INTEGRATION OF RACEMISATION FOR THE CHMO CATALYSED SYNTHESIS OF OPTICALLY PURE LACTONES

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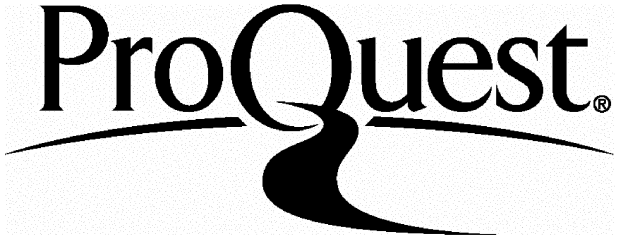

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ABSTRACT

Traditional chemical procedures can be difficult to perform stereoselectively. Many reactions result in racemic products which require resolution and therefore a maximum of fifty percent product yield - to produce a single enantiomer. The temperature, pressure and reagents involved in many of these reactions make the scale-up of the process from bench to production scale difficult.

Biotransformations can offer an alternative route to optically pure products from racemic starting materials, although they often suffer from low yields. The combination of a bioconversion and chemical racemisation could produce a step increase in the yield of enantiomerically pure products. This idea was explored within the research detailed in the thesis.

A representative system in which a bioconversion and racemisation step can be combined to produce material with high enantiomeric excess and improved yield was chosen. The Baeyer-Villiger reaction involves the conversion of ketones (or cyclic ketones) to esters (or lactones). The reaction can be catalysed by the monooxygenase group of certain enzymes. The cyclohexanone monooxygenase (CHMO) catalysed bioconversion of a 2-substituted cyclic ketone was used as a model reaction for the combined process.

The integrated process was compared with the traditional chemical procedure and the increasingly popular biotransformation. The integrated process was performed in two different type of reactor, a linked reactor and a membrane bioreactor. The process in the linked reactor was found to have no advantage over the chemical or biovconversion process, whilst the process performed in the membrane bioreactor had a significant advantage (in terms of yield and enantiomeric excess) over the chemical and bioconversion route.

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List of Schemes.

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4.1 $\text{Rate} = k\,[\text{substrate}]\,[\text{catalyst}]$

5.1 $J_a = N_a / A$

5.2 $N = ka\,A\Delta\,C = ka\,A\,(C^{ao} - C^{ai})$

5.3 $N_{ov} = N_{film1} = N_{mem} = N_{film2}$

5.4 $N_{ov} = K_{ov}\,[\,C^{b1} - C^{b2}]$

5.5 $N_{film1} = K_{film1}\,[\,C^{b1} - C^{i1}]$

5.6 $N_{film2} = K_{film2}\,[\,C^{i2} - C^{b2}]$

5.7 $K_{mem} = P^{mem}_{aq}\,D_{mem} / \delta_{mem}$

5.8 $N_{mem} = (P^{mem}_{aq}\,D_{mem} / \delta_{mem})\,[C^{i1} - C^{i2}]$

5.9 $[C^{b1} - C^{b2}] = [C^{b1} - C^{i1}] + [C^{i1} - C^{i2}] + [\,C^{i2} - C^{b2}]$

5.10 $N_{ov} / K_{ov} = (N_{film1} / K_{film1}) + (N_{mem}\delta_{mem} / P^{mem}_{aq}D_{mem}) + (N_{film2} / K_{film2})$

5.11 $R_m = 1/K_a$

5.12 $1/K_{ov} = (1/K_{film}) + (\delta_{mem}/P^{mem}_{aq}D_{mem}) + (1/K_{film2})$

5.13 $P^{mem}_{aq} = [