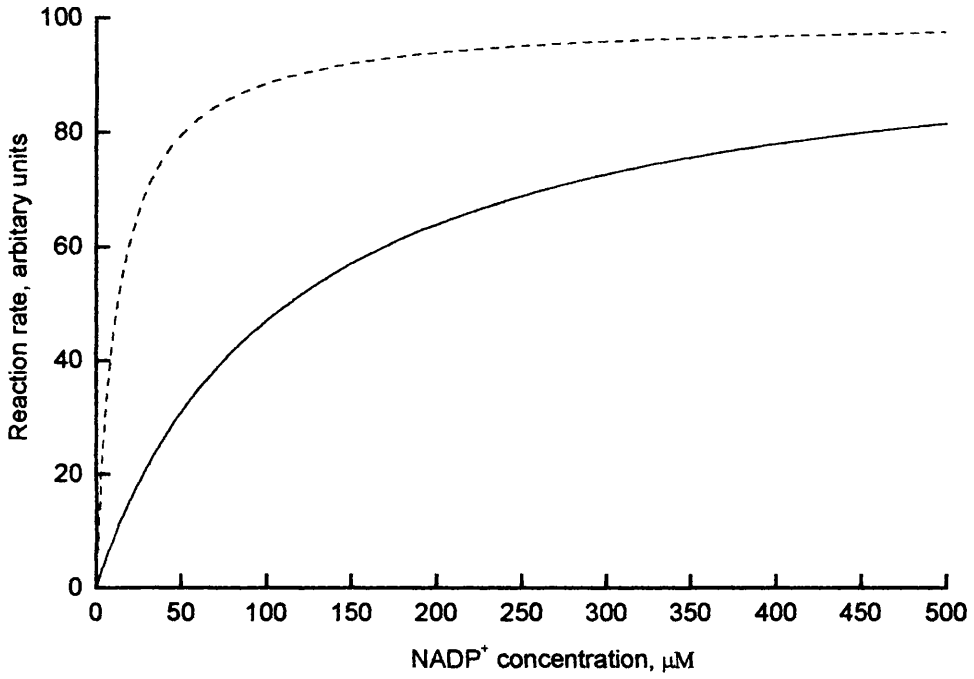


Figure 3.7 Reaction rate as a function of cofactor concentration for TBADH(---) and TAGDH(—), (reconstructed from the work of Lamed and Zeikus, 1981 and Smith *et al*, 1989).

3.6 Discussion

3.6.1 Advantages and disadvantages of TBADH and TAGDH

The two enzymes under investigation would seem on paper to both be suitable for this application; both are relatively inexpensive, using readily available substrates as well as being quoted as possessing excellent stability under a range of conditions. However the experiments performed indicate that their properties under conditions suitable for effective 5-MOP synthesis by CHMO differ considerably and illustrates in more general terms the need for systematic investigations in such cases where there is the option to pursue more than one recycling strategy.

TBADH allows two potential process variations, the use of a sacrificial substrate (**Figure 3.2**) or a two step conversion beginning with alcohol derivative of either 4-MCH or 2-HCP (**Figure 3.3**). The latter process has several attractive features; firstly it would remove substrate inhibition from CHMO by continuous generation of the ketone at a controlled rate and secondly would remove product inhibition from TBADH by the action of CHMO. In practice this is not possible due to the low specific activity shown toward 4-methyl cyclohexanol. This is probably due to the size of the molecule, causing steric hindrance in gaining access to the active site of TBADH (Lamed and Zeikus, 1981). A similar effect is likely to be seen with 2-hexyl cyclopentanol. Most industrially useful molecules tend to be large. Therefore this process variant is not a general solution to supplying CHMO with NADPH, whereas the use of 2-propanol is unaffected by the choice of ketone substrate in terms of NADPH productivity and is more generic.

The pH profiles of the two dehydrogenases are very different from each other, TAGDH preferring neutral conditions and becoming less active in alkaline solution, almost no activity is seen above pH8.5. TBADH has a broader activity profile with a maximum activity at approximately pH 8 showing high activity in the region pH 8-9.

Activity drops off rapidly toward pH7. The overlap with CHMO is thus also variable; high specific activity (and high stability) for CHMO is between pH 8 and pH10, with very little activity under neutral conditions. The pH compromise for lactone stability and CHMO stability/activity has previously been defined as pH 8 in chapter 2; thus TBADH performs better under these criteria, TAGDH showing a specific activity only 25% of it's potential maximum at pH8.

In this specific case there is some overlap of activities for all three enzymes, there is no pH in the region considered where there is no realistic overlap. It is quite possible that there may be a case when the enzymes under consideration in an analogous system would have much narrower pH/activity bands such that there is absolutely no overlap and it would be impossible for both to have activity in the same reactor; the pH trend is thus an extremely important consideration when choosing a recycle enzyme.

TBADH shows a much higher affinity for the cofactor than TGADH, the K_m values are 0.013mM and 0.113mM respectively, the difference being almost an order of magnitude (Smith *et al*, 1989; Lamed and Zeikus, 1981), this becomes readily apparent when the original data is reconstructed (shown in **Figure 3.7**). The analysis of the figure indicates that the use of TAGDH would require much higher concentrations of cofactor to produce high reaction rates, making high values of TTN unrealistic. Higher concentrations of cofactor would also potentially cause problems in lactone recovery after the reaction is complete, it is important to keep the aqueous phase as free of 'impurities' as possible when conducting downstream recovery. The requirement for TTN would be less restrictive if the non-phosphorilated form NADH was accepted by CHMO as it is more stable and much cheaper than NADPH, there are also many more NADH accepting enzymes commercially available.

The consequence of using CHMO in an industrial process under high substrate and product concentrations in that inhibition is seen by both. Dehydrogenases in general are also known to be particularly affected by their products (Chenault and Whitesides, 1988). This was found to be the case for both enzymes, high levels of

inhibition were seen in the case of TAGDH to such an extent that specific activity was only 5% of it's potential at a gluconate concentration of 30mM effectively limiting lactone concentrations achievable to this amount because of the stoichiometry of the reaction. The effect of acetone on TBADH follows a similar pattern; activity falls to a level of 15% in the region 30-50mM but seems to level off at this point. Although inhibition is observed by the products, the effect was not denaturation in both cases as longer exposure did not result in accelerated denaturation rates. TBADH and TAGDH were also unaffected by 2-propanol and glucose at concentrations of 50mM and 300mM respectively.

The phenomenon of inhibition would appear to apparently limit the lactone concentration achievable to levels that are commercially unrealistic without the addition of large amounts of dehydrogenase. The only way to solve this is to remove the product from the system by ISPR. For removal of gluconate there are two problems. Firstly gluconate could realistically only be removed by adsorption onto a solid support. Secondly the gluconate is a lactone and as such any ISPR technique may not be very selective in distinguishing between 5-MOP/6-HTPO and gluconate making highly pure lactone formulations expensive and difficult to achieve. It has been suggested that the dehydrogenation reaction of glucose could be pulled to completion because the gluconate product itself is prone to non-enzymatic hydrolysis in aqueous solution (Stewart, 1998). It is unclear however if the rate of degradation is satisfactory to prevent gluconate accumulation or if the hydrolytic product is itself inhibitory to TAGDH.

The removal of acetone from solution is much easier and would integrate well with the proposed process design. The supply of oxygen to CHMO will be via sparging of air at 30°C where acetone has a vapour pressure of 283mmHg (boiling point 56.2°C) (Marsden, 1963) showing high volatility. The effect of this would be to perform a gas stripping of the acetone out of the aqueous phase at a rapid rate such that it is likely to keep the instantaneous concentration very low. In this respect TBADH would be much simpler to use than TAGDH, requiring no modification of the process design other than the addition of a downstream solvent trap.

Toxicity of the model ketones and lactones on both enzymes was shown to be largely non existent however TAGDH underwent significant denaturation in the presence of 50mM 4-MCH, loosing half it's activity in 24 hours compared to a control. No kinetic inhibition was seen against either enzyme at the levels used. The toxic effect was unexpected due the reported stable nature of the enzyme; possibly the reason TBADH is less affected is that it processes alcohols and ketones and so may naturally be more resistant to higher concentrations of ketone functionalities than TAGDH.

It was found that 2-propanol exclusively was inhibitory to CHMO at low concentrations, prompting an investigation into the time related stability to determine if the effect was in fact coupled to denaturation. No toxic effect could be seen after 24 hours, all the samples retained approximately the same activity they began with. This inhibitory effect does not prompt any change in the proposed process as 4-MCH would be presented to the enzyme by controlled feeding, 2-propanol could simply be added in the same way by preparing a mixture of the two in the correct molar proportion and feeding as before. With 2-HCP the substrate will form a second phase, 2-propanol can simply be fed separately although it may partition to some degree into the organic phase.

The issues raised within these specific studies have highlighted the different suitability of the two enzymes considered. **Table 3.4** lists the key points and which enzyme best fits with the use of CHMO. It is evident that TBADH is more suitable from these criteria and so will be used exclusively for further work.

Experimental criteria	TBADH	TAGDH
pH/activity profile overlap with CHMO	○	●
Inhibition by product	●	●
Ease of ISPR	○	●
Toxicity/inhibition on CHMO	●	○
Affinity for $NADP^+$	○	●
Effect of CHMO on enzyme action	○	●
Overall suitability	○	●

Table 3.4. Suitability of TBADH and TAGDH to recycle NADPH for CHMO based syntheses of 5-MOP and 6-HTPO. (●) = unfavourable; (○) = favourable

3.6.2 General remarks

This investigation has highlighted the significant experiments that shaped the choice between the two enzymes. These questions are general to the problem of cofactor recycling using a second enzyme, particularly the effect of product inhibition on dehydrogenases. In this example it is proposed that it will be overcome by using the volatility of acetone to remove it from the system. A similar effect has been used in the synthesis of L-*tert*-leucine (Kragl *et al,* 1993) by recycling NADH using formate dehydrogenase that produces carbon dioxide which exits as a gas. The important questions that need to be asked of a potential recycle enzyme can be listed so that the screening of a number of candidates can be quickly achieved and the unsuitable ones rapidly identified. The proposed key questions are:

- Cost of sacrificial substrate (in the context of the synthetic product value).
- pH/activity profile compatability.
- Effect of product inhibition/ease of ISPR.
- Affinity for cofactor (K_m) - defines the lowest reasonable [cofactor].
- Toxic or inhibitory effect on the synthetic reaction
- Effect of the synthetic reaction on recycle enzyme

Using this list it becomes quickly apparent which of the enzymes will be the most suited to the desired application, and those highly unsuited rapidly discarded from future unnecessary consideration. No attempt is made to assign priority to any of the questions over the others as in order to make a confident judgment between several possible enzymes the data relating to all six questions should be available. For example in the introduction to this chapter is was shown that FDH is widely accepted to be enzyme of choice for NADH based reactions due to the formation of carbon dioxide. These studies show that the kinetics shown against the cofactor are of crucial importance. The mutated form of FDH that accepts NADPH has a high K_m of 0.4mM (Rissom *et al*, 1997), over 30 times higher than TBADH.

The criteria for suitability have been based on the proposed process from chapter 2, itself a product of the proposed structured design methodology. In this case the design from chapter 2 has for the purposes of suitability been considered to be fixed and inflexible, the recycle enzymes had in effect to fit in with this design. A more effective technique would be to identify if a isolated biocatalyst would be needed early in the design procedure and consider the recycle of the cofactor as an integral part of the structured design approach. It is clear that the structured design procedure fails to take this into account at present.

3.7 Summary

In this chapter the selection of a method to recycle NADPH to CHMO was considered. Non-enzymatic methods were not included, the choice being between two dehydrogenase enzymes. The ability of these enzymes to recycle NADPH effectively was investigated in relation to the proposed design of a CHMO based process. It became evident that TBADH fitted the criteria most effectively and becomes the choice of recycle method for further studies.

A proposed checklist of important issues that need to be addressed in selection of cofactor regeneration was defined for similar future processes that has generic applicability. It was also proposed that in the case of the evaluation of future redox biocatalysis where the requirement for cofactors is critical the definition of recycle method (whole cell, second enzyme) needs to be made early on and the selection of the appropriate enzyme must be made *within* the process design.

The application of TBADH to the process raises issues of process optimisation in that the system contains two catalysts that must both work effectively whilst minimising the requirement for cofactor. The next chapter will consider this problem both within the context of CHMO biocatalysis and in more general terms for redox biocatalysts.

4.0 Modelling of the CHMO/TBADH reaction

4.1 Introduction

In the previous chapter the selection of TBADH as the method to recycle NADPH was made and justified in the context of a structured design methodology. A proposal was made of the important features of a potential recycle enzyme that had to be satisfied in order for it to be considered a useful method. Having established these parameters and chosen TBADH for this application it is necessary to identify the operational region that will be the most productive for both enzymes, and therefore the optimal solution of producing lactones in high titres whilst minimising the use of cofactor.

It is the aim of this chapter to use quantitative techniques to examine the effect of changing process variables in this system in order to locate the region of most efficient operation for a number of criteria (enzyme efficiency, low cofactor requirement); through the solution of the equations describing the system. Secondly having identified the region of operation it will then be possible to quantify the possible reactor modes identified in chapter 2; in an attempt to verify the conclusions reached and to see if the structured design methodology correctly guided the process design procedure to the correct conclusions from the constraints identified.

Finally the possibility of combining the heuristic and qualitative structured design approach with quantitative process models will be discussed; to produce more effective design procedures for application to other systems.

4.2 Description of the mathematical model

4.2.1 General issues

The use of linearised differential rate equations in reaction engineering is a valuable tool for example to be able to design non isothermal plug flow reactors for a particular conversion (Fogler, 1992) or investigate the repression of side reactions using different reactor configurations (Levenspiel, 1999). In the first example the rate equation of the particular reaction will change with increasing temperature from external heating, the reaction rate being a function of distance travelled along the reactor.

This system, where two catalysts (CHMO and TBADH) are competing for a limited resource (cofactor) and the substrate of one is the product of the other, lends itself to be investigated mathematically by simultaneous solution of the rate equations of the two enzymes. Modelling of enzymatic reactions has previously been demonstrated, for example with the synthesis of sorbitol (Ikemi and Ishimatsu, 1989) and L-*tert*-leucine (Kragl *et al*, 1996).

In this chapter a simple unsteady state model of the system should allow the optimisation of the process in terms of enzyme use, total cofactor needed for both satisfactory product yield and TTN. The model will also be used to define the steady state proportions of the two forms of the cofactor (essential in identifying the useful pH range for cofactor stability) which is a strong function of solution pH (Chenault and Whitesides, 1987; Lowry *et al*, 1961).

In this biocatalytic system the reaction occurs in a stirred tank, the rate equations of the competing enzymes change as a function of time as the proportions of the two cofactor forms vary. An added complication occurs as both enzymes suffer inhibition

caused by the form of the cofactor they produce (Ryerson, *et al*, 1982; Ford *et al*, 1993).

4.2.2 Assumptions

The simulation of the interaction between CHMO and TBADH is assumed to occur with no oxygen limitation to CHMO such that the monooxygenase is considered to have only two substrates (ketone and NADPH). This was assumed to occur as no value for oxygen K_m is available, and it has been suggested (Walsh and Chen, 1988) that the affinity for oxygen is high enough to consider the reaction rate to be largely independent of oxygen concentration, providing it is continually supplied. A sparged stirred tank reactor would satisfy this criterion.

For simplification purposes the enzymes are not considered to degrade over time, as calculating the degradation kinetics, with many factors contributing, is difficult and prone to errors. With an immobilised biocatalyst this assumption is likely to be correct over the limited timescales considered within the scope of this investigation.

The 4-MCH/5-MOP system will be used solely in this chapter, partly due to the inhibitory nature of both molecules on CHMO (a common feature of many enzymes) making for a more general investigation into cofactor recycling systems and also due to the availability in literature (Walsh and Chen, 1988) of the kinetics describing the behaviour of CHMO with this ketone. The drawback is to (potentially) limit the usefulness of this technique to asymmetric synthesis, although it should be possible to draw some general conclusions.

4.2.3 Design Equations

4.2.3.1 Rate law

Both enzymes have been shown to react following a sequentially ordered mechanism such that the substrates must bind in a specified order for reaction to occur. In the case of CHMO the order of addition is NADPH, oxygen and ketone (Ryerson, *et al*, 1982). For TBADH it is $NADP^+$ followed by 2-propanol (Oestreicher *et al*, 1996). The value of Ks_B is approximated to $Km_{B(app)}$ as this parameter is not published for CHMO. Previous work (Zubay *et al*, 1995) has established that the rate law of a two substrate sequential ordered mechanism is as follows:

$$Rate = \frac{V_{max}[A][B]}{Km_B Km_A + Km_A[B] + Km_{B(app)}[A] + [A][B]} \qquad (8)$$

$$Km_{B(app)} = Km_B\left(1 + \frac{[I]}{Ki}\right) \qquad (9)$$

Table 4.1 shows the relevant kinetic data needed to solve this equation for the two enzymes in this system (Ryerson, *et al*, 1982; Pereira *et al*, 1994; Walsh and Chen, 1988).

Constant	TBADH		CHMO	
	Component	Value, mM	Component	Value, mM
Km_A	2-propanol	0.341	4-MCH	0.016
Km_B	$NADP^+$	0.013	NADPH	0.020
K_i	NADPH	0.023	$NADP^+$	0.038

Table 4.1. Kinetic parameters for TBADH and CHMO

4.2.3.2 Empirical rate equations

Previous studies (Pereira *et al*, 1994) have shown that TBADH suffers product inhibition, confirmed in the previous chapter. CHMO also is characterised by substrate and product inhibition. In order to incorporate these factors into the model it was necessary to derive equations governing the rate of reaction of both enzymes as a function of concentration. The four effects not described in equation (8) are

1) Inhibition of TBADH by acetone (Figure 3.5)
2) Inhibition of CHMO by 2-propanol (Figure 3.6)
3) Inhibition of CHMO by 4-MCH (Table 2.4)
4) Inhibition of CHMO by 5-MOP (Figure 2.8)

Using the mathematical function fitting facility of Microcal^{TM}Origin (See Appendix 1) data manipulation software a mathematical relationship was derived for each condition, relating the percentage of the maximum enzyme activity (at zero concentration of the particular component) to the actual concentration. Excellent agreement was found between the function and the graphical trend in all four cases.

The equations are shown below for the different conditions. It should be noted that the mathematical functions derived are purely empirical and represent the best fit to the data the software could produce.

$$1)\ \text{Percentage activity} = 13.6 + 86.5e^{-0.17[\text{acetone}]} \quad (10)$$

$$2)\ \text{Percentage activity} = 100 - 0.37[propanol] - 0.01[propanol]^2 + 0.000075[propanol]^3 \quad (11)$$

$$3)\ \text{Percentage activity} = 37.5 + 68.3e^{-0.03[4\text{-MCH}]} \quad (12)$$

$$4)\ \text{Percentage activity} = -30.9 + 103.6e^{-0.003[5\text{-MOP}]} + 27e^{-0.063[5-MOP]} \quad (13)$$

4.2.3.3 Acetone removal

The reaction rate of TBADH is dependent on the aqueous acetone concentration, the removal from solution will be by a gas stripping mechanism due to aeration. In order to accurately model this effect the mass transfer characteristics of the system need to be defined. The rate of mass transfer is governed by the interfacial area of the gas/liquid interface and the mass transfer coefficient. In considering the transfer of oxygen to solution it is convention to define the product of these two values, the volumetric mass transfer coefficient, k_La (Stanbury and Whittaker, 1995). An analogous constant can be considered for acetone transfer from solution to the gas phase, in the opposite direction to oxygen.

Using a 2L LH series fermenter containing a 20mM acetone solution in Tris-HCL buffer (50mM, pH 8, 30°C) agitated at 500rpm and aerated at 2vvm, samples of the aqueous phase were taken periodically and analysed by GC. Using equation (14) below the value of $k_La_{(acetone)}$ could be identified from the gradient of a graph of log[acetone] as a function of time (shown as **Figure 4.1**), assuming the gaseous

acetone concentration approximates to zero compared to the concentration in solution.

$$\frac{-d[\text{acetone}]}{dt} = k_L a_{(acetone)}[\text{acetone}] \qquad (14)$$

A $k_L a_{(acetone)}$ value of $0.024 min^{-1}$ was calculated using the condition stated above. This allows the use of equation (14) in the model.

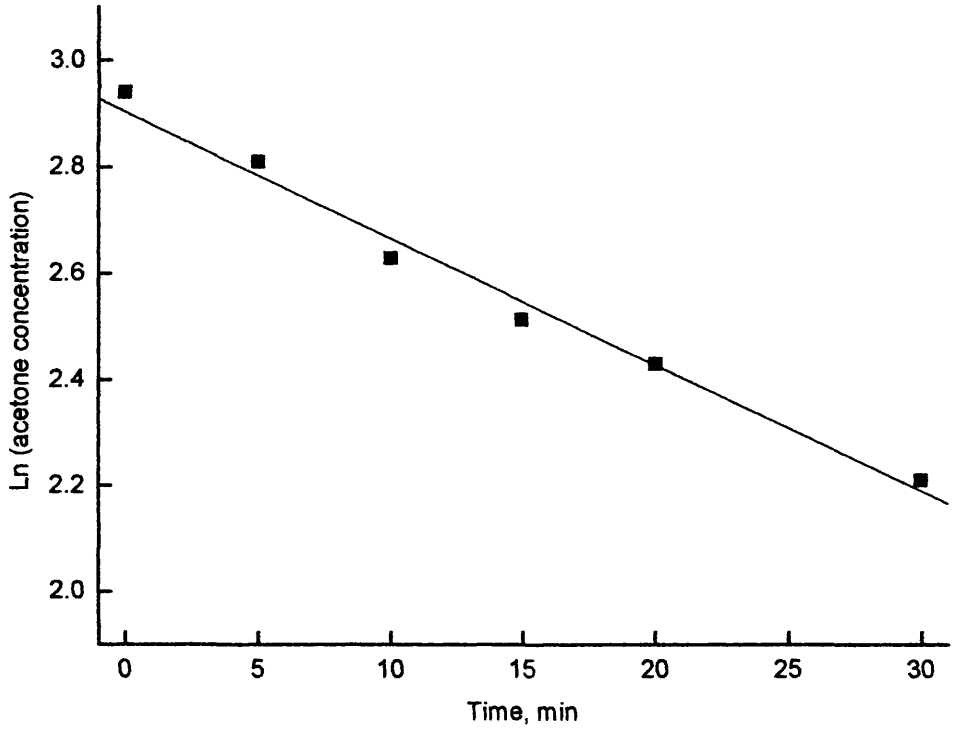


Figure 4.1 Logarithmic plot for the calculation of acetone volumetric mass transfer coefficient

4.2.3.4 Cofactor stability

NADPH is unstable under acidic conditions, but stable in alkali and vice-versa for $NADP^+$ (Wong and Whitesides, 1981). In general the oxidised form is more stable. Degradation has been shown to proceed by a first order mechanism and is accelerated in the presence of phosphate ions (Chenault and Whitesides, 1987; Meyer *et al*, 1997). The difference in the pH stability of the two forms will mean there will be conflicting requirements, and that overall stability will be a strong function of the equilibrium position of the two forms which in turn leads to stability being a strong function of the relative enzyme activities present. Decay constant data of the two forms of the cofactor is available. The kinetics follow first order decay and values for the rate constant $k_{(obs)}$ are reproduced in **Table 4.2** below (Chenault and Whitesides, 1987).

pH	$k_{(obs)NADPH}$, h^{-1}	$k_{(obs)NADP^+}$, h^{-1}
7.0	0.0158	0.000316
7.5	0.00794	0.000398
8.0	0.0025	0.0005
8.5	0.001	0.000794
9.0	0.000316	0.00199
9.5	0.000178	0.0063
10.0	0.000126	0.0158

Table 4.2 Degradation constants of NADP(H)

It is possible to estimate an "overall" k_{obs}, a weighted average of the two rate constants in relation to the species steady state concentrations. From this the half life, $t_{1/2}$ of the total cofactor can also been calculated as shown below:-

$$\frac{-d[\text{total cofactor}]}{dt} = k_{obs}[\text{total cofactor}] \qquad (15)$$

$$k_{obs}[\text{total}] = \frac{[\text{NADPH}]}{[\text{NADPH}]+[\text{NADP}]} k_{obs(NADPH)}[\text{NADPH}] + \frac{[\text{NADP}]}{[\text{NADP}]+[\text{NADPH}]} k_{obs(NADP)}[\text{NADP}] \qquad (16)$$

$$t_{1/2} = \frac{\ln 2}{k_{obs}[\text{total}]} \qquad (17)$$

4.2.4 Numerical Algorithm

Using Euler's one step method to linearise the rate equations over a small time interval, Δt, (Appendix 3) it is possible to solve the differential enzyme rate equations (8), the acetone mass transfer equation (14) and the cofactor stability equations (15 and 16). This method is simple and rapid to implement and so was used for these reasons.

Within the solution of the model it is necessary for clarity to define the proportion of enzymes used. The CHMO fraction is based on total catalytic activity within the reactor of both enzymes. Basing the enzyme fraction on activity is of more use than using protein concentration, as specific enzyme activity (Umg^{-1}) can vary.

The quoted efficiencies of the two enzymes are defined as the fraction of the potential maximum (V_{max}) rate that the particular enzyme is currently working at. A total system efficiency defined as the average of the two individual enzyme efficiencies is a useful guide to overall performance.

4.2.5 Data Collection

The model was solved using Euler's numerical algorithm within Microsoft™ Excel and data of cumulative time against concentration for all the reaction components using Visual Basic™ Macros. For any given reaction the data was then available in tabular form. The influence of pH, enzyme loading and cofactor loading could easily be assessed and the step time, Δt, altered at will.

4.3 Results of the model

4.3.1 Steady state analyses

4.3.1.1 General Issues

In all the conditions investigated (cofactor concentration 0-2mM; CHMO fraction 0.1-0.95) the system settled to steady state in a matter of minutes when using a Δt of 1 second (to minimise the error inherent in the numerical solution). This is not surprising as it can be seen from the rate equations for the two enzymes that the rate is a strong function of cofactor concentration, both as a true substrate and due to inhibition. It is the strong inhibition that introduces a large amount of negative feedback to the system, driving it to reach steady state.

4.3.1.2 Effect of CHMO activity fraction

The model was run using varying enzyme fractions, from a CHMO fraction of 0.1-0.95 and a constant cofactor concentration of 0.8mM, until steady state concentrations of the two cofactor forms had been reached. The enzyme efficiencies at steady state were noted. **Figure 4.2** illustrates the effect of enzyme fraction on the catalyst efficiency. The overall efficiency goes through a maximum at a CHMO fraction of 0.5, with the individual efficiencies increasing as their activity fractions decrease.

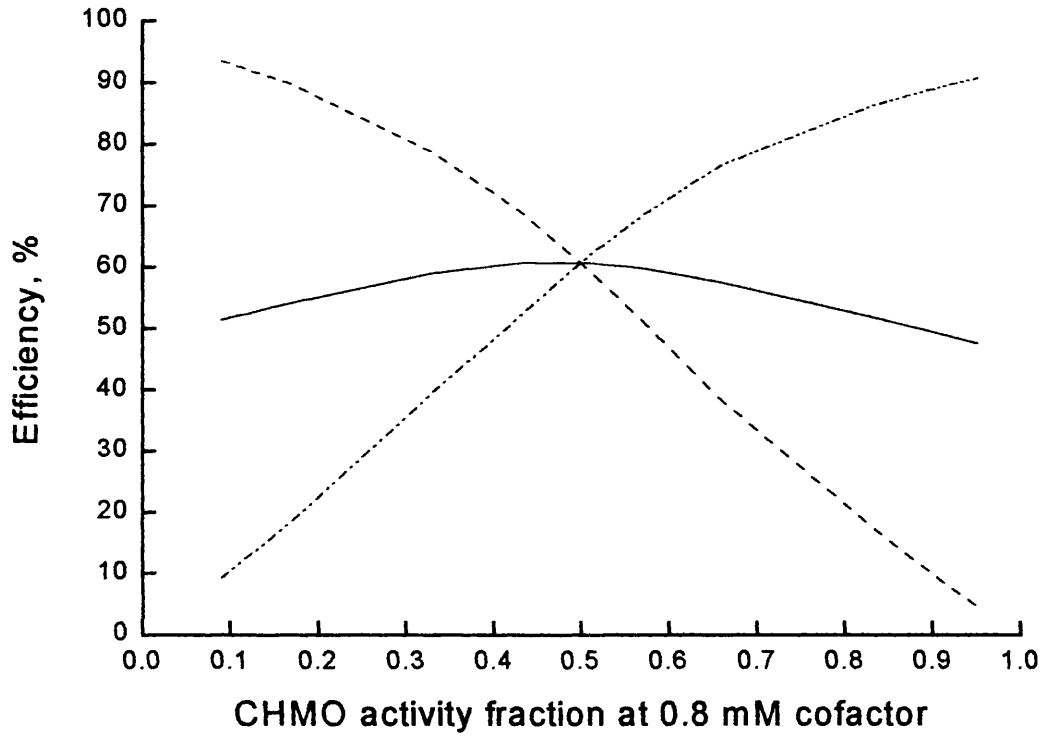


Figure 4.2. The effect of CHMO fraction on the activity efficiency of the system (—), TBADH(‴) and CHMO(–) assuming a total cofactor concentration of 0.8mM.

4.3.1.3 Effect of total cofactor concentration

In order to examine the effect of varying the total cofactor concentration, the model was run at a constant CHMO fraction of 0.5 and increasing cofactor concentrations up to 2mM. **Figure 4.3** shows that the overall efficiency reaches an asymptote in the region of 0.1mM total cofactor and is a first order function beneath this value.

One solution that would seem to make sense if it was desirable to operate with high CHMO productivity is to merely increase the total cofactor concentration within the vessel so that although the relative proportions of the two cofactor forms would remain unchanged the actual concentration of NADPH would be enough to saturate the monooxygenase. This is true up to a point as illustrated in **Figure 4.3**. Up to a cofactor concentration of 0.1mM the efficiency rises sharply, until both enzymes tend to the saturated region of operation. A maximum is encountered above 0.1mM, due to the saturation effect and dominance of competitive inhibition from the "other" form of the cofactor. This has important economic significance as it defines both the limit which the cofactor has no further effect on reaction rate, and the lower limit beneath which the rate is too low.

Total cofactor concentration also defines total turnover number for a given product yield. Assuming a reasonable product concentration of 0.1M an inverse relationship is seen with decreasing TTN as [cofactor] increases on **Figure 4.3**. A compromise has to be found to give both an acceptable product yield and high cofactor turnover.

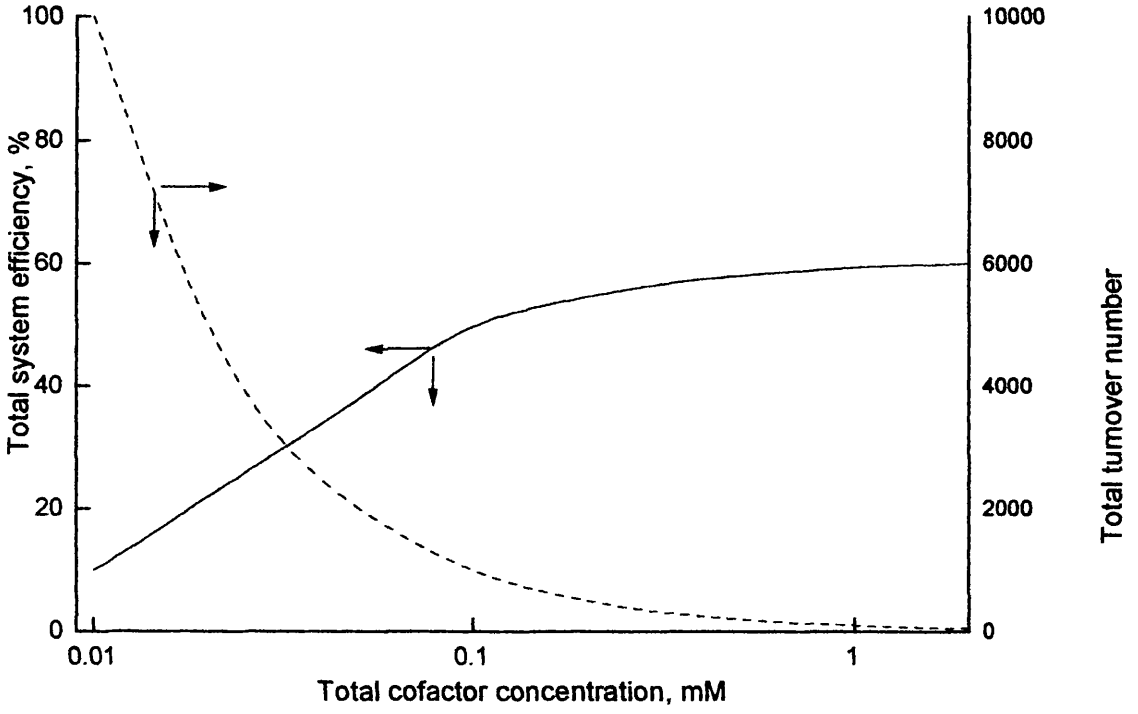


Figure 4.3 The effect of total cofactor on the overall system efficiency(—) and total turnover number for a 0.1M conversion (--) at a constant CHMO activity fraction of 0.5

4.3.1.4 Effect of removing $K_{i(cofactor)}$

From **Figure 4.3** it can be seen that there is a limit to the overall efficiency that can be generated due to the inhibitory effect of the form of the cofactor that each enzyme produces. One possible way of removing this limit is the possible future protein engineering of one or both enzymes to reduce this effect. Similar predictions to that in section 4.3.1.3 were made using the model, removing in turn the K_i effect of CHMO and TBADH, followed by the removal of the term from both rate laws. This is shown in **Figure 4.4**.

It is evident that removal of cofactor competitive inhibition has an effect on increasing the system efficiency achievable. Both enzymes show different degrees of improvement, removal of inhibition on TBADH produces a greater improvement than CHMO. This is to be expected as the value of K_i for TBADH is smaller than for CHMO. This shows that the initial aim of protein engineering should be focused on TBADH. If both enzymes are altered then the limit on efficiency is totally removed, and system efficiency approaches an asymptote at 100%.

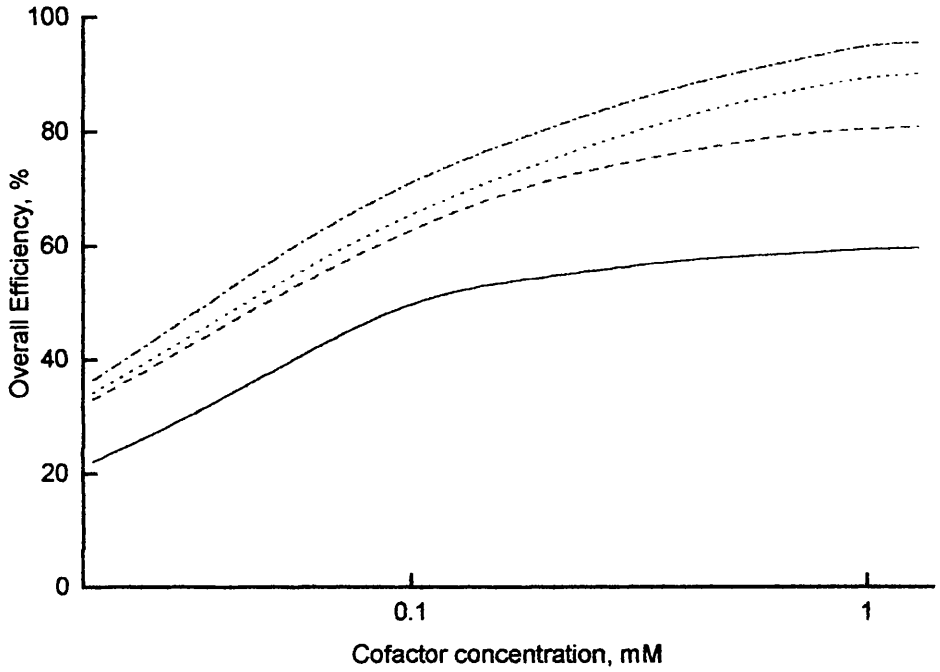


Figure 4.4 The effect of total cofactor on the overall system efficiency(—), efficiency after removal of inhibition of CHMO(---), efficiency after removal of inhibition of TBADH(····) and efficiency after removal of inhibition of both CHMO and TBADH(-·-·)

4.3.1.5 Cofactor stability as a function of pH and CHMO fraction

As well as minimising the amount of cofactor needed by looking at the efficiency it is also necessary to examine the stability of the cofactor, as both forms are inherently unstable in an aqueous environment. A graph of half life as a function of CHMO fraction and pH can be generated as a 3D surface (**Figure 4.5**).

A twisted "saddle" shape is generated with two regions of highest stability; the first seen at a pH of 9-9.5 and a CHMO fraction of less than 0.3 and the second at a pH of 7.5-8.5, with CHMO fraction above 0.7. The two maxima are explainable as in the first instance the CHMO fraction is low, the relative value of [NADPH] will be high and the pH is in the stable alkaline region. The second region will have high relative [$NADP^+$] and be largely in the neutral-weakly alkaline pH band. A line of lower, yet still notable stability joins these two areas diagonally between the entire range of enzyme fraction and pH (8-9). If this or a similar process were to be run for extended periods these data would clearly reduce the need for addition of new cofactor, as the conditions could be chosen such that extremely high stability is achievable. Even in shorter batch processes this is important as it could be possible to re-use the aqueous phase several times, if the product could be largely removed.

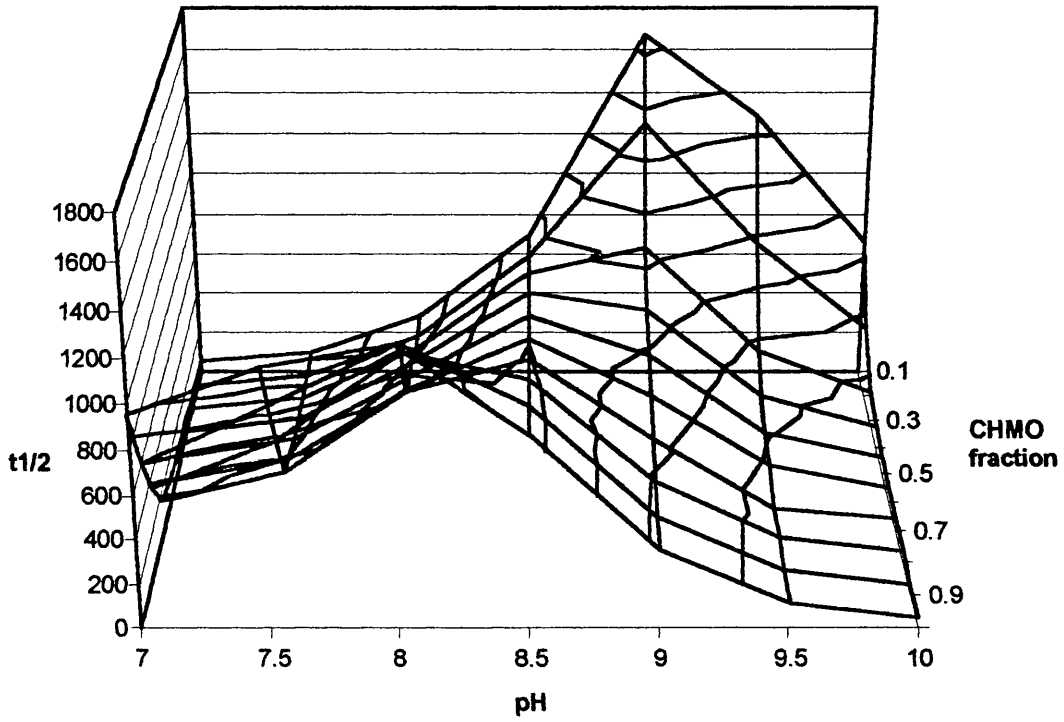


Figure 4.5 Total cofactor half life as a function of CHMO fraction and pH

4.3.1.6 Model Validation

To use this model confidently it is necessary to confirm that it is accurately representing this system, particularly as several assumptions were necessary in order to generate the rate equations. In order to assess the accuracy of the model reaction, performance was predicted at identified values of CHMO fractions from 0.1 to 0.7 (total cofactor concentration of 0.2mM). The prediction was tested by following the concentration of NADPH as a function of time in a UV spectrophotometer (Uvikon 922) equipped with a time drive. NADPH absorbs light strongly at a wavelength of 340nm whereas $NADP^+$ does not. Initially all the cofactor was in the form of $NADP^+$; this is the form that would be used in practice as it is much less expensive than NADPH. The total reaction volume was 1mL. The absorbance data was converted to actual concentration data using the published value of the extinction coefficient ε. The experimental values of CHMO fraction were calculated by assaying both enzymes in isolation. The value of cofactor concentration was chosen so as to avoid the possibility of the absorbance of UV light entering the non-linear region. The predicted and actual data for a representative CHMO fraction of 0.25 is shown in **Figure 4.6**. As expected the concentration of NADPH rises sharply and approaches equilibrium parabolically. The prediction overestimates the steady state cofactor concentration by 10%. It is possible that this is attributable to contaminating enzyme activities present since the TBADH, although commercially sourced, is not pure and CHMO is used as a crude cell homogenate from recombinant *E.coli*. Confirmation is given by the partial conversion of $NADP^+$ to NADPH before addition of 4-MCH/2-propanol. Also it is possible that the TBADH is partially undergoing the back reaction, producing small amounts of 4-methyl cyclohexanol.

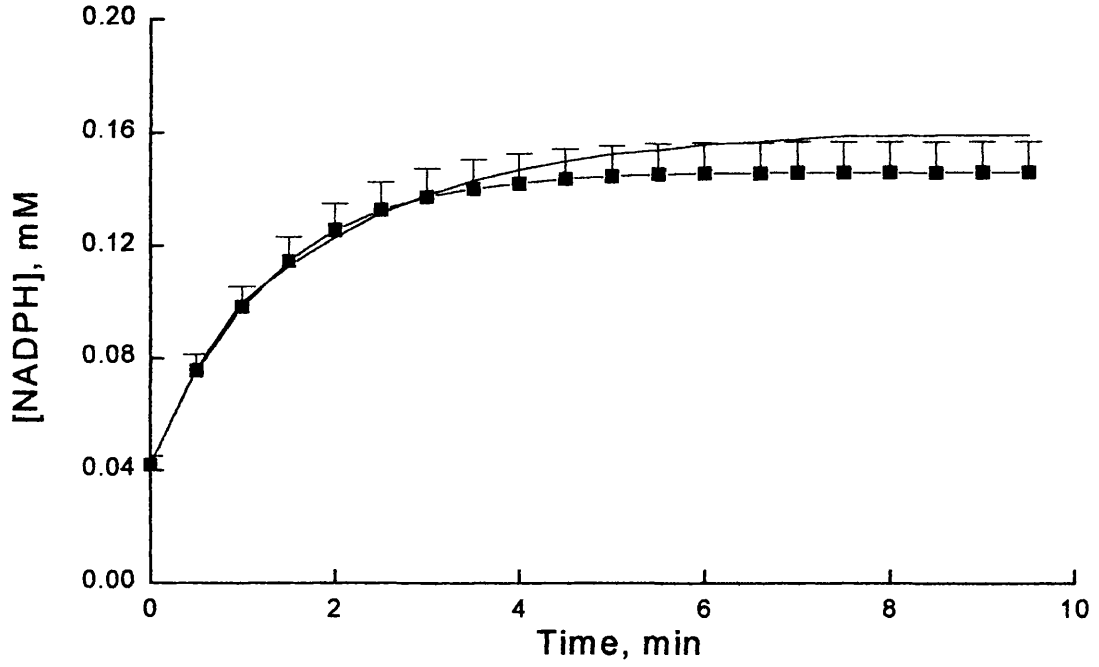


Figure 4.6 The time course of the predicted(—) and observed(■) NADPH concentration at a CHMO fraction of 0.25.

Figure 4.7 compares the experimental values of [NADPH] with those predicted, for a range of CHMO fractions. A completely accurate model would generate a plot on this graph with an gradient of unity.

The line through the experimental points has a gradient of 0.9, showing a consistent error of 10% across the range of enzyme fractions. This value however is not the true error in modelling CHMO productivity. In order to derive this a sensitivity analysis comparing the CHMO rate using the predicted and measured values of cofactor concentration was performed using the published rate equation. The average error in CHMO velocity was found to be 7.3%.

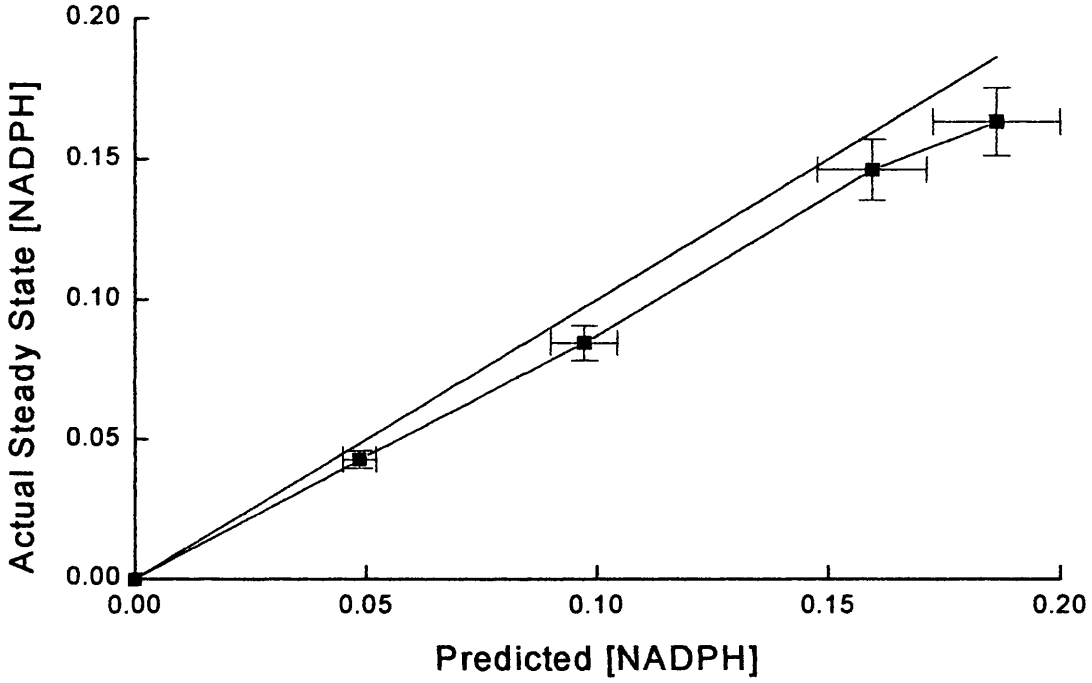


Figure 4.7 Parity plot comparing the line of parity(-) and experimental value(■) of the steady state NADPH concentration.

4.3.2 Predictions of system performance

4.3.2.1 Effect of enzyme and cofactor loading on product yield

Using the model it was possible to assess the productivity of the system as a function of cofactor and enzyme loading. Reactions were modeled in this instance at a constant 4-MCH and 2-propanol concentration of 20mM to approximate a well controlled fed-batch regime. Cofactor concentration was varied from 0.02-0.4mM, enzyme loading from 250 UL^{-1} (for each enzyme) to 1250 UL^{-1}. This upper limit of enzyme loading was set to a level just below where oxygen supply limitation would begin to take an effect (Lynch, 1994). **Figure 4.8** illustrates the results from these model runs.

As would be expected from the earlier steady state experiments the STY is a strong function of cofactor concentration below 0.1mM, with the response becoming largely independent above this value for all the enzyme loadings, the response becoming more marked as the enzyme loading increases.

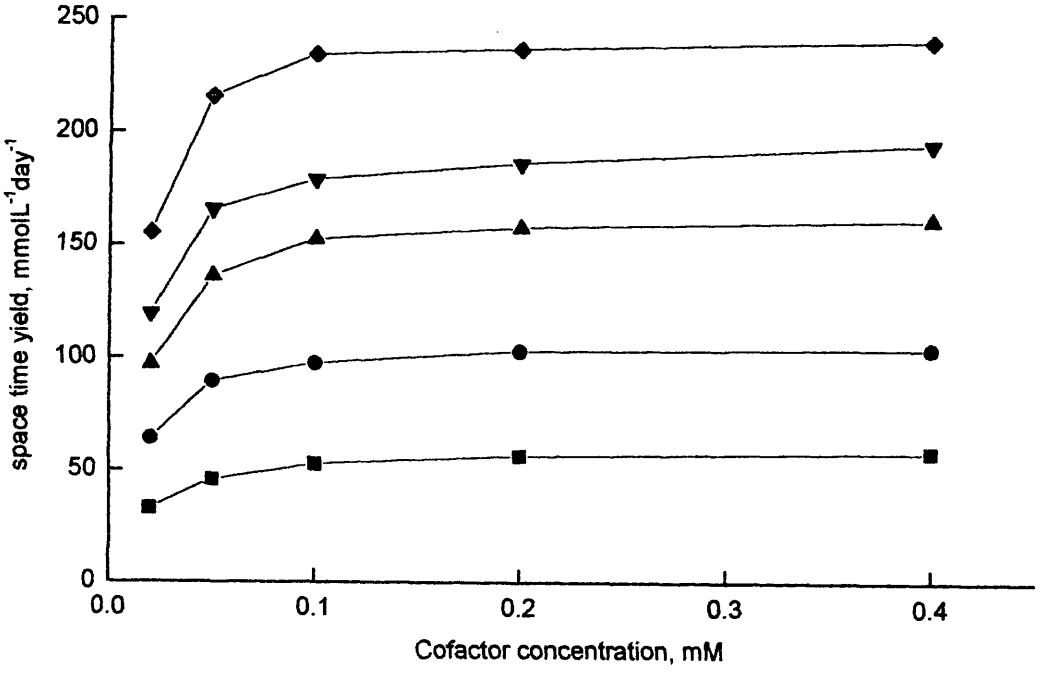


Figure 4.8 The STY of the reaction system as a function of enzyme and cofactor loading for 250 UL^{-1}(■), 500 UL^{-1}(●), 750 UL^{-1}(▲), 1000 UL^{-1}(▼) and 1250 UL^{-1}(◆). All enzyme loadings quoted are for each enzyme

4.3.2.2 Influence of process configuration on product yield

The model runs described in **Figure 4.8** illustrate the ability of the model to predict performance as a function of the concentration of the reaction components for a given reactor configuration. Equally it is valuable to be able to investigate the performance of a number of different reactor modes to define their comparative performance. This approach allow the *quantification* of the proposed designs from chapter 2, which were selected on purely *qualitative* grounds.

The condition of 250 UL^{-1} enzyme and 0.1mM cofactor was chosen as representative of the conditions investigated. Several process design variants were considered, including a 100mM batch reaction (4-MCH and 2-propanol), controlled fed batch (as in section 4.3.2.1), linear fed batch (0.002mMs^{-1} chosen to match the enzyme loading) and also the effect of product removal techniques (to remove CHMO product inhibition). ISPR was simulated by assuming a certain proportion of the product formed was removed from the viscinity of the reaction; degrees varied from 50% (considered poor) up to 95% (considered good). The results are shown in **Figure 4.9**.

The data were normalised to the productivity of the simplest reactor mode (batch); indicated by an arbitrary productivity of 100. It is evident from inspection that product removal techniques have a limited effect on improving yields, 95% product removal in batch yields only a 36% improvement in yield. The best improvement is seen when a fed batch system is employed over a batch reaction. Productivity in the reaction increases 253% if a linear feed is used. Fed batch productivity also increases only marginally if ISPR is used.

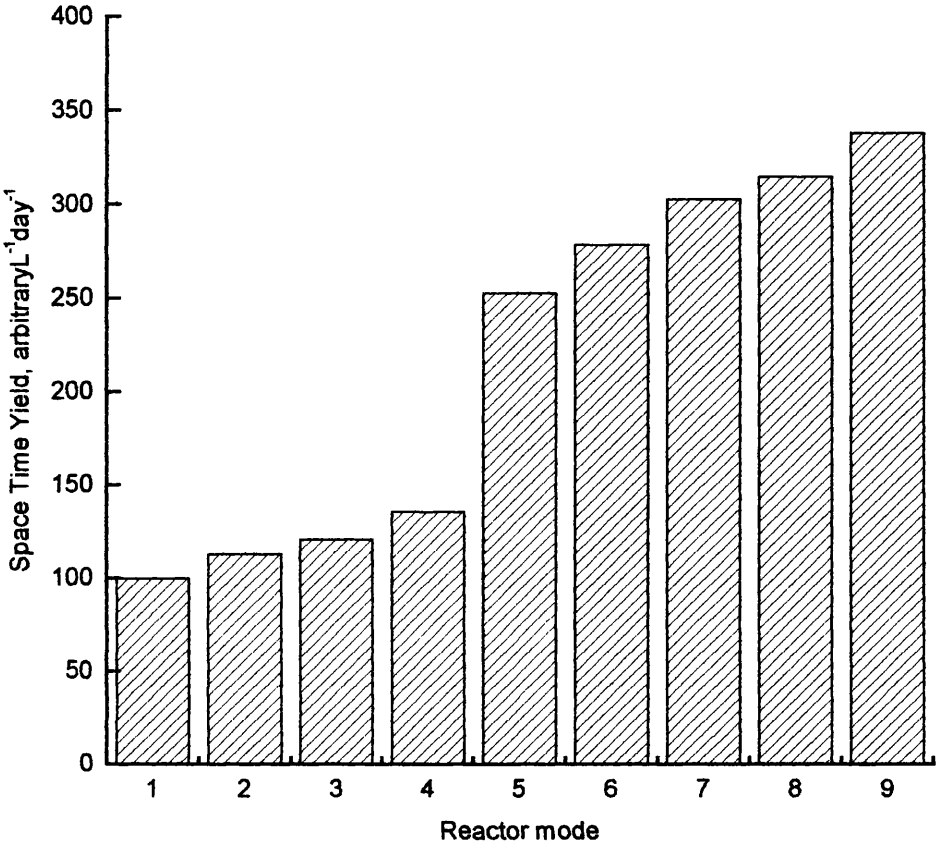


Figure 4.9 Yields of the reaction system as a function of condition:

1) 100mM batch
2) 100mM batch + 50% ISPR
3) 100mM batch + 70% ISPR
4) 100mM batch + 95% ISPR
5) Linear fed batch
6) Controlled fed batch, 10mM 2-propanol, 5mM 4-MCH
7) Controlled fed batch + 50% ISPR
8) Controlled fed batch + 70% ISPR
9) Controlled fed batch + 95% ISPR

4.4 Discussion

4.4.1 Steady state analyses

4.4.1.1 General Implications

The model helps highlight a number of important issues that relate to the economic feasibility of using this system to produce chiral lactones on a preparative scale. It is clear that the performance of the system is greatly influenced by the proportions of the two enzymes used (enzyme fraction) and the cofactor concentration (which defines TTN). A compromise must be found between producing enough lactone and minimising cost. The added issue of cofactor instability as a function of pH adds a further level of complexity. The inhibition kinetics relating to cofactor must also be considered, efficiency at a given cofactor concentration is improved by having a low value of K_i. Reducing this must be an important target for future protein engineering.

This interaction between cost and possible process can be examined more easily using four typical economic scenarios that consider the enzymes as having different costs associated to them in relation to each other. These scenarios assume that the cost of cofactor is always high; which is realistic for NADPH. Although the work reported here is specific to the system under investigation the principle can apply to other linked recycle systems, merely substituting the enzymes of interest for CHMO and TBADH. Comparison of the scenarios is best achieved using the principle of windows of operation (Woodley and Lilly, 1996) plotting key variables to show the feasible limits of the variables plotted in each case. The limits are defined here on economic grounds. The window plotted for each scenario relates enzyme fraction to pH for a given objective function. It is then possible to see the area or "window" of feasible operation for the particular variables, helping to define process design.

In all cases it is necessary to generate a high TTN, typically greater than 500. This precludes the use of cofactor concentrations above 0.1mM (assuming 0.1M product). Total efficiency also defines a lower limit at 0.05mM below which overall rate is unacceptable. Cofactor stability considerations define limits of operational pH, two values of 400 and 800 hours for half life are chosen arbitrarily. These issues are general for the four scenarios in question as they relate to the assumed high cost of cofactor. In relation to the conclusions reached in chapter 2 it was shown that there is a upper limit to pH due to lactone instability which is not shown on the window for clarity.

Scenario 1 (CHMO more expensive than TBADH)

In this case the process must be geared toward maximising the use of available CHMO and so a high CHMO efficiency is needed making the CHMO fraction no greater than 0.5. Excess TBADH can be used as it is cheaper. A lower limit must be set at a CHMO fraction of 0.1 since below this the overall system will suffer a loss of activity. These factors are plotted as operational windows in **Figures 4.10** and **4.11**.

Scenario 2 (TBADH more expensive than CHMO)

In this situation the expensive enzyme is TBADH, so that the CHMO fraction must be greater than 0.5 to make best use of the TBADH and no greater than 0.9 for the reason outlined in scenario 1. This can be seen in **Figures 4.12** and **4.13**. Within the scope of the thesis this is the scenario nearest to reality; TBADH is expensive due to it's commercial source whereas CHMO is produced much more cheaply in house.

Scenario 3 (CHMO and TBADH of equal (high) cost)

The lower and upper limits to CHMO fraction apply due to loss of overall efficiency, important when use of both enzymes must be maximised. However the window is larger than either scenario 1 or 2 because both enzymes are considered to be of equal (high) cost. This can be seen in **Figure 4.14** and **4.15**.

Scenario 4 (CHMO and TBADH of equal (low) cost)

When both enzymes are considered to contribute a significantly small amount to the overall cost then the window becomes much larger, allowing the enzyme fraction to be selected towards increasing cofactor stability and reaction rate. In this case although the window is much larger any process would tend to be operated with a large excess of TBADH to increase lactone production, as shown in **Figures 4.16** and **4.17.**

4.4.1.2 Process constraints

Figures 4.10-4.17 indicate that having enzymes of comparable cost allows for a more flexible approach as the operational windows are larger than in the other cases considered. Most notably is the wider range of pH, from 7-10 (depending on the enzyme fraction used). In scenario 1 the limits are pH 7.5-10 and for scenario 2 they are 7-9.25 when considering a half life of 400 hours. It is also important to note that in order to run the process economically both the window(s) described here and the constraints identified in section 2.9.1 must be satisfied; illustrating the importance of these detailed studies in NADPH recycle systems.

Comparing the windows for each scenario at the two values of acceptable half life it becomes apparent that the window is strongly influenced by this parameter. This is best illustrated using scenario 1; with a half life of 800 hours the window of

operation is divided into two very restrictive areas, compared to a much less restrictive window at 400 hours. This has implications when running this (or similar) processes in continuous mode where the largest overall running cost is likely to be attributable to cofactor recharging.

The cost of cofactor is by far the biggest constraint imposed on all the scenarios; a situation that at present has limited the application of such systems. In future the cost of cofactor may decline and the available windows of operation should become less restrictive.

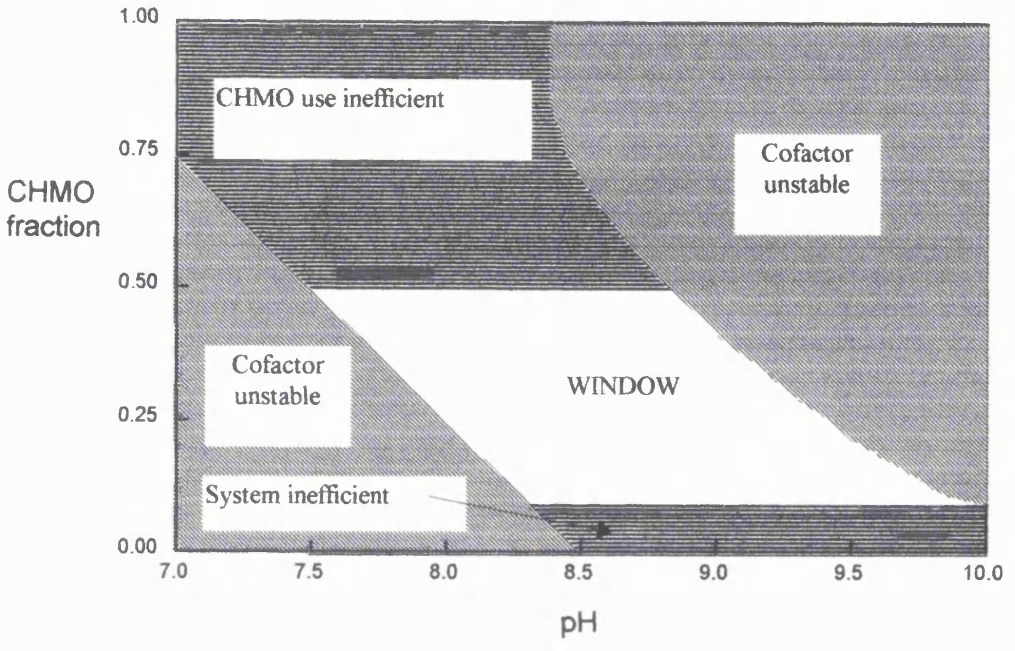
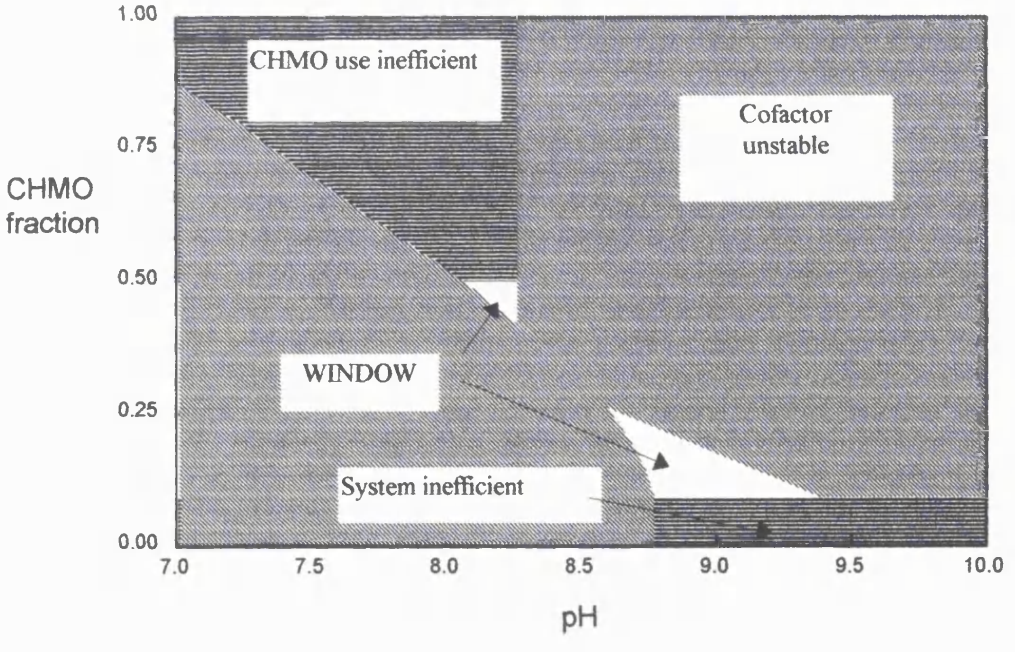


Figure 4.10 Operating window for economic scenario 1, half life 400 hours.
Figure 4.11 Operating window for economic scenario 1, half life 800 hours.



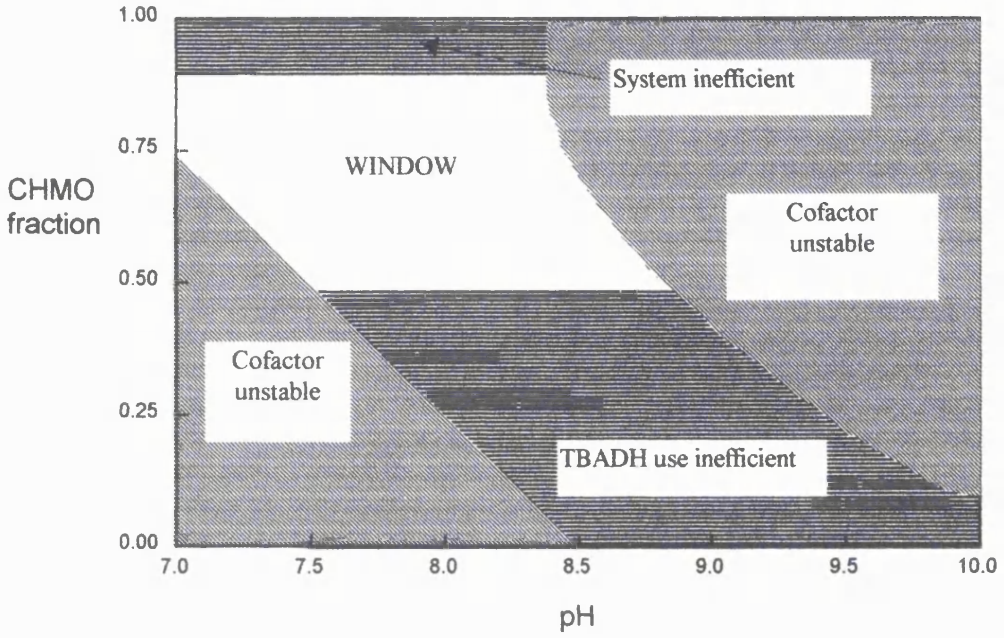
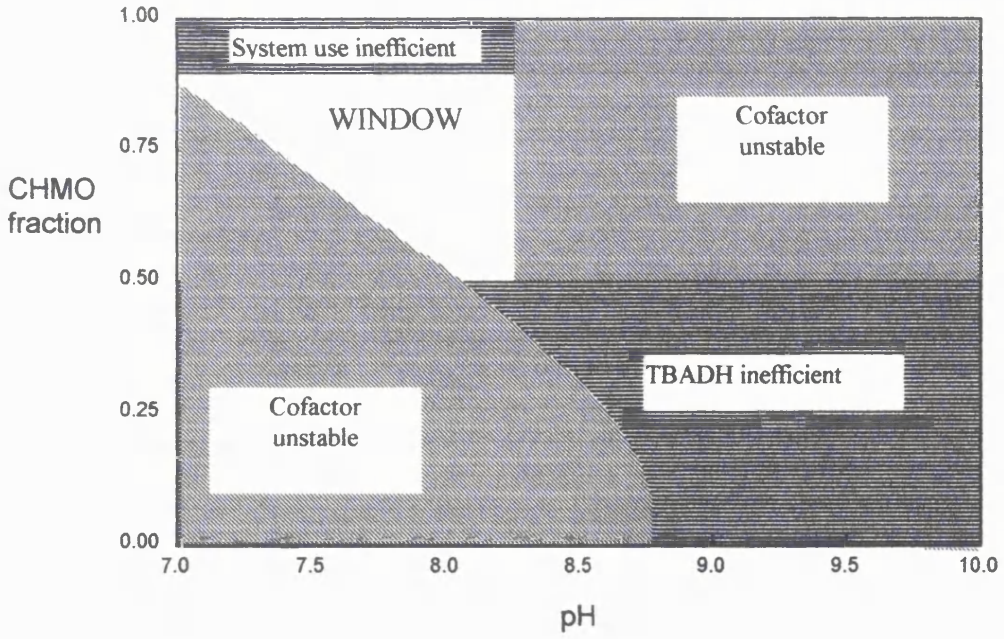


Figure 4.12 Operating window for economic scenario 2, half life 400 hours.
Figure 4.13 Operating window for economic scenario 2, half life 800 hours.



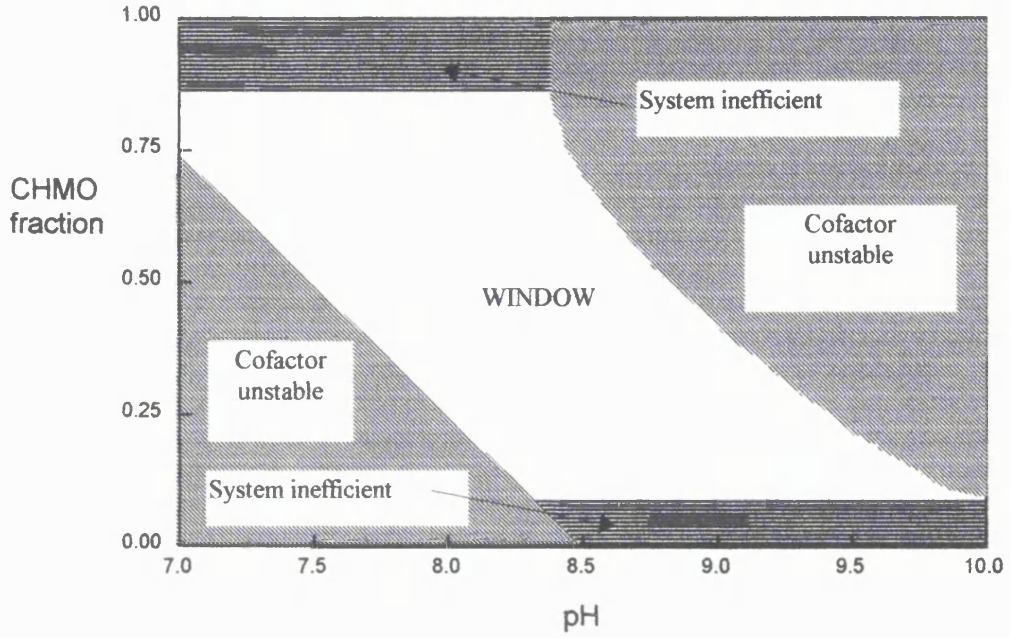
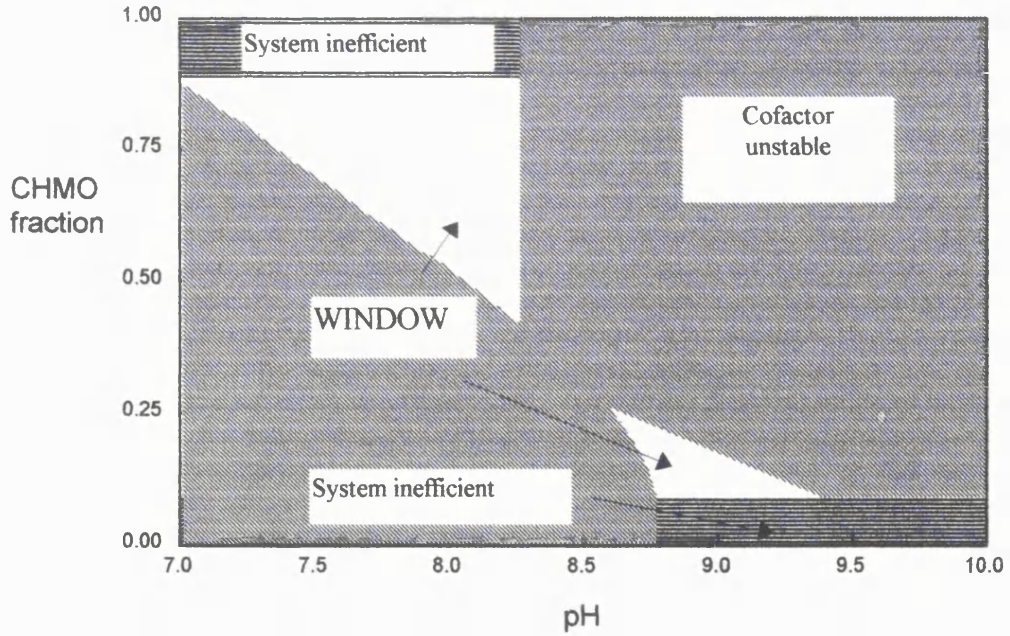


Figure 4.14 Operating window for economic scenario 3, half life 400 hours.
Figure 4.15 Operating window for economic scenario 3, half life 800 hours.



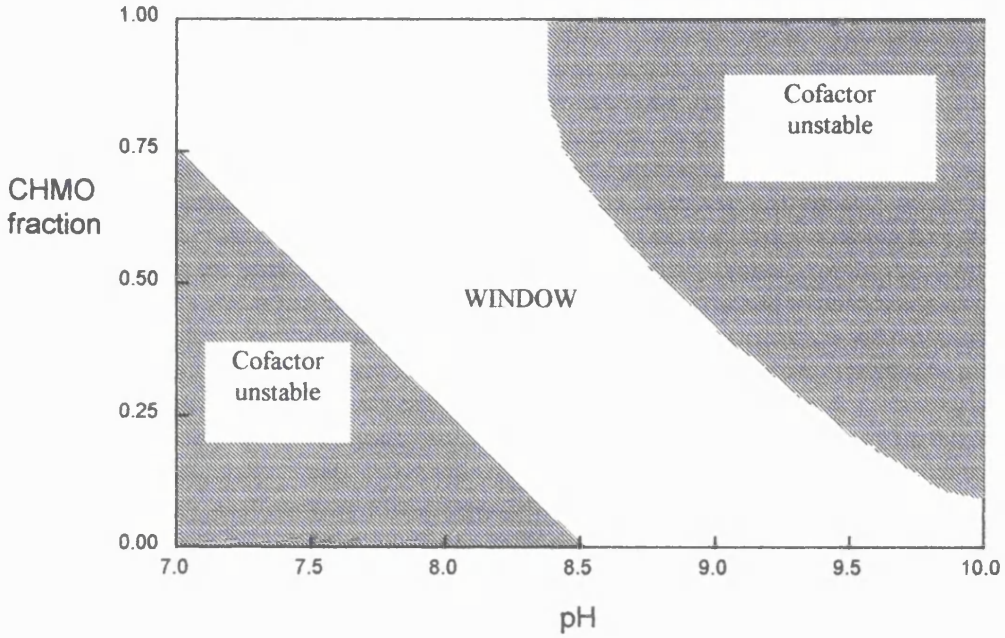
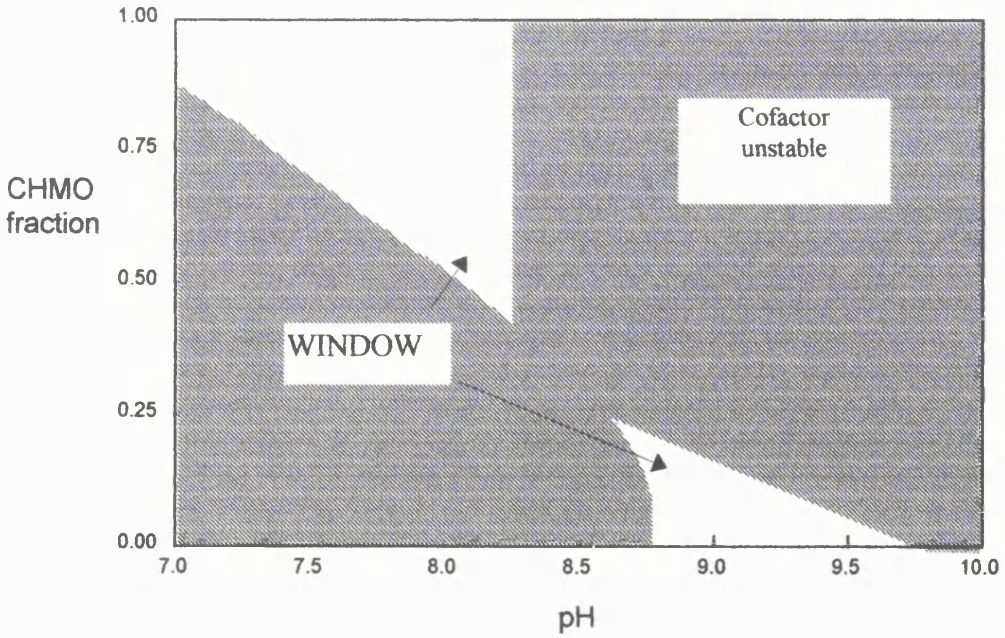


Figure 4.16 Operating window for economic scenario 4, half life 400 hours.
Figure 4.17 Operating window for economic scenario 4, half life 800 hours.



4.4.2 Predictions of system performance

The data gained from **Figure 4.8** gives a valuable indication as to the potential productivity of the system. Without the ability to model the reaction this would be difficult to estimate due to the large number of variables that need consideration and the interaction between them would compound the issue. As was predicted from **Figure 4.3** (plot of overall efficiency as a function of cofactor loading) the STY is a strong function of cofactor concentration below 0.1mM, above which the STY increases only marginally. This modelling approach would be of use to the designer in a case for example where there is a design specification for STY and it is known at which TTN the process will be economic. The amount of enzyme can thus be easily calculated.

In chapter 2 it was proposed that the most suitable process to perform the synthesis of 5-MOP from 4-MCH was a fed-batch stirred tank reactor, with a certain question mark over the need for product removal to remove inhibition and toxicity on CHMO (the model does not consider the toxic effect on CHMO as the assumption was made that the enzyme would be immobilised, and this effect would be removed). The model was used to provide quantitative design information on the possible reactor designs, the results of which form **Figure 4.9**. It is clear that the transition from batch to fed-batch processing has a dramatic effect on productivity, which under these conditions rises by some 250%. Surprisingly the instigation of ISPR has a much more limited impact on productivity unless it is extremely selective.

In chapter 3 the effect of acetone inhibition upon TBADH was demonstrated to be highly significant. It was observed that during the assessment of the nine process variants shown in **Figure 4.9** acetone concentration present after a simulated 24 run time rose steadily from 1mM to 3.5mM in cases 1-9 respectively as the reaction rate increased. This is potentially the next bottleneck on process development and not oxygen supply as would be expected. It is possible to increase the driving force to remove acetone from solution by raising the reaction temperature however this will

possibly have deleterious effects on enzyme and cofactor stability as well as lowering the aqueous oxygen saturation concentration.

These data are vital to the process design procedure as it is able to verify the design proposed in chapter 2, a design choice arrived at via a non-numerical heuristic approach. The *absolute* value of productivity of a FBSTR performing this reaction can now be compared to the other designs considered, whereas before it could be only estimated at best.

4.5 Summary

The use of a numerical solution has been shown to be effective in solving the rate laws for the CHMO/TBADH process, allowing it to be optimised to reduce the cost of the expensive cofactor, and to get the maximum activity from the enzymes. This has implications for processes that require expensive cofactors (a general problem for redox enzymes) that would make the use of these enzymes as isolated catalysts economically feasible. The identification of a number of possible economic scenarios has highlighted the windows of operation possible for differing situations.

This technique has also allowed the productivity of various process configurations to be assessed. These data coupled with the structured design procedure in chapter 2 has produced an improved and more complete design procedure with greater confidence in it's final output.

The final experimental chapter will show how the results of chapter 2 and 3 (the use of a FBSTR and cofactor recycle by TBADH) and the modelling data of this chapter (particularly the required enzyme ratio and cofactor concentration for efficient and economic operation) can be brought together to perform syntheses of 5-MOP and 6-HTPO. The use of immobilised vs soluble enzyme will be compared as catalysts, in particular the STY and TTN achievable with each.

5.0 Preparative scale bioconversions

5.1 Introduction

In the previous chapter the use of numerical techniques allowed the quantification of various process options to be achieved. The model was applied to the synthesis of 5-MOP but the approach is valid for 6-HTPO synthesis. It should also be possible to apply this technique to other biocatalytic systems.

In this chapter the feasibility of CHMO based lactone syntheses will be demonstrated, by considering the use of both free and immobilised enzymes to produce 5-MOP and 6-HTPO. The characterisation of CHMO in chapter 2 demonstrated the potential processing problems of using free enzyme, namely the denaturating effects of the substrates and products and the damaging effect of aeration on CHMO. This chapter will investigate the suitability of immobilisation as a technique to overcome these processing problems. The advantages and disadvantages of the two catalyst forms will also be considered.

5.2 Materials and methods

5.2.1 Bioreactor

100mL reactions were carried out in 1L conical shake flasks at 30°C shaken at 200rpm in an orbital shaker (New Brunswick Scientific, Eddison, USA). Reaction pH was kept at pH 8 by buffering with 50mM Tris-HCL.

1.5L reactions were performed in a LH 2L series 210 bioreactor fitted with a ring sparger and 2 Rushton turbines for agitation. Control of solution pH was achieved by automated titration of 4M *ortho*-phosphoric acid/sodium hydroxide. GC analysis of the reactants and products was performed as described previously. Quantification of 2-propanol and acetone was not performed. All reactions used cofactor initially in the form of $NADP^+$ as it is approximately an order of magnitude cheaper than NADPH.

5.2.2 Eupergit C

Eupergit C was a kind gift from Dr Thomas Boller, Rohm Pharma GmbH, Germany.

Eupergit C was selected as a suitable support for immobilising CHMO and TBADH due to previously reported successful immobilisation of TBADH (Keinan *et al*, 1986). The attachment procedure is in general straightforward, enzyme solution is introduced to the beads and the mixture left for 24-96 hours for the reaction take place (Faber, 1992).

Eupergit C is described as a porous polymer of methacrylamide N,N'-methylene-*bis*(methacrylamide) with a particle diameter of 150μm, pore diameter 10-30nm and a specific surface area of $183m^2g^{-1}$(dry). The attachment reaction consists of the opening of epoxide groups on the bead and reaction with amino residues on the enzyme. The reaction can proceed under mild conditions and does not change the protein charge distribution, due to the reaction being an alkylation process. The covalent nature of the attachment prevents subsequent leakage. However enzyme activity is invariably lost due to conformational change and steric hindrance. As a rule of thumb each bond between carrier and enzyme reduces activity by 20% (Faber, 1992). The covalent attachment in many cases produces a catalyst of superior stability compared to the native enzyme (Brocklebank *et al*, 1999). **Figure 5.1** below illustrates the binding mechanism.

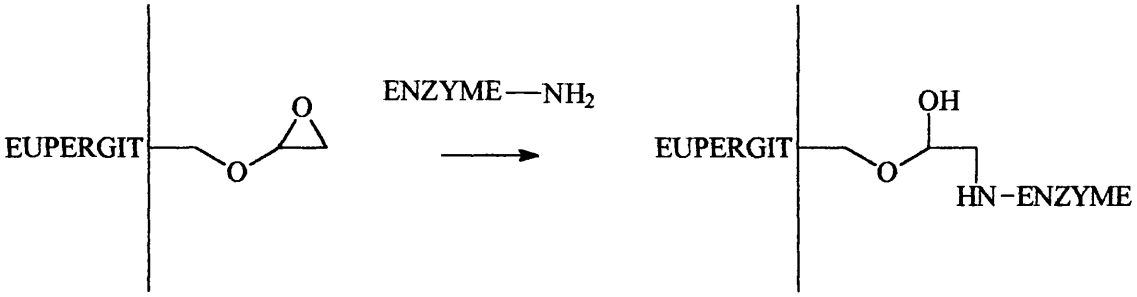


Figure 5.1 Typical reaction between Eupergit C and enzyme (Gerhartz, 1990)

5.2.3 Eupergit bound enzyme activity assay

5.2.3.1 Cyclohexanone monooxygenase

Immobilised CHMO (0.5-1g) was incubated and gently stirred at 30°C for 5 minutes in 10mL 50mM Tris-HCL buffer (pH 9) containing 1.6mM NADPH, the reaction started with the addition of cyclohexanone to a concentration of 2mM. 0.1mL aliquots were taken every 2 minutes for 20 minutes and diluted into 900μL of 50mM phosphate buffer (pH7) pre-chilled to 4°C. The samples was centrifuged at 15000rpm for 1 minute to remove traces of Eupergit C and the absorbance of the solution measured at 340nm. $\Delta A(340nm)/\Delta t$ was calculated over the linear region, and the enzyme activity calculated as in section 2.2.4.2

5.2.3.2 Alcohol dehydrogenase

The procedure was similar to that described in section 5.2.3.2, however the reaction mixture contained 1.6mM $NADP^+$ and the reaction initiated by the addition of 2-propanol to a final concentration of 150mM.

5.2.4 Preparation of isolated monooxygenase

E.coli JM107 pQR210 was grown as described in section 2.2.5. Cell harvest was performed in a Beckman J2 centrifuge at 4°C, 10000rpm for 30 minutes. Supernatant was discarded and the cells re-suspended in 50mM Tris-HCl buffer, pH 9. Cell disruption was performed in a Manton-Gaulin Lab 40 high pressure homogeniser for

samples of 30-40mL (3 passes, 1200bar), or a Manton-Gaulin Lab-60 homogeniser for samples of 1-5L (5 passes, 500 bar). Removal of cell debris to generate a clarified lysate was accomplished by re-centrifuging as before, except the centrifugation time was 60 minutes. Lysate was typically stored overnight at 4°C with minimal loss of activity before use.

5.2.5 Free enzyme bioconversions

5.2.5.1 100mL reactions

128U of CHMO and 85U of TBADH were used to perform a batch reaction with 50mM 4-MCH and 55mM 2-propanol (used in 10% excess to limit the effect of evaporation). As discussed in chapter 4 a cofactor concentration of 0.1mM was used.

101U of CHMO and 35U of TBADH were used to perform a batch reaction. Initially 2-HCP and 2-propanol were added to the aqueous solubility limit of the ketone, 2.5mM. After two hours additions of 2-HCP and 2-propanol to a final concentration of 25mM and 27.5mM were made respectively. The ketone forming a second well dispersed phase. A cofactor concentration of 0.05mM was used.

5.2.5.2 1.5L reactions

300U of CHMO and167U of TBADH were used to perform a reaction initially with 15mM 4-MCH and 16.5mM 2-propanol. A cofactor concentration of 0.1mM was used. Agitator speed was limited to 250rpm and air flow to 1vvm. Above this level of agitation induced foaming was excessive. After 9 hours a further substrate addition was made to raise the substrate concentration by 20mM.

180U of CHMO and 65U of TBADH were used to perform the conversion of 2-HCP to 6-HTPO with a cofactor concentration of 0.05mM. Initially 2-HCP and 2-propanol were added to a concentration of 25 and 28mM respectively, the ketone forming a second phase. Air was supplied at 1vvm, and agitation at 250rpm

5.2.6 Immobilised enzyme bioconversions

1000U of CHMO were immobilised onto 35g of Eupergit C as described in section 5.4. 170U of TBADH were similarly immobilised on 3g of beads. After thorough washing the beads were transferred to the 2L bioreactor containing 1.5L of RO water held at pH 8 containing 0.1mM $NADP^+$. The reactor was charged with 4-MCH to a concentration of 20mM and 2-propanol to 25mM. Air was supplied at 1vvm, and agitation at 500rpm. After 8 hours further additions were made, increasing the concentrations of 4-MCH by 45mM and 2-propanol by 49mM. The progress of the reaction was monitored by GC.

The immobilised enzyme used was identical to that used in the 1.5L scale synthesis of 5-MOP. Cofactor was added to a concentration of 0.05mM. 2-HCP was added as a second phase to a reactor concentration of 99mM, 2-propanol was added at time zero (to 8.5mM), 4 hours (a further 17.1mM rise) and 9 hours (a further 17.1mM rise).

5.3 Results of free enzyme bioconversions

5.3.1 100mL reactions

5.3.1.1 4-methyl cyclohexanone

The reaction is shown in **Figure 5.2**, the reaction rate became linear after approximately 2 hours. The final yield was a 5-MOP concentration of 42mM ($5.4gL^{-1}$) The TTN produced was 433. 4-methyl cyclohexanol was detected toward the end of the reaction, the concentration not exceeding 0.4mM indicating that as expected the back reaction of TBADH was not significant.

5.3.2.2 2-hexyl cyclopentanone

After 2 hours the reaction was complete. (**Figure 5.3)** Residual ketone of 50% was detected (the unreacted enantiomer). Feeding excess ketone above the solubility limit produced more lactone, to a final concentration of 12.3mM, several times greater than the aqueous solubility limit of 6-HTPO. The dissolved protein in the reaction was seen to become opaque and an emulsion formed due to the second phase. GC samples were taken from the bulk liquor as the second phase was seen to be dispersed. The final yield was a 6-HTPO concentration of 12.3mM ($2.3gL^{-1}$) The TTN produced was 253.

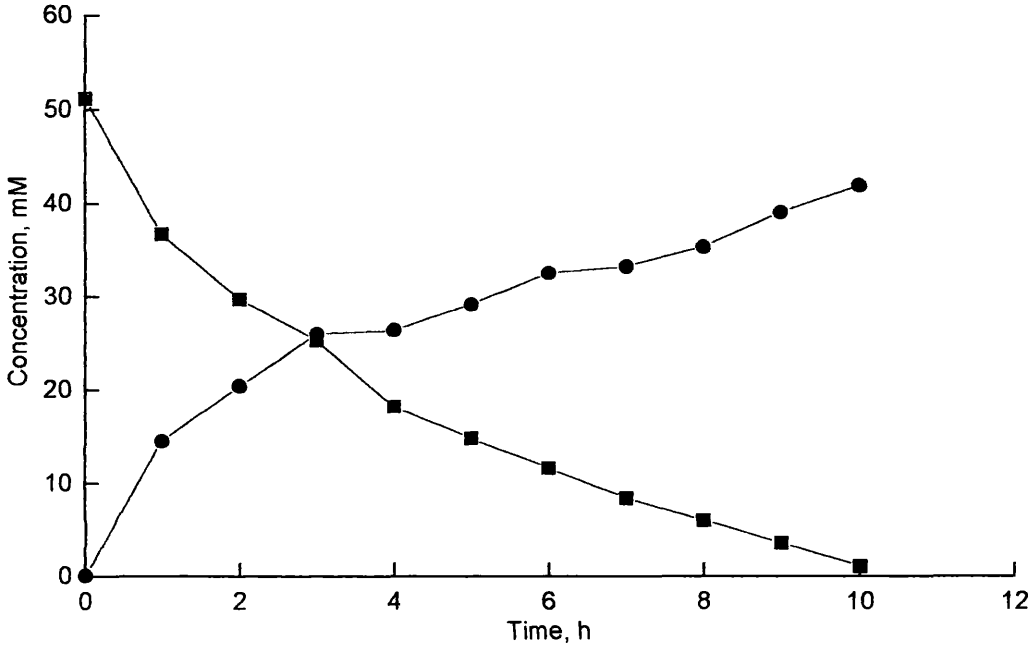


Figure 5.2 100mL scale bioconversion of 4-MCH(■) to 5-MOP(●)

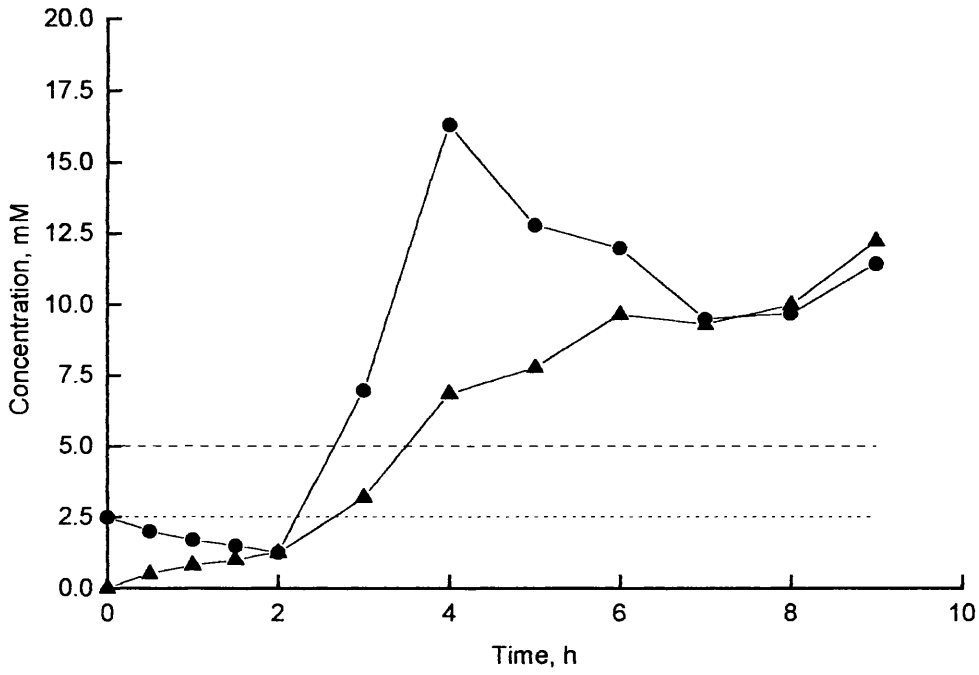


Figure 5.3 100mL reaction consuming 2-HCP(●) and producing 6-HTPO(▲). Aqueous solubility's of 2-HCP(····) and 6-HTPO(---) are also shown.

5.3.2 1.5L reactions

5.3.2.1 4-methyl cyclohexanone

The results are shown in **Figure 5.4**. The final yield after 26 hours was a 5-MOP concentration of 31.5mM ($4.1gL^{-1}$) The TTN produced was 321.

5.3.2.2 2-hexyl cyclopentanone

The reaction can be seen in **Figure 5.5**. The reaction was observed to cease after five hours, the reaction mixture turned slowly from a clear yellow colour to an opaque brown, the second organic phase apparently causing precipitation of dissolved protein. The final lactone yield was 3.72mM ($0.68gL^{-1}$) and the TTN was 77.

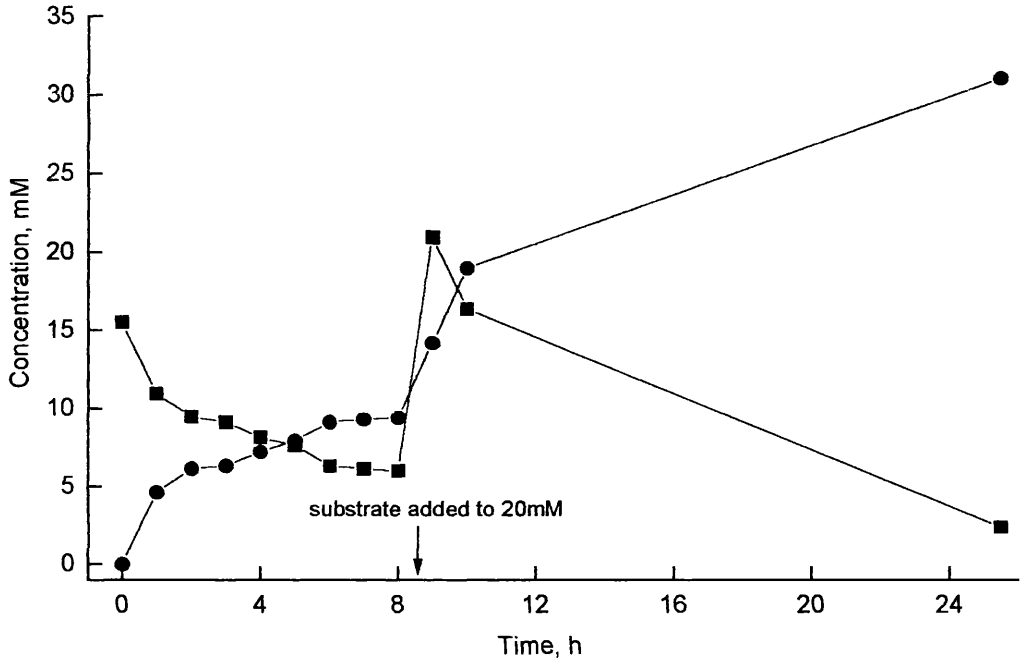


Figure 5.4 1.5L reaction showing 4-MCH(■) and 5-MOP(●).

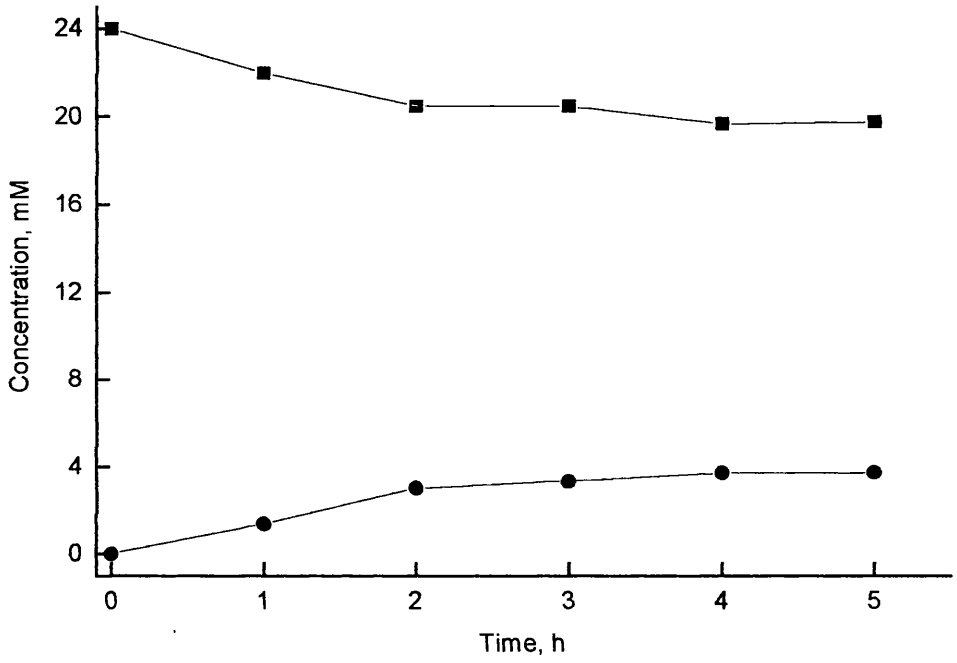


Figure 5.5 1.5L reaction showing 2-HCP(■) and 6-HTPO(●)

5.4 Immobilisation of enzymes

5.4.1 Cyclohexanone monooxygenase

5.4.1.1 General considerations

Eupergit C product literature (Rohm Pharma GmbH) indicates that the immobilisation of enzymes can be accomplished from a protein purity of 100% down to crude clarified cell lysates. Any temperature from 4-30°C and immobilisation time from 24-96 hours can be used, and a phosphate buffer concentration of 1M is recommended. Protein loads of 10-200mg protein per gram of dry Eupergit are also suggested. It should be stressed that these are guidelines only and the nature of the enzyme environment is critical in determining the efficiency of binding and the conditions required by any particular enzyme are in general not predictable. The conditions quoted above are thus only a suggested starting point.

As the preparation of immobilised enzyme is time consuming it was decided to attempt immobilisation of CHMO directly from *E.coli* homogenate, and TBADH from the commercial preparation with no further purification.

5.4.1.2 Optimisation of the immobilisation conditions

The stability of the enzyme in solution under the immobilisation conditions is critical in generating an active immobilised catalyst as the timescale for binding can be up to four days. This was considered to be an appropriate starting point in defining the immobilisation conditions, particularly the temperature to be used. Crude clarified lysate of CHMO (20U, 5mL) was prepared in 1M phosphate buffer, pH 9 and left to

stand in glass universals at room temperature at 4°C. The residual CHMO activity was monitored over four days. The results of this study are shown in **Figure 5.6**.

It is evident that the storage of enzyme at room temperature results in rapid deactivation in 48-72 hours, as would be expected from similar studies performed in a lower molarity Tris-HCL buffer in chapter 2. Storage at 4°C also results in activity loss. However after 96 hours there is still detectable activity. Due to the use of crude homogenate there is the possibility of microbial contamination. The samples at room temperature became visibly more turbid over time suggesting this was the case. This was confirmed by observing the sample through a light microscope after gram staining.

Due to the enhanced stability of CHMO at 4°C *c.f.* room temperature the immobilisation trials were conducted at this temperature exclusively.

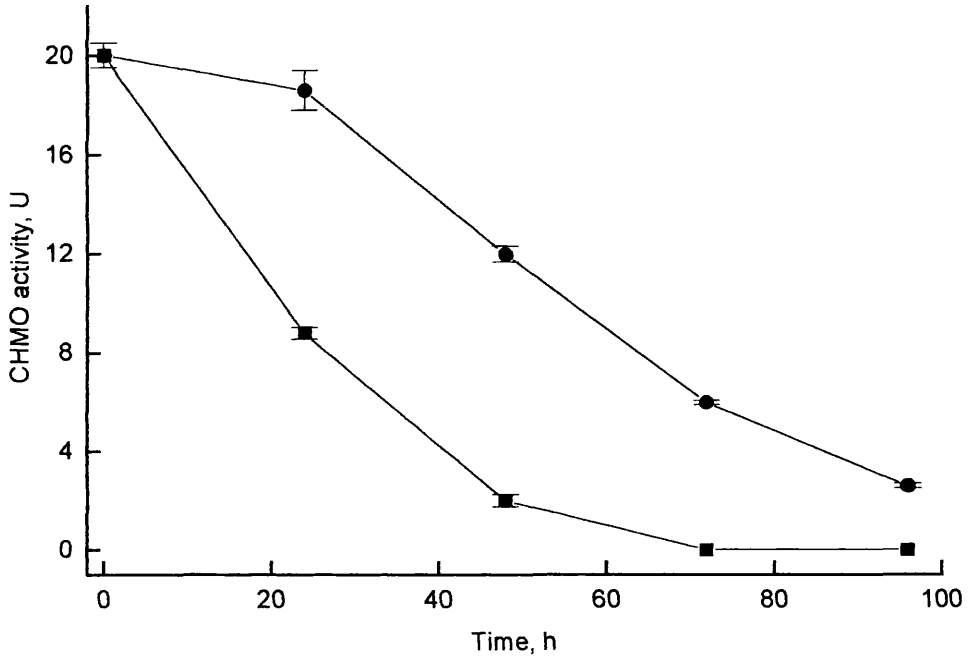


Figure 5.6 The stability of soluble CHMO under typical immobilisation conditions at room temperature(■) and 4°C(●).

Immobilisation trials were conducted at a range of phosphate buffer concentrations from 0.25-1M all at pH 9. The protein loading was kept at 90-100mg protein, 18-20U CHMO (5mL) per gram of dry Eupergit C (mid-way between the lower and upper limits for bead loading as recommended in the product literature). The time of exposure was (with one exception) kept at 72 hours, also as recommended. The residual activity of the beads (defined as the activity of the beads as a percentage of the total enzyme activity in solution at time zero) is shown below in **Table 5.1**.

Buffer concentration, M	**Residual CHMO activity, %**
0.25	1.02
0.5	1.35
0.75	3.17
1.00	3.70
1.00*	3.55

*Immobilisation ran for 96 hours

Table 5.1 Residual activities as a function of buffer concentration

Residual protein levels were in the range 40-50% and remaining CHMO activity in solution 10-15% after 72 hours. This indicates that the protein is binding to the beads. The residual activity of the beads was very low compared to the initial amount in solution; no greater than 3.7% was observed. The residual activity did seem however to be a function of buffer concentration as residuals increased as the buffer molarity increased. Increasing exposure to 96 hours also appeared to have no effect.

It has been suggested that enhanced retained stability after immobilisation can be due to inclusion into the reaction mixture of low concentrations of the target enzyme's substrate(s) (Thomas Boller, Rohm Pharma GmbH, personal communication). This is due to maintaining the correct protein conformation during

the formation of covalent attachments to the beads. This is one possible reason for low residual CHMO activity particularly as no protein binding problem is observed. A previous attempt to immobilise CHMO via covalent attachment onto poly(acrylamide-*N*-acrylsuccinimide) (Abril *et al*, 1989) used trace quantities of NADPH (0.2mM), cyclohexanone (0.07mM) and dithiothreitol (0.15mM). The latter compound providing increased protection to CHMO from oxidation. No data on the residual activity of the immobilised CHMO was given however.

The immobilisation trials were repeated using the quoted concentrations of the three compounds. The results of these trials are shown in **Figure 5.7**, also shown are the results of the original trials for comparison. Similar levels of protein binding and remaining soluble CHMO were observed. Retained CHMO activity was typically 3 times greater for a given concentration of buffer using the new method suggesting the importance of retaining CHMO tertiary structure during the binding procedure.

The highest activity was seen using a buffer concentration of 0.5M, this became the condition used for all subsequent preparations of immobilised CHMO producing a catalyst with a specific activity in the range 1-1.2Ug^{-1} dry beads. Further development was not considered as the retained activity of 12% was sufficient for studies within the scope of this thesis. The efficiency of the binding procedure is a complex function of temperature, pH, time, buffer molarity, protein loading, protein purity and trace compound concentration. There remains much in the way of potential areas of experimentation for improving CHMO activity retention.

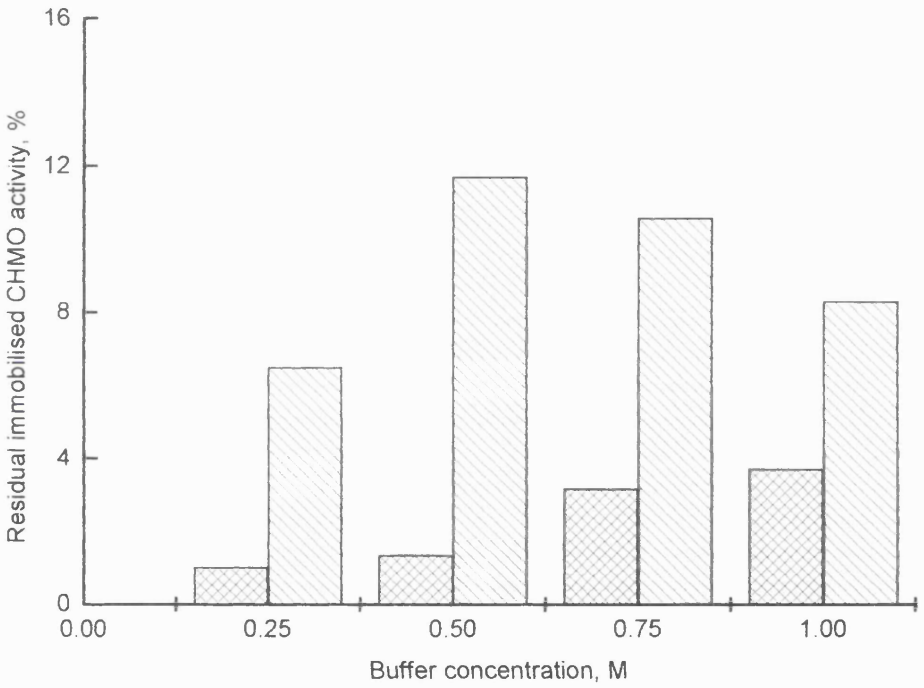


Figure 5.7 The CHMO activities retained after immobilisation, showing the original method(**XXX**) and the improved method(\\\).

5.4.1.3 Stability of immobilised monooxygenase

Immobilised CHMO was prepared as previously described, and incubated at 30°C in 50mM Tris-HCL buffer, pH 8 containing 50mM 4-MCH, 50mM 5-MOP and 0.1mM NADPH to simulate typical reaction conditions. The beads were periodically washed and assayed for activity over a period of 14 days. This is shown in **Figure 5.8**.

It is evident that the immobilisation procedure imparts greater stability to the enzyme than is seen in soluble form. After 14 days the enzyme retains approximately 70% of it's original activity, a loss of 2-3% per day. From studies of product and substrate toxicity it can be estimated that the enzyme would become completely deactivated in 1-2 days under the same conditions. This is approximately a 23 fold increase in potential operational use.

5.4.2 Alcohol dehydrogenase

5.4.2.1 Original Method

The method used to prepare immobilised TBADH was reproduced from the literature (Keinan *et al*, 1986). The enzyme preparation used was a partially purified commercial extract from *T.brockii*. The procedure was carried out using 0.1M phosphate buffer containing 1mM mercaptoethanol, pH7 for 24 hours at room temperature. Protein loading was 10mg of protein per gram of dry Eupergit C (approximately 33U). The procedure was carried out on a 5mL scale, using 1g dry Eupergit. A residual activity of 12.5% was obtained compared to 15% quoted (Keinan *et al*, 1986).

The difference between the literature value and the experimental value here could be for several reasons Firstly the source and purity of the TBADH was different and secondly the commercial preparation contained traces of dithiothreitol which could affect the protein binding.

5.4.2.2 Improved method

The immobilisation time used in the original method of 24 hours is at the lower limit of the suggested time from the Eupergit product literature. In an attempt to increase the retained activity the binding time was increased to 72 hours, whilst keeping other parameters constant. The procedure produced a catalyst with an improved retained activity of 41.4% and a specific activity of 13.6Ug^{-1} dry Eupergit C.

5.4.2.3 Stability of immobilised alcohol dehydrogenase

Stability of TBADH immobilised onto Eupergit C has previously been reported (Keinan *et al*, 1986). Continuous reactions were performed at 37°C for over a month with no observable loss of TBADH activity. Storage at 4°C for four months also resulted in no loss of activity.

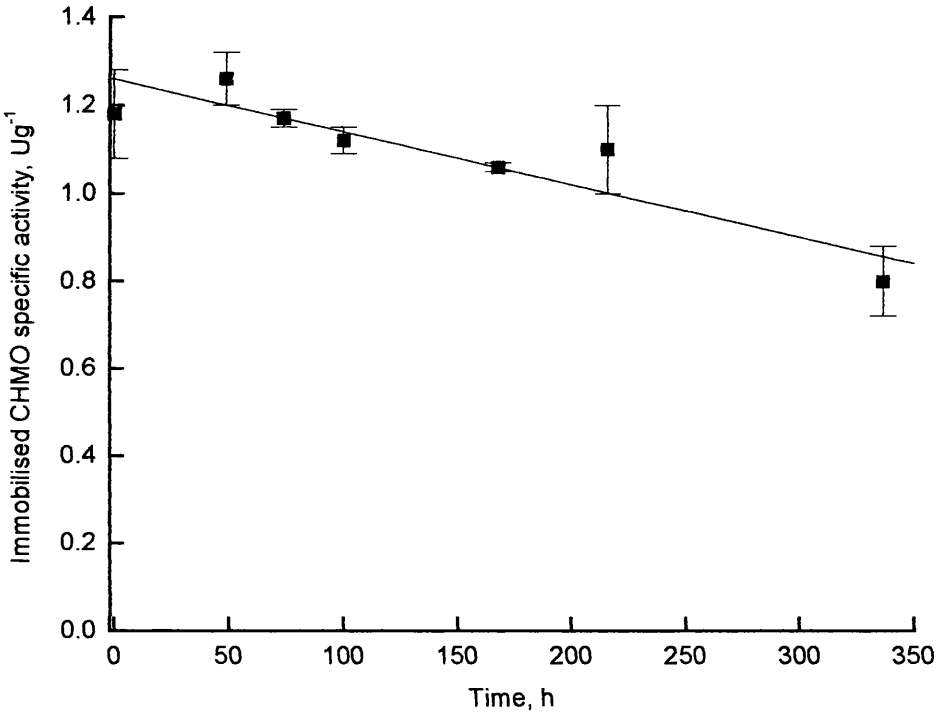


Figure 5.8 The activity of immobilised monooxygenase as a function of time under simulated reaction conditions.

5.5 Results of immobilised enzyme bioconversions

5.5.1 4-methyl cyclohexanone

The reaction profile is shown in **Figure 5.9**. Product accumulation was seen to occur approximately linearly. After 26 hours the beads were recovered by sedimentation and washed. The final yield of 5-MOP was a concentration of 60.1mM ($7.7gL^{-1}$). The TTN produced was 620.

5.5.2 2-hexyl cyclopentanone

The reaction profile is shown in **Figure 5.10**. Product appeared to be generated linearly. The final yield of 6-HTPO was 36mM ($6.6gL^{-1}$), a TTN of 742 was generated.

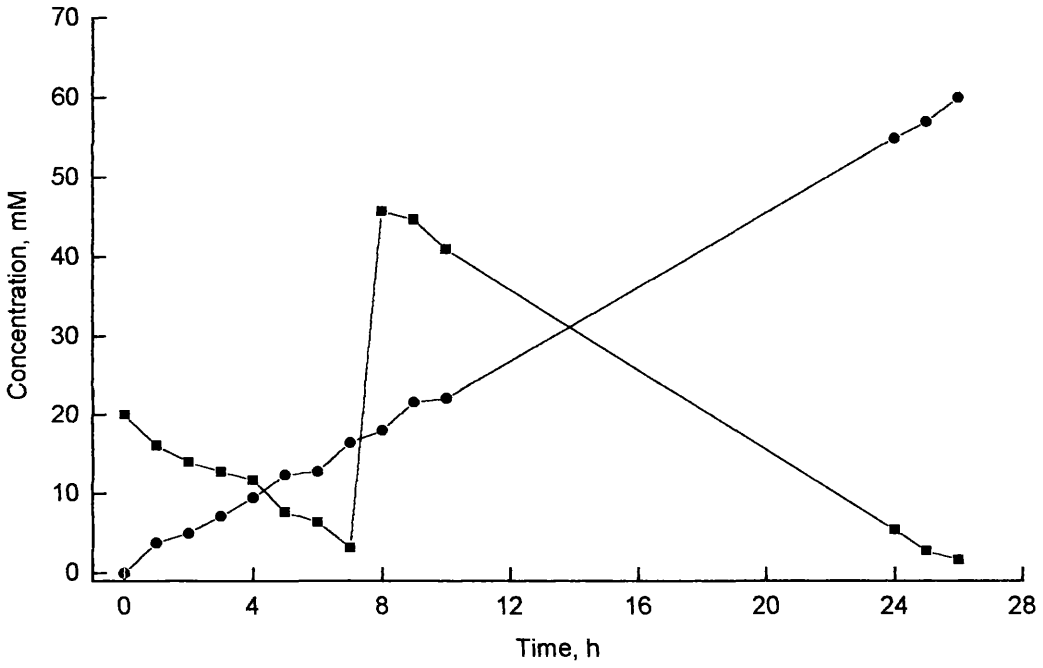


Figure 5.9 1.5L immobilised enzyme reaction showing 4-MCH(■) and 5-MOP(●).

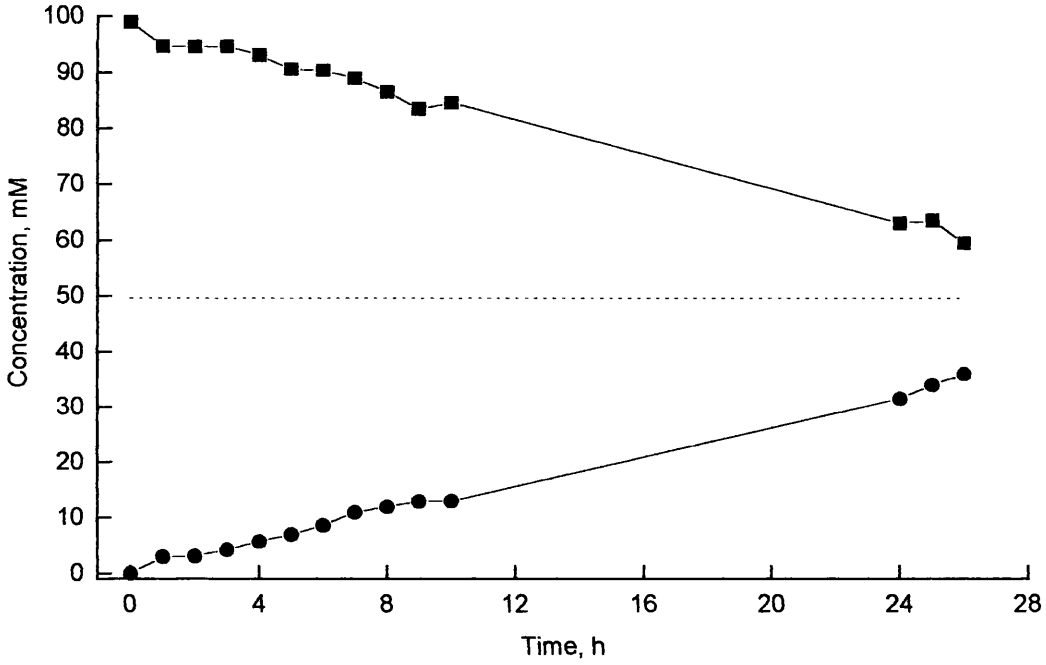


Figure 5.10 1.5L immobilised enzyme reaction showing 2-HCP(■), 6-HTPO(●) and the theoretical reaction limit(····).

5.6 Co-immobilisation of both enzymes

5.6.1 Rationale

In order to use the CHMO/TBADH system as a commercially viable process it would be necessary to have a recombinant source of TBADH due to it's expense. This has been achieved in *E.coli* with expression 30 fold higher than in *T.brockii* (Peretz *et al*, 1997). The ideal situation would be an engineered strain containing both the gene for TBADH and CHMO. If these genes were on the same promoter and expression system then it follows that the polypeptide chains would be expressed in equimolar amounts assuming no limitation in the supply of amino acids. Knowing the specific activity of the pure enzymes and their molecular weight it is possible to calculate the fractional activities (as used in chapter 4) that would be present in a recombinant organism. The calculation for this is shown in Appendix 4. If the assumptions quoted above are true the CHMO fraction (in terms of activity) would be 44.1%; TBADH being 55.9%.

An immobilised catalyst containing both activities can then theoretically be prepared from the homogenate of the CHMO/TBADH clone in one procedure. The difficulty in this procedure is the conflicting environment required by the two enzymes for efficient retained activities and so a compromise must be reached that may not be entirely satisfactory.

5.6.2 Procedure

A simulated homogenate of an engineered *E.coli* strain containing both enzyme activities was prepared firstly by quantifying CHMO levels within clarified lysate and dissolving commercially prepared TBADH into it in the correct activity proportion. In 5mL of lysate CHMO activity was assayed and found to be 28.4U in total. This required the addition of 36.6U of TBADH (32mg). 1g dry Eupergit was added and the immobilisation conditions used were those that favoured CHMO activity retention, as detailed in section 5.4.1.2 (TBADH is more likely to immobilised with greater retention of activity under non-ideal conditions than vice versa).

After 72 hours it was found that of the 152mg of protein initially present in solution, 12.56mg was still soluble, a binding efficiency of 91.7%. Soluble enzyme activities after this time could not be accurately quantified due to interference from the other enzyme within the assay.

5.6.3 50mL reaction

The beads were washed and suspended in 50mL of 50mM Tris-HCL buffer, pH 8 containing 0.1mM $NADP^+$, 10mM 4-MCH and 12mM 2-propanol. The reaction mixture was shaken at 30°C and samples periodically taken for GC analysis. The reaction profile is shown in **Figure 5.11**. Product was formed at a rate of 0.036 $mmol.h^{-1}$ equating to a specific activity of $0.6Ug^{-1}$ dry Eupergit C.

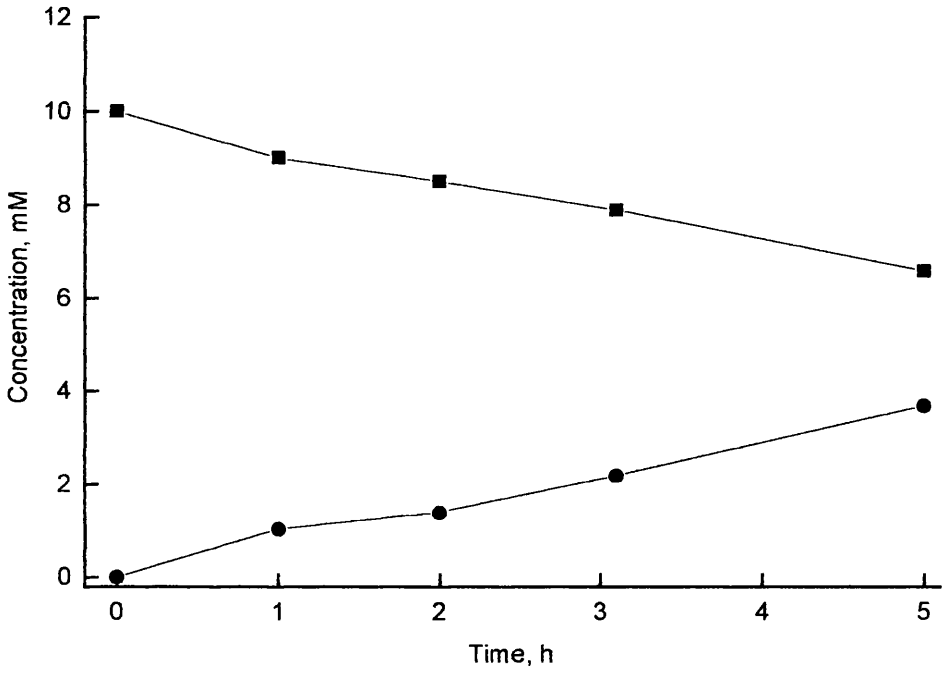


Figure 5.11 Reaction using co-immobilised enzymes, showing 4-MCH(■) and 5-MOP(●).

5.7 Discussion

5.7.1 Free enzyme studies

From the studies conducted using free enzyme it is clear there are a number of benefits to this form of catalyst over whole cell and immobilised enzyme. The reaction rates seen were much closer to the maximum possible *c.f.* whole cell studies (where reaction rates were found to be very low). There is however the extra expense of additional cofactor and of the second enzyme. On 100mL scale using 4-MCH as substrate the reaction rate was observed to be initially rapid, becoming more linear. This may be due to a limitation in the removal of acetone from solution, particularly in shake flasks where the mass transfer rate may be low. Similar experiments using 2-HCP, which forms a second phase did not indicate the slowing of product formation. This may be because acetone partitions in some degree into the organic phase and removes some of the inhibitory effect on TBADH. The use of a second phase was successful in raising product yields above the aqueous solubility limit. The formation of an emulsion was observed, which was not unexpected as there was a significant amount of dissolved protein. This did not appear to affect the formation of product at 100mL scale although the purification of 6-HTPO is made more challenging than from a whole cell or immobilised enzyme reaction. It is also unclear as to the mass transfer characteristics of the system, the rate of mass transfer from the organic to aqueous phase may or may not be limiting and so measurement of the mass transfer coefficient should be a target for future studies. At 1.5L scale the increased agitation and aeration coupled with a second liquid phase seemed to cause the rapid deactivation of CHMO, the yield produced from the 2-HCP reaction was very low and the reaction mixture became visibly more opaque. The single phase of the equivalent 4-MCH reaction did not seem to cause the same effect on the protein, and the yield produced was more satisfactory. There was however the limitation on agitation rate in both reactions due to excessive foaming above 250rpm, a serious

bottleneck in supplying oxygen to the reaction. Using homogenate also presents the problem of maintaining sterility, homogenate is extremely vulnerable to microbial attack, the implication is that the reaction may be halted prematurely limiting the yield, the process may also be difficult to validate on a large scale.

5.7.2 Immobilised enzyme studies

Initial attempts to immobilise CHMO were not successful, retained activities upon immobilisation were disappointingly low. The inclusion of trace amounts of reaction components acted to preserve the integrity of the active site during the immobilisation procedure, although the retained activities only improved to a maximum of 12%. The optimisation of all the variables that affect the retained activity (temperature, pH, time, buffer molarity, protein loading, protein purity and trace compound concentration) is very much still a 'hit and miss' procedure with little in the way of a systematic approach. The retained activity of 12% therefore still represents an unoptimised system but it does permit the use of the immobilised enzyme to perform a number of studies. Judicious selection of the immobilisation conditions can increase activity of the enzyme upon immobilisation, such as Transketolase from *E.coli* (Brocklebank *et al*, 1996) which initially produced residual activities of 20% and after a period of development work this had increased to 60%. The specific activity of immobilised CHMO produced was only in the region 1-1.2Ug^{-1} which is also low. Possibly this is due to the dilute nature of CHMO within the clarified lysate, where it represents *ca*1% of the total protein. Immobilisation of both enzymes simultaneously from one solution produced a catalyst with a specific activity of 0.6Ug^{-1}, this is lower due to the compromise in conditions that have to be used for both enzymes resulting in lower binding for both. However this does illustrate the potential for immobilising a catalyst in a single procedure from a lysate of a recombinant *E.coli* strain containing both enzyme activities.

The stability of the immobilised form of CHMO illustrated the increase in stability that is often generated by this technique. Enzyme activity decreased only gradually in the presence of elevated levels of both 4-MCH and 5-MOP. This compares well with soluble enzyme, which under similar conditions would be completely deactivated in 1-2 days. However this increase in stability must be offset against the drop in activity seen during the immobilisation procedure. The overall effectiveness of either form of the enzyme is thus the amount of product it will form per unit of activity initially produced within the cell. This can be calculated by integrating under a curve of activity as a function of time. This is shown in **Figure 5.12**. The basis used is 1 unit of CHMO. This becomes 0.12 units after immobilisation. Although the immobilised form is likely to be active beyond the 14 days of the study, this was considered the limit of the use of the immobilised form, as it is unwise to extrapolate the activity beyond the timescale actually measured.

Free enzyme theoretically will produce 1.44mmol product per unit of activity whereas immobilised enzyme produces 1.88mmol, an increase of 30%. The increase is only modest due to the loss of activity upon immobilisation. If the residual activity of the immobilised enzyme were to be increased to e.g. 45% the increase in productivity rises to 360%. The enzyme was only assayed over 14 days and from inspection of the stability plot it is more likely that the figure of 30% would rise if a longer stability trial were conducted. This is not the entire picture as free enzyme can realistically only be used once whereas the immobilised form allows more flexible processing by introducing the possibility of repeated batches.

Losses of immobilised TBADH activity over time are reported to be extremely low under processing conditions (Keinan *et al*, 1986) such that periodically a percentage of the catalyst would have to be re-charged to make up for declining levels of immobilised CHMO. TBADH was immobilised with much higher residual activity (42%) and to a higher specific activity ($13.6Ug^{-1}$). The higher retained activity is to be expected as the protein is highly resistant to denaturation and conformational change. The specific activity is higher as the source of the protein was initially much purer than the preparation of CHMO.

Reactions performed with the immobilised enzymes illustrate some of the potential benefits and disadvantages of using CHMO in this form. Separation of catalyst is much less problematic than using soluble enzyme. The beads of Eupergit C easily settle out from solution and product rich liquor can simply be decanted. The product stream thus only contains a small quantity of salts (from pH control) and cofactor. Enzyme kinetics often change when immobilised due to mass transfer limitations of the substrates gaining access to the enzyme within the bead, for example the value of apparent K_m often rises. In the case of cofactor the K_m value was shown to be critical in running a recycle system efficiently (chapter 4), any increase in this value due to immobilisation (for either CHMO or TBADH) may necessitate running the reaction with additional cofactor to compensate, lowering the TTN achievable. The low retained activity could also be due to a change in the kinetics, a 4-MCH concentration of 2mM is used in both the soluble and immobilised assay. This is far in excess of the K_m value for soluble enzyme (0.016mM) but the value for immobilised enzyme may be higher and the assay may be kinetically limited. Upon closer inspection this is unlikely because the 2L reactions contained higher 4-MCH concentrations and the reaction rate did not appear to be significantly higher. The distribution of immobilised protein has previously been shown to occur evenly throughout an Eupergit C bead (Brocklebank, 1998) this offers CHMO protection from gas/liquid and liquid/liquid interfaces. Higher agitation rates can be used without fear of foaming or protein damage, the result being a 'de-bottlenecking' of the supply of oxygen to the reaction. The rapid deactivation of CHMO in the 2-phase agitated reaction would also not occur.

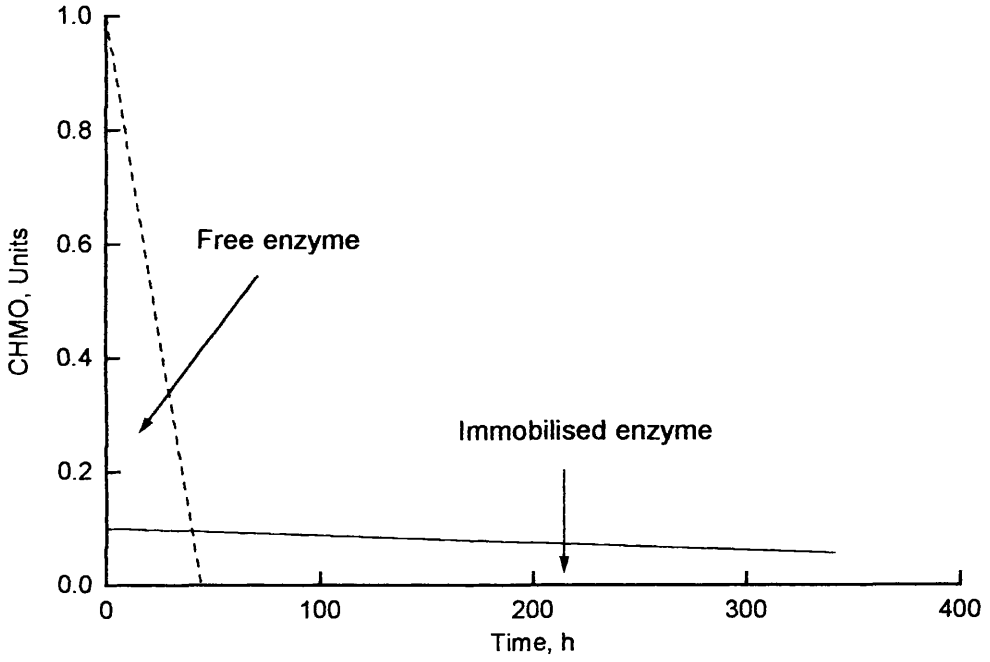


Figure 5.12 Comparison of the productivity of free and immobilised CHMO

It is seen from both 1.5L experiments that the TTN produced is above the proposed level of economic operation of 500. The synthesis of 5-MOP produced a TTN of 620 and that of 6-HTPO 742. 6-HTPO partitions almost completely into the organic phase comprising the unreacted enantiomer of 2-HCP. The separation of the organic phase leaves an aqueous phase containing little ketone and lactone together with most of the initial cofactor. It is thus possible to re-use most of the cofactor to perform another batch. The TTN will in effect increase for every batch completed with the same initial aqueous phase. A small addition of cofactor will be needed after every batch to account for degradation and processing losses during phase separation. The TTN becomes the overall product accumulation over *n* batches in relation to the total cofactor added over *n* batches. From the results of chapter 4 the half life of the cofactor can be estimated, knowing the pH used and the relative activities of the two enzymes. Assuming an arbitrary loss of 10% of the aqueous phase upon phase separation and that the reaction time is constant the value of TTN as a function of batch number can be calculated. The calculation is laid out in Appendix 5. Equation(18) below describes the relationship between batch number, TTN, yield of product in organic phase and cofactor recharge rate.

$$TTN_n = \frac{Yn}{C + R(n-1)} \qquad (18)$$

The theoretical increase in TTN over 15 batches is shown in **Figure 5.13**. After 15 batches the TTN rises to 3400. It can be seen that the overall TTN begins to approach a limiting value. This is because the bottom half of the equation increases proportionally faster than the top half compared to the value at batch number 1. The TTN of the overall reaction may increase in another way. If the unreacted R-enantiomer of the ketone also had a useful lactone derivative it could be prepared by subsequent chemical 3-CPBA oxidation, as the stereochemistry will be preserved. Effectively the TTN applies equally to this lactone as well as the S-enantiomer that was synthesised directly by CHMO. All TTN values could in effect be twice as much as they appear.

This re-usability of the aqueous phase in theory could apply to the synthesis of more water soluble compounds such as 5-MOP if recovery could be made directly from solution. As long as the cofactor remains active and in solution this technique would have the same effect on TTN.

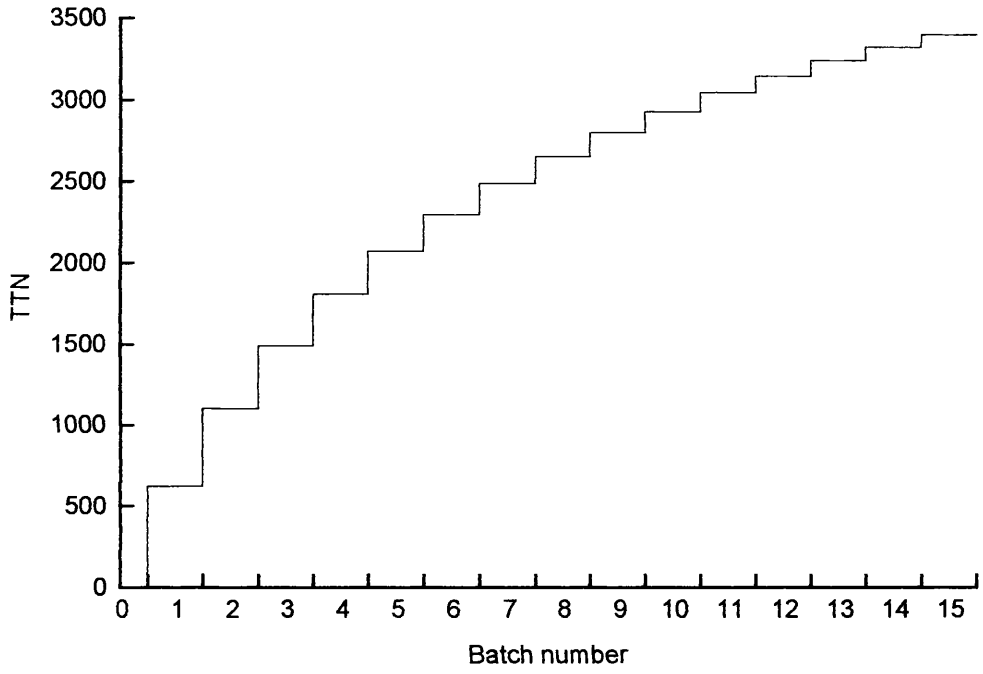


Figure 5.13 The effect of batch number on TTN in the synthesis of 6-HTPO.

5.8 Summary

In this chapter TBADH was employed to supply CHMO with reduced cofactor in a number of reactions at both 100mL and 1.5L scale using the poorly soluble substrate 2-HCP and the more soluble 4-MCH. Reactions were carried out using both soluble and immobilised enzymes and the yield and TTN recorded for each. These are summarised below in **Table 5.2**.

Substrate	Catalyst	Scale, L	TTN	[product], gL^{-1}
4-MCH	Soluble	0.1	433	5.4
2-HCP	Soluble	0.1	253	2.3
4-MCH	Soluble	1.5	321	4.1
2-HCP	Soluble	1.5	77	0.7
4-MCH	Immobilised	1.5	742	6.6
2-HCP	Immobilised	1.5	620	7.7
2-HCP	Immobilised	1.5	3400*	7.7

*Theoretically after 15 batches

Table 5.2 Summary of CHMO syntheses

The required TTN value of >500 was only achieved in both of the larger scale immobilised enzyme reactions.

Immobilisation confers superior stability to CHMO and TBADH, although the productivity of CHMO upon immobilisation only increases by 30% due to the low retained activity. Immobilisation protects CHMO from interfacial damage, particularly in the case of liquid/liquid interfaces when 2-HCP is the substrate. Immobilised enzyme also simplifies product recovery, and introduces the possibility of re-use of the cofactor over several batches

.

6.0 General Discussion

In section 1.5 the aims of this thesis were laid out, in particular the applicability of the structured design approach was to be investigated in relation to redox biocatalysis using the Baeyer-Villiger oxidation of ketones by cyclohexanone monooxygenase as a representative example. This class of enzymatic catalysis has a number of distinct features, most notably the requirement for NAD(P)(H) cofactors. It was proposed that this feature may make redox biocatalysis a special case, and as such the structured approach may not be appropriate as for other classes of biocatalysis. The choice of two model substrates, a water soluble asymmetric reaction and a poorly soluble resolution were made in order to investigate as wide a range as possible of the type of molecule the enzyme may be presented with. It was initially thought that the difference in reaction class (asymmetric vs resolution) would have the biggest influence on the way the process would have to be operated, for example the buildup of the unreactive enantiomer in resolution reactions is a feature that would on inspection be significant with implications for inhibition and toxicity. It was found that the most significant feature of the reactants and products was the solubility. This feature defines the effects on the process that the molecule will have, for example the poorly soluble system of 2-HCP/6-HTPO did not cause inhibitory or toxic effects on CHMO precisely because the concentration in the enzyme's environment was very limited. The solubility of the molecules also make the class of reaction unimportant, poorly soluble reactions will have to be operated with a second liquid phase which in the case of a resolution reaction acts as a reservoir for the unreactive enantiomer. The system behaviour with a second phase thus approximates to an asymmetric reaction. The reversal of this argument also applies as a resolution reaction with a highly soluble substrate also makes the unreactive enantiomer less important as there is no constraint of low productivity caused by solubility. Toxicity due to the unreactive enantiomer has also been drastically reduced by immobilisation of CHMO, as has the denaturating effect of the second liquid phase. Substrate feeding in more soluble reactions is a likely feature to

overcome inhibitory effects, with poorly soluble reactions the substrate is also fed but it occurs automatically from the second phase.

From these discussions it is clear that the structured approach has been of use in identifying those process options that clearly were not suitable whilst suggesting those options that should be studied further (FBSTR for soluble reactions, TLPBSTR for poorly soluble reactions). The reactant/product solubility was identified as the characteristic that has the greatest influence on process suitability (see **Figures 2.6** and **2.7**). What did present itself from these studies was that no alternative to whole cell catalysis was suggested (in that free enzyme cannot function without reduced cofactor). This is of particular concern because the whole cell form was shown to be impractical as a catalyst. Isolated enzymes themselves are generally not problematic to use however with redox enzymes the need for cofactor is paramount, NADPH is simply too expensive to be supplied stoichiometrically. Although the structured approach suggests a cell free system the 'special case' of redox enzymes is not addressed at all by this procedure and is a serious omission. The process is defined with this one vital piece of the design left out, and cannot be operated at all unless the method of cofactor supply is in place. Particular attention to the form of catalyst is necessary in future studies of other redox systems. It was decided to increase the scope of the characterisation studies embedded within the structured approach to address cofactor supply, however the study was limited to enzymatic methods, which are generally accepted as the only current practical option.

There are a number of ways this could have been approached, but it makes the most sense to apply the same methodology as has been used in the earlier investigations. In that way any recommendations or modifications to the formal design procedure should be easier to implement. The rationale behind the choice of approaching this by a detailed investigation into the applicability to two dehydrogenase enzymes has been discussed elsewhere. On inspection both enzymes appeared to be as suitable as each other to recycle NADPH to CHMO. On closer inspection the suitability of the enzymes became divergent as the characteristics of them were examined and dissected, showing that the suitability on paper is often misleading, TAGDH looked a very attractive option however under conditions required for economic operation

(i.e. low cofactor concentration, pH 8 for CHMO activity) the situation looks very different. This stresses the importance of a methodical investigation into this kind of problem and the current vacuum of knowledge in this area of rational choice of recycle enzyme.

The most important lesson that comes out of the investigation of TBADH and TAGDH is the need to collect the data on these types of enzymes in order to have the ability to form a judgment to decide if any of the candidates are suitable for the application of interest. It is proposed that there are six key areas that need investigation, these being:

- Cost of sacrificial substrate (in the context of the synthetic product value).
- pH/activity profile compatability.
- Effect of product inhibition/ease of ISPR.
- Affinity for cofactor (K_m) - defines the lowest reasonable [cofactor].
- Toxic or inhibitory effect on the synthetic reaction
- Effect of the synthetic reaction on recycle enzyme

The hierarchy of the questions is left open as different processes may have different priorities (due to the economics of the case in question) and in any case knowledge of all the data listed above would help to make a choice in context. One of the proposed benefits of the structured approach is the speed at which design can be completed. If there is the need for cell free recycling of cofactor it would seem that the additional experiments required are considerable and the suitability of this approach questionable. On inspection however the first four of the characteristics listed above are independent of the target reaction, only the last two are specific to the application being investigated. This generality of most of the data required suggests that it would be possible and prudent to gather this data first (a great deal of which may be available in the literature) to form a 'library' of data to refer to on a range of candidate enzymes. The extra experimentation required to form a judgment

on the most suitable enzyme to the target application would thus be rapid. It may also be possible to use this library of information alone to 'pre-select' a few of the more promising enzymes for further evaluation. One thing to note in this discussion is that the proposals suggested are based on this one single example of redox biocatalysis and the investigation of only two candidate recycle enzymes, in order to have greater confidence in these proposals a number of other examples should be given the same systematic treatment to validate the technique and increase confidence.

The problem of cofactor supply was considered only after the initial characterisation work was completed, resulting in the inevitable situation that the process conditions were in effect fixed and inflexible, e.g. the reaction pH/oxygen supply via aeration was laid in stone; the two dehydrogenases had to fit into this inflexible design brief. This clearly is not the ideal approach to process development as the introduction of artificial inflexibility may exclude some processing options from consideration, and inefficient design may result. Clearly a better approach would be to identify if cell free operation is required early in the design procedure for redox enzymes, the subsequent assessment of process options would keep the need for a second enzyme in mind, ideally attempting to develop the process as consisting of two catalysts from the outset.

Another stated aim of this research was to develop numerical tools to allow the designs proposed by the structured approach to be quantified without detailed experimentation. This is an important consideration as the confidence in the design will be greater if it can be shown in absolute terms what the yield will be, particularly in relation to other process options. Clearly it is also possible to consider other objective functions such as product concentration but the study is limited to total product produced in the scope of this thesis. This issue of comparative yield becomes more important in the context of the need for a second enzyme, as any data on process performance is more difficult to interpret when there is another equally vital catalyst present. The need to identify windows of operation that satisfy yield and cofactor turnover would also be made easier if numerical techniques were developed, particularly as one of the chief reasons for disappointing performance

from recycle systems has been suggested as poor choice of reactor environment due to a lack of information concerning the system (Lee and Whitesides, 1985).

The identification of the windows of operation for different required cofactor stabilities highlights the danger in this type of system for underperformance. In some cases the window was very restrictive in both CHMO fraction and operational pH. The windows again illustrate how close to uneconomic operation these processes may be operating. Although the economic analysis used the CHMO/TBADH system, and some of the scenarios considered were not realistic to this specific case the substitution of other redox systems into the analysis may involve these scenarios as more representative of the actual process economics.

The predictive work carried out using the 4-MCH system allowed the process design proposed in the earlier chapters to be assessed in relation to its productivity compared to other designs. It was shown that the choice of a FBSTR was justified, producing significantly higher yields than batch processing. The modelling studies also illustrate another area of design that could use this approach, that being the assessment of process improvements. The modelling studies illustrate how the incorporation into the process of ISPR may improve yields. It may be decided that there is a figure of yield improvement below which it is not worth implementing such a technique. The assessment of yield improvement with varying degrees of ISPR efficiency (as carried out in chapter 4) would give access to this information.

These data illustrate the importance of quantitation but also raises a series of questions relating to the use of models. For example what is the best use to be found for a potentially powerful tool and to what specific goals should it attempt to reach? What is the role of windows of operation? At what point in the design procedure should they be implemented? In the studies undertaken within this thesis the use of windows was to define the chemical/physical environment within which the process should be operated, whilst models both generated the data for the windows and provided a measure of comparative process performance. This may not be the best use of these techniques and it is possible that in many cases models should be introduced earlier or later in the design procedure for greater efficiency. The

structured design procedure aimed to highlight unsuitable options quickly so that rapidly the choice of process becomes a decision between only a small number of options. The introduction of models too early in the procedure requires a potentially large volume of detailed kinetic information to be gathered and the time taken to implement the models may be better spent elsewhere. A more efficient use of time would be to use the structured approach as before and then use models on a much smaller number of options. An overall design procedure must be both *rapid* and *accurate*. Models can provide accurate data, but may not be rapid whereas qualitative selection tools may not be as accurate as models but can quickly screen process options. This is illustrated diagramatically in **Figure 6.1**. The most effective design procedure provides confident design in as short a time as possible. An unstructured approach will involve time lost in unnecessary experimentation which is improved by following a structured approach. Further improvement is seen when this is combined with quantitative analyses. Clearly process design should incorporate the best features of each whilst minimising their drawbacks, essentially deciding where qualitative selection ends and other techniques begin.

The role of windows of operation in the design procedure is also of importance, as has been demonstrated in assessment of the conditions required for prolonged cofactor stability in chapter 3. The data used to generate these particular windows were provided by steady state modelling. In all cases the data gathered from characterisation studies can be used to generate windows, for example in the CHMO system the window of operational pH is also a function of lactone stability and enzyme activity. The role of windows would seem to be to complement both the characterisation studies conducted within the structured approach (by expressing them in a graphical form) and quantitative analyses. The windows produced are effective at identifying the physical environment that the process must possess. A further application of windows is to use them in conjunction with quantitative analyses aimed at assessing process options and possible future process improvements. Any potential alteration to a design to improve performance must still operate at all times within the imposed constraint of the window(s). In some systems the operating window may be completely 'closed' unless a constraint is removed, for example product inhibition/equilibrium effects may be dominating the reaction

kinetics. Quantitative analysis can determine the improvements to productivity that ISPR may offer but the window must at the same time provide an area of feasible operation.

Using the conclusions reached from the rational investigation into cofactor recycling, coupled with the data gathered from the model, the structured design procedure can be improved for biocatalysis in general and also special considerations arising from redox biocatalysis can be addressed, such that the procedure is improved. This should produce process options through qualitative assessment which can be verified for their relative performance by process models. The design procedure will be more robust, and the process design can be implemented with greater confidence. Pilot scale trials will be reduced in number using this approach, this may be especially significant where the catalyst or reaction substrate is extremely scarce and such pilot scale trials may be prohibitively expensive. Process improvements can also be easily piloted using the modelling approach. The proposed modified approach is best illustrated diagramatically by comparing the original design procedure (**Figure 1.4**) with the equivalent modified figures, **Figure 6.2** being for biocatalysis in general and **Figure 6.3** for the special case of redox biocatalysis.

The modified approach for 'general' biocatalysis differs from the original flowsheet at the point where several candidate process options are identified. The model is the tool used to make the final process choice. This offers the best compromise between accuracy and speed of design as discussed previously. The point at which qualitative assessment ends and quantitative modelling begins is the critical factor. The process model can then be used to assess the potential for process improvements that can be implemented at a later date. The flowsheet for redox reactions is based upon this, however at the point that process constraints are defined the identification of the process options is made by application of rules of thumb as before coupled with the 'library' of knowledge containing data on potential recycle enzymes. The need for a second recycle enzyme is dependent on the whole cell catalyst proving to be unsuitable and thus the form of catalyst must be identified early on. The process choices can be defined with greater confidence, and the use of models then applied to identify operating windows and calculate process performance as before. Simple

steady state data can define the operating conditions and windows whilst models describing entire process runs define the relative performance of reactor modes. Within the flowsheets the definite decision paths are shown as filled lines whereas other decisions that may or may not be appropriate are shown as dotted lines

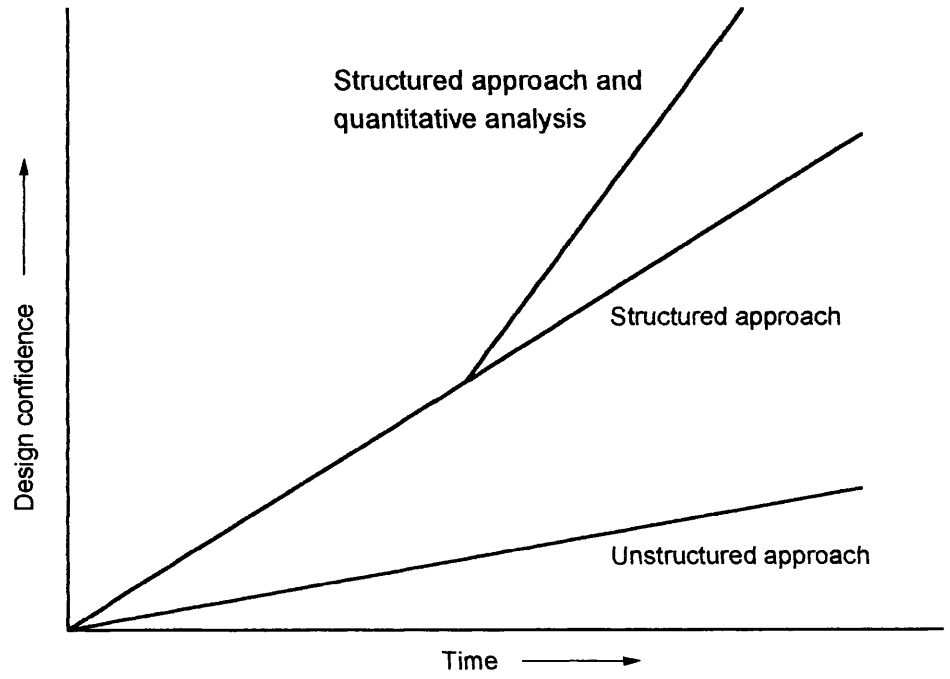


Figure 6.1. Relative effectiveness of design approaches.

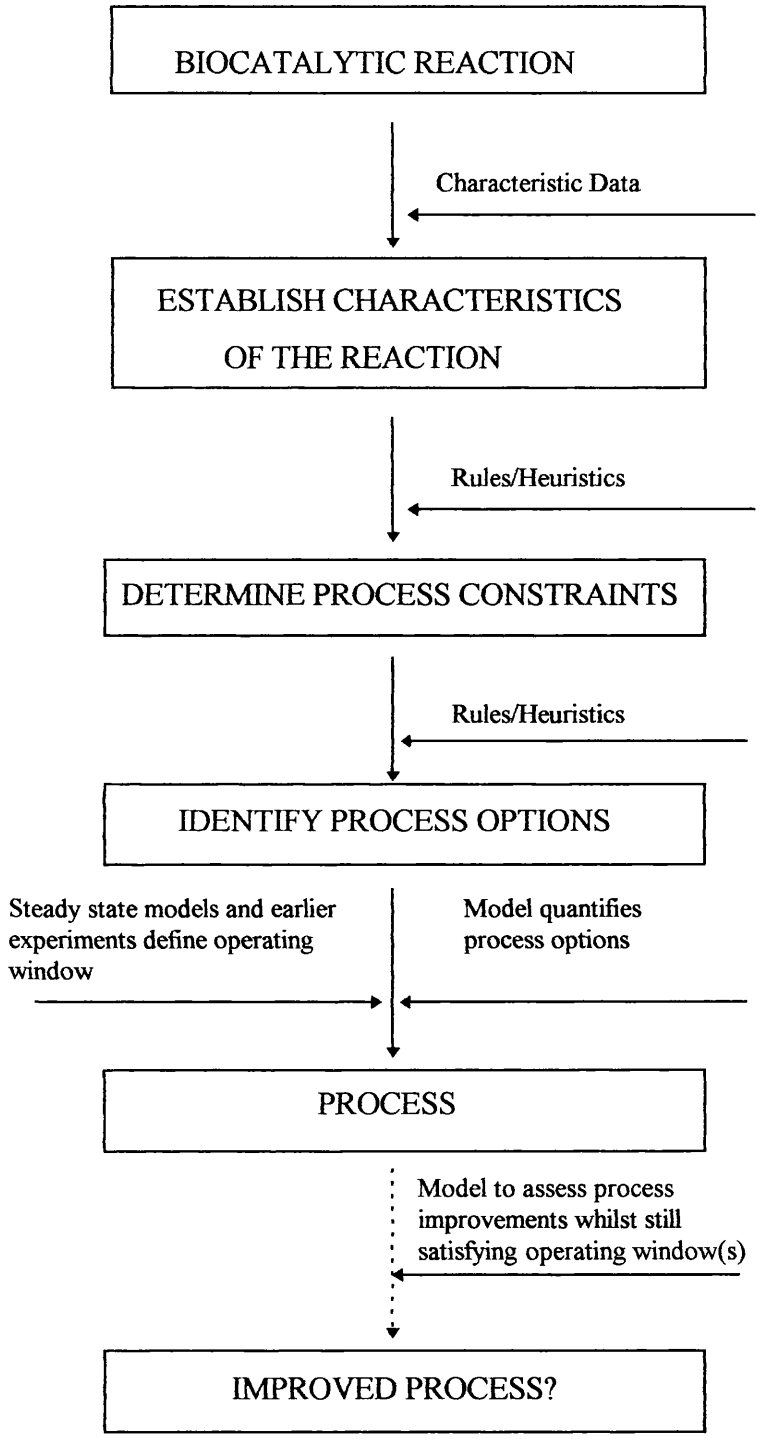


Figure 6.1. Improved biocatalytic process design methodology

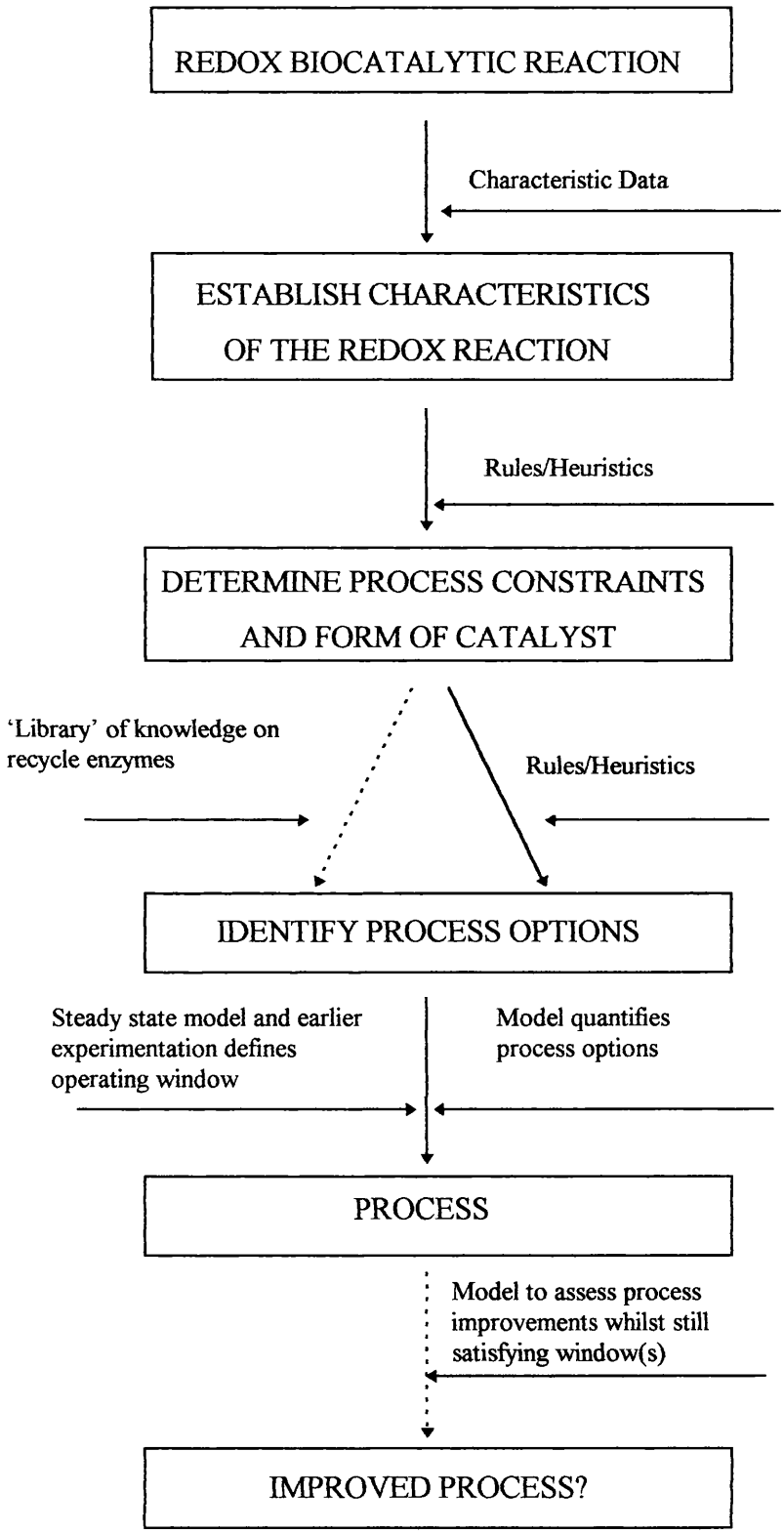


Figure 6.2. Improved biocatalytic process design methodology for redox reactions

The data gathered from the characterisation and modelling studies has demonstrated the feasibility of CHMO based lactone syntheses. However it is important to demonstrate the technology and produce multi-gram quantities of the two lactones under consideration. The limit imposed upon the scale of reaction was dictated by the cost of TBADH from a commercial source but the reactions carried out within a 2L agitated bioreactor demonstrate the potential of the system. Of major importance to the overall cost of operating the process is the TTN value produced. Although the value required is subject to a lack of definition in the literature, values quoted range from 10^2-10^3 and so 500 was chosen as a benchmark. This target was exceeded with both substrates using immobilised enzymes, TTN could easily rise to 3400 when 6-HTPO is produced if the aqueous phase is recycled. The feasibility of immobilising both enzymes from a single cellular homogenate is also achievable, if both genes were present within a recombinant organism. Although the specific activity of the immobilised catalyst was low this was largely due to low enzyme titres within the recombinant organism, which could be addressed by either an improved expression system within the host or downstream purification of the target protein.

Enantiomerically enriched 5-MOP and 6-HTPO were both synthesised at the 10g scale, this would easily rise to a kg scale process if a bioreactor of approximately 150L was employed and the design presented in this thesis was implemented. This process, consisting of NADPH recycle and oxygen supply with two redox enzymes immobilised upon a single support demonstrates the value of structured process development and process models to biocatalysis as a practical industrial option in fine chemicals and pharmaceuticals manufacturing.

7.0 Conclusions

7.1 The biocatalyst

1. Cyclohexanone monooxygenase from recombinant *E.coli* JM107 pQR210 was obtained by batch fermentation at 2L scale on a glycerol carbon source and complex media. Productivity was in the range 500-700UL^{-1}

2. The pH/activity profile is broad with maximum activity at approximately pH 9.0. CHMO is stable above pH 7.5, below this value stability decreases.

3. CHMO is monomeric with a tightly bound FAD prosthetic group that does not dissociate. NADPH is an essential cofactor that provides reducing power.

4. Enzyme stability is adversely affected by the presence of oxygen and gas/liquid interfaces. Residual activity is 20-30% lower after 24 hours exposure. Stability is higher under anaerobic conditions and in the presence of >0.1mM NADP(H). After 24 hours activity remains at 90-95% of it's initial value

7.2 The model bioconversion

1. 4-methyl cyclohexanone and 2-hexyl cyclopentanone were selected as model substrates and a GC method developed to separate and detect them.

2. The model ketone substrates were stable within the range pH7-10. Lactone products were labile in alkali, the rate of degradation increasing with increasing alkalinity. Residual levels varied from 100% at pH 7 to 30% at pH 10 after 24 hours. All model reaction components are liquids under ambient conditions and were not significantly volatile.

3. 2-HCP/6-HTPO were soluble in aqueous to less than 1gL^{-1}. 4-MCH/5-MOP were both soluble to greater than 20gL^{-1}.

4. 2-HCP/6-HTPO were not toxic or inhibitory to the enzyme up to the solubility limit. 4-MCH/5-MOP were inhibitory above 2mM and caused enzyme deactivation to occur at a level of 50mM. The rate increased with

increasing concentration. At 100mM 5-MOP, CHMO deactivation was observed to be 80-90% in under 5 hours.

5. Whole cell bioconversions at pH 8 produced reaction rates <2% of the potential product formation rate for both model systems. Intracellular enzyme was completely deactivated after 5 hours. Reactions conducted with 5% ethanol and 1% toluene failed to increase reaction rate.

6. A FBSTR was proposed as a reactor for 4-MCH based synthesis and a TLPBSTR for 2-HCP reactions. Both included sparged aeration and isolated CHMO as the catalyst.

7.3 Recycling of NADPH

1. Four dehydrogenase enzymes were selected as possible candidates for recycling of NADPH to CHMO from $NADP^+$. Two thermostable enzymes were selected (TBADH and TAGDH) on the basis of cost for further study.

2. TBADH showed a broad pH/activity profile with an optimum pH of 8. TAGDH showed a narrow response with a maximum at pH 7.

3. Both enzymes were characterised by product inhibition above 1mM, acetone on TBADH and gluconate on TAGDH. No substrate inhibition was seen up to 300mM. No toxic effect was seen by reaction components on either enzyme. CHMO was found to be kinetically inhibited by 2-propanol.

4. The model CHMO reaction components had no effect on either enzyme except 50mM 4-MCH on TAGDH, causing 50% irreversible activity loss after 24 hours.

5. TBADH had an affinity for cofactor an order of magnitude greater than TAGDH; $K_m(NADP^+)$ being 0.013mM and 0.113mM respectively.

6. TBADH was selected to perform cofactor recycling to CHMO. Selection criteria for recycle systems have been developed.

7.4 Process modelling

1. A model was developed describing 5-MOP synthesis was based around the differential rate equations of TBADH and CHMO. The model was solved by iterative numerical solution using a spreadsheet. The effects on TBADH of acetone, the effect of 4-MCH and 2-propanol inhibition on CHMO and gas stripping of acetone from solution were incorporated into the model by empirical function fitting.

2. NADP(H) stability was calculated from pH dependent degradation kinetics. Cofactor stability was found to be a strong function of pH and relative enzyme activities present. Highest stability occurred between pH 8-9 and a fractional total CHMO activity of 0.3-0.7.

3. Most efficient use of the two enzymes was found to occur at an activity ratio of 1:1. Increasing cofactor concentration increased reaction rate for both enzymes up to 0.2mM where the response became linear. Overall enzyme usage could not exceed 60% of the maximum possible.

4. Removal of competitive inhibition from the enzymes caused by the form of the cofactor produced by each theoretically raises the potential efficiency from 60% to 95% at a total cofactor concentration of 1mM.

5. Validation of the model under steady state conditions described the model as underestimating the concentration of NADPH by 10% over a range of relative enzyme proportions.

6. Theoretical 5-MOP yields were calculated as a function of both cofactor and enzyme loading using the CHMO\TBADH system.

7. Quantification of the performance of a range of processing strategies was performed for 5-MOP synthesis using the model. Fed-batch processing was calculated to be 2.53 times more productive than batch.

7.5 Preparative bioconversions

1. Synthesis of 5-MOP and 6-HTPO were achieved at 2L scale using TBADH to supply cofactor to CHMO using soluble enzymes. Yield of 5-MOP was $5.4gL^{-1}$ .Yield of 6-HTPO was lower at $0.7gL^{-1}$ due to protein damage caused by liquid/liquid and gas/liquid interfaces.

2. Immobilisation of CHMO and TBADH was performed onto Eupergit C. Retained activities of 12% and 42% were achieved respectively. Specific activities were $1.2Ug^{-1}$ (CHMO) and $13.6Ug^{-1}$ (TBADH). Co-

immobilisation of both enzymes on the same sample of Eupergit produced a catalyst with an activity of $0.6Ug^{-1}$.

3. Immobilised CHMO showed increased stability against 4-MCH and 5-MOP. Incubation in a 50mM solution of both compounds caused a drop in activity of 30% over 14 days. Immobilised CHMO theoretically produces 30% more product than soluble enzyme before the enzyme is deactivated.

4. 2L reactions using immobilised enzymes produced multi-gram quantities of both lactones with TTN values above 600. Reuse of the aqueous fraction in 2-HCP based reactions would result in an overall TTN of 3400 after 15 batches.

8.0 Future Work

Further work in the following areas would be of benefit:

The Baeyer-Villiger system:

- Development of a rapid and accurate method for determining the enantiomeric excess of the ketones and lactones in both model systems. This would require the synthesis of both enantiomeric forms of each compound in order to validate the assay.
- Cloning of the CHMO gene into several different hosts such as *Pseudomonas* to produce a practical whole cell catalyst.
- Cloning of both CHMO and TBADH into the same host at high levels of expression to further develop cell free catalysis
- Protein engineering of CHMO to accept NADH as cofactor and to reduce competitive inhibition caused by $NADP^+$.
- Detailed kinetic analysis of immobilised CHMO and TBADH. Investigate the mass transfer characteristics of the two phase system to determine process limitations.
- Assess the ee of the two lactones produced with the immobilised catalyst to see if the immobilisation procedure changes enantioselectivity.
- Continue studies to improve the retained activity of both enzymes upon immobilisation.
- Investigate methods of product removal for the 5-MOP system to leave the aqueous cofactor intact to allow the TTN to be improved.

The design tools:

- Apply the tools developed to other redox systems, to determine the effectiveness of the approach and to highlight areas in need of improvement.
- Develop selection criteria for other systems requiring different cofactors (e.g. ATP) in a similar way as that presented in this thesis.
- Develop models to describe other biocatalytic systems that have been previously investigated with the structured approach to see if the designs chosen are the most appropriate.

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Appendix 1 List of Suppliers

Kontron Instruments	Blackmoor Lane Coxley Business Park Watford WD1 8XG UK
Merck kGaA	64271 Darmstadt Germany
New Brunswick Scientific	Edison New Jersey USA
Alltech Associates	Carnforth Lancashire LA5 9XP UK
BDH Laboratory Supplies	Poole Dorset BH15 1TD UK
MSE Ltd	Crawley Sussex UK
Perkin-Elmer Corporation	761 Main Avenue Norwalk Ct 06859-0010 USA
Heraeus Instruments Ltd	9 Wates way Brentwood Essex CM15 9TB UK
Lancaster Synthesis	Eastgate White Lund Morecombe UK
Avocado Research Chemicals Ltd	Shore Road Heysham Lancashire UK

Fluorochem Ltd	Old Glossop Derbyshire UK
Acros Organics	Geel West Zone 2 Janssen Pharmaceuticalaan 3a 2440 Geel Belgium
Sigma-Aldrich	Gillingham Road Poole Dorset UK
Microcal Software Inc	1 Roundhouse Plaza Northampton MA 01060 USA
Beckmann Instruments	2500 Harbour Boulevard Fullerton CA 92634-3100

Appendix 2 - Calibration Curves and chromatograms

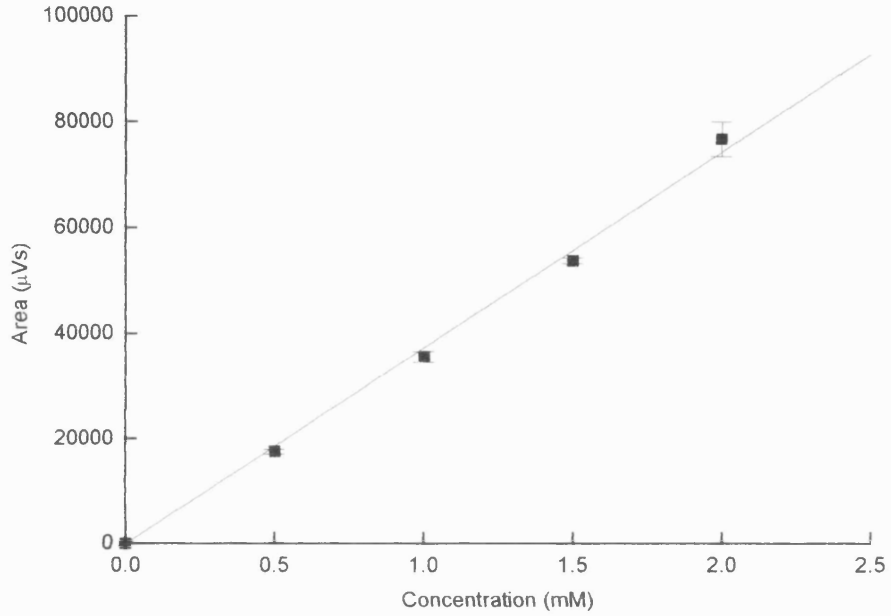


Figure A1 Calibration curve for GC peak area for 2-hexyl cyclopentanone.

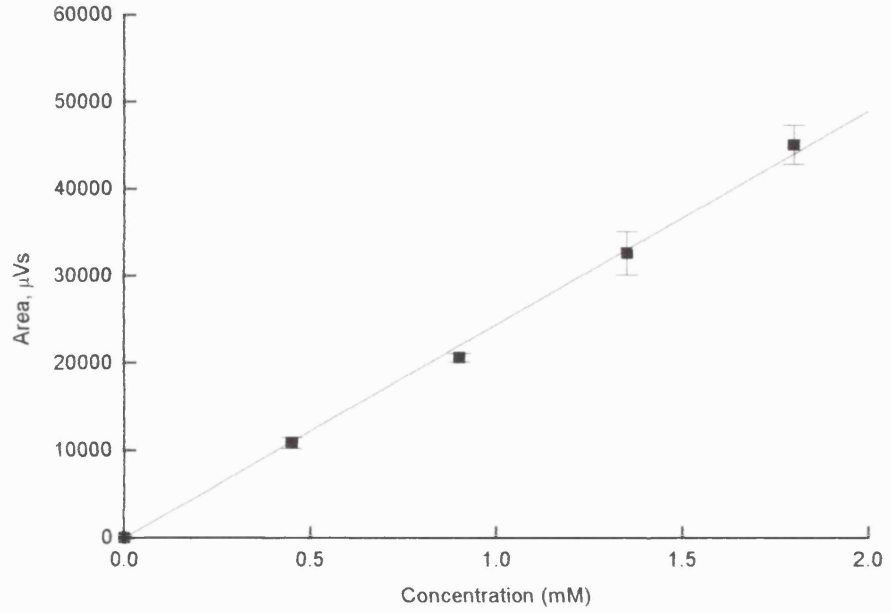


Figure A2 Calibration curve for GC peak area for 6-hexyl tetrahydropyran-2-one.

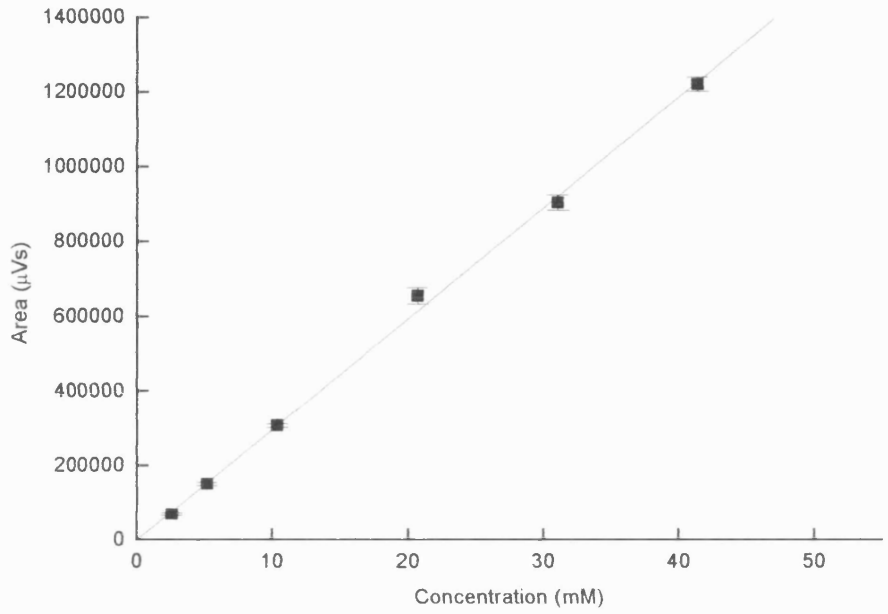


Figure A3 Calibration curve for GC peak area for 4-methyl cyclohexanone.

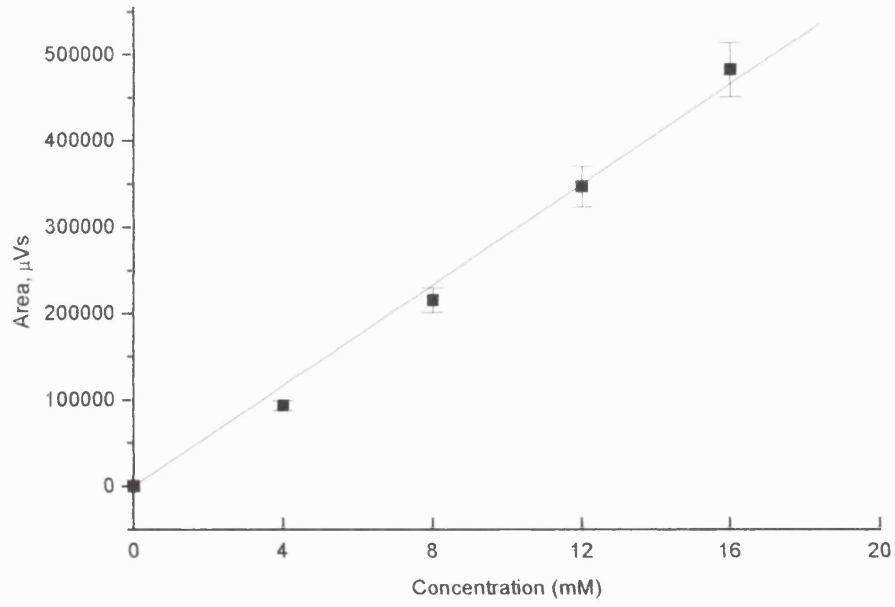


Figure A4 Calibration curve for GC peak area for 5-methyl oxepan-2-one

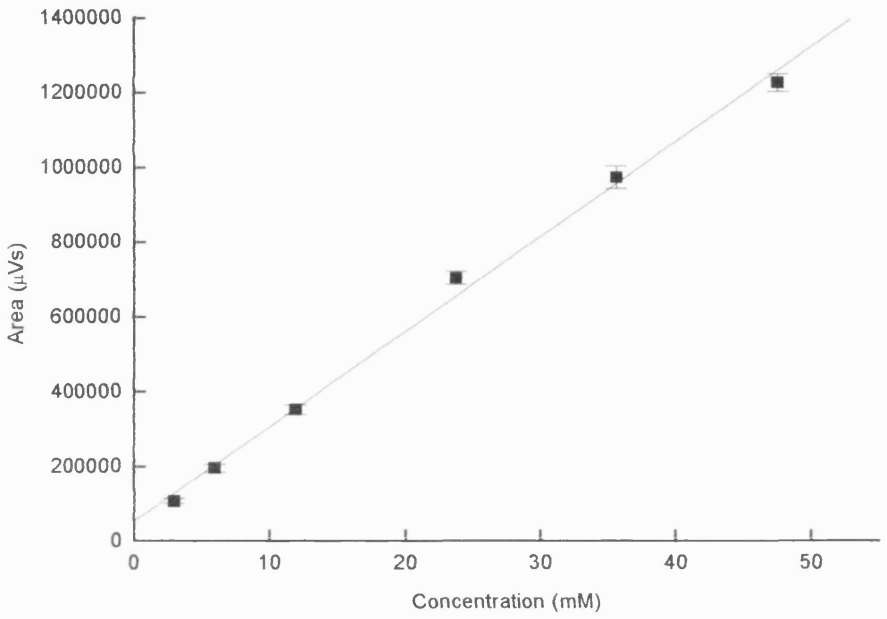


Figure A5 Calibration curve for GC peak area for 4-methyl cyclohexanol.

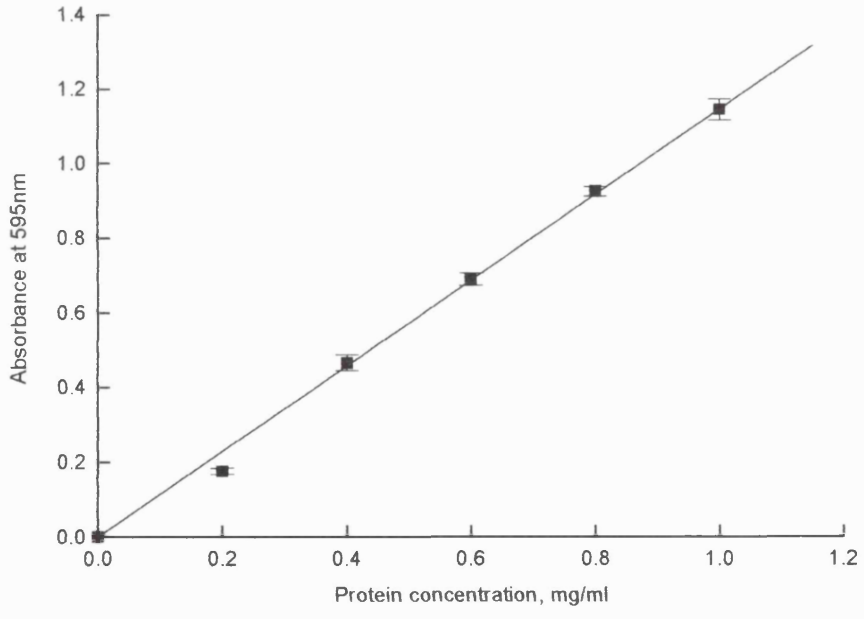


Figure A6 Calibration curve for determination of protein concentration.

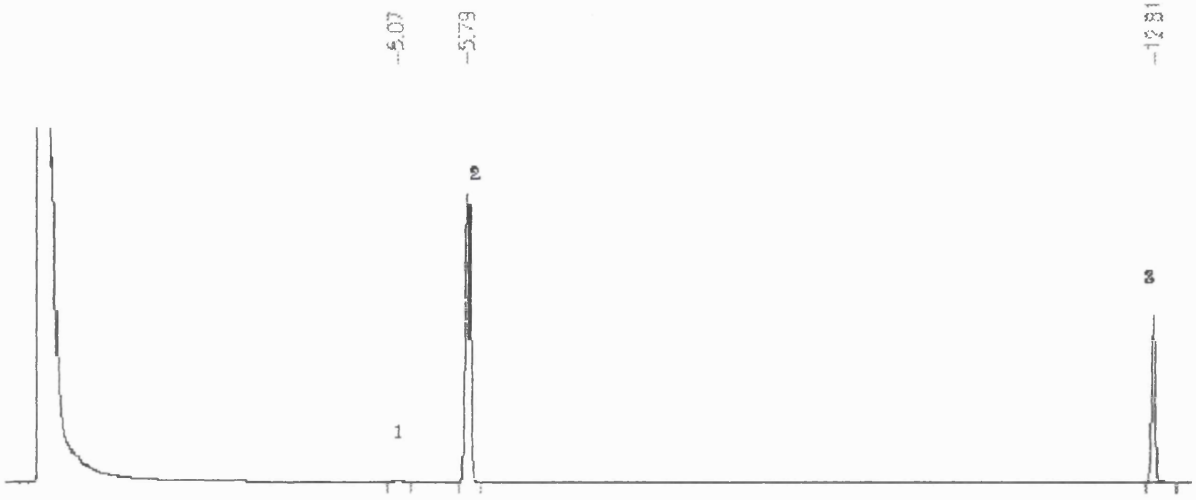


Figure A7 Gas chromatograph showing: 1 = 4-methyl cyclohexanol
2 = 4-methyl cyclohexanone
3 = 5-methyl oxepan-2-one

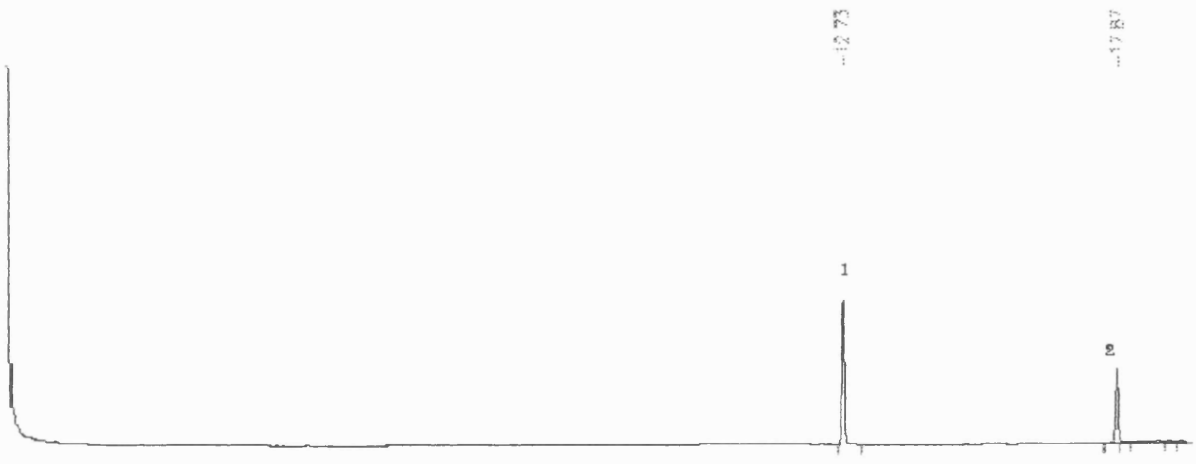


Figure A8 Gas chromatograph showing 1 = 2-hexyl cyclopentanone
2 = 6-hexyl tetrahydrpoyran-2-one

Appendix 3 - Euler's one step rule

With a differential equation in the form:

$$dy/dx = f(x,y) \tag{19}$$

Euler's method with a linear solution:

$$y_1 = y_0 + (x_1 - x_0) f(x_0,y_0) \tag{20}$$

or more conveniently defining h, the integration step length as $(x_1 - x_0)$

The equation becomes

$$y_1 = y_0 + h f(x_0,y_0) \tag{21}$$

Design equations

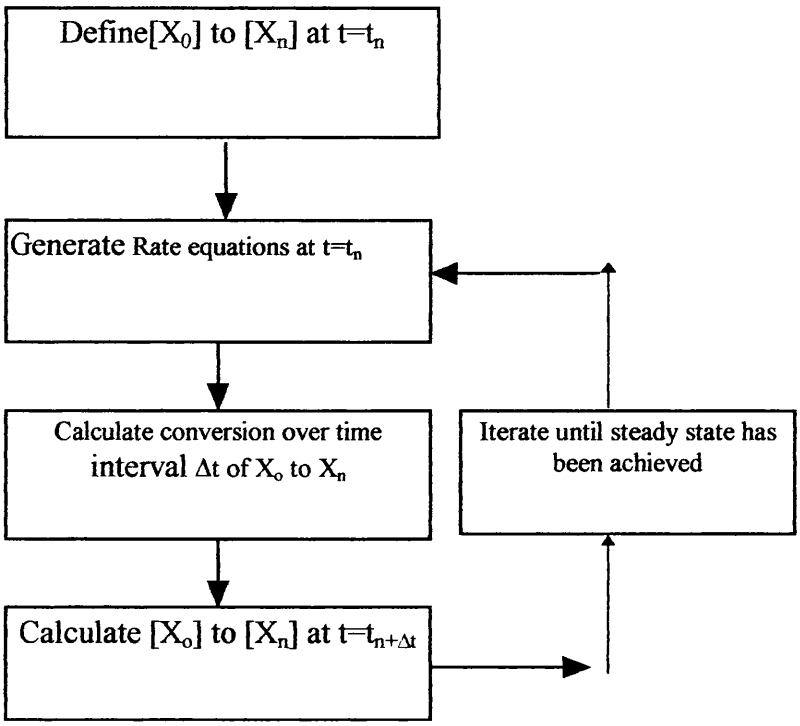
Applying this to the system under investigation

$$d[NADPH]/dt = rate_{ADH} - rate_{CHMO} \tag{22}$$
$$d[NADP]/dt = -rate_{ADH} + rate_{CHMO} \tag{23}$$

Applying Euler's rule:

$$[NADPH]_{T+\Delta T} = [NADPH]_T + \Delta t(rate_{ADH} - rate_{CHMO}) \tag{24}$$
$$[NADP^+]_{T+\Delta T} = [NADP^+]_T + \Delta t(rate_{CHMO} - rate_{ADH}) \tag{25}$$

Algorithm:


Define[X_0] to [X_n] at $t=t_n$
Generate Rate equations at $t=t_n$
Calculate conversion over time interval Δt of X_o to X_n
Iterate until steady state has been achieved
Calculate [X_o] to [X_n] at $t=t_{n+\Delta t}$

Appendix 4 - Relative enzyme activities within a recombinant organism

Specific activities of pure enzymes (Lamed and Zeikus, 1981; Trudgill, 1990)

CHMO $21 Umg^{-1}$ (Molecular weight=59000)

TBADH $41.8 Umg^{-1}$ (Molecular weight=37500x 4(homotetrameric))

Therefore:

CHMO $21 \times 59000 = 1239000\ Ummol^{-1}$ of polypeptide

TBADH $41.8 \times 37500 = 1567500\ Ummol^{-1}$ of polypeptide

$$\text{CHMO activity fraction} = \frac{1239000}{1239000+1567500} \times 100 = \mathbf{44.1\%}$$

Appendix 5 - Theoretical increase in TTN by reuse of cofactor

At pH 8, and CHMO fraction of 0.63, $k_{(obs)}x = 0.00104\ h^{-1}$

$$[cofactor]_{t=24} = 0.05e^{24*0.00104} \qquad (26)$$

$$[cofactor]_{t=24} = 0.0487 mmolL^{-1}$$

Assuming 10% lost during processing:

recharge rate = 0.05 - 0.9*0.0487 = $0.0062 mmolL^{-1}$

Final 6-HTPO concentration in reactor = $36 mmolL^{-1}$

Aqueous concentration approximately $5 mmolL^{-1}$(solubility limit)

$\therefore$ Recoverable yield = $31 mmolL^{-1}$

TTN is defined as product formed/cofactor added

$$\therefore \quad TTN_n = \frac{31n}{0.05 + 0.0062(n-1)} \qquad (27)$$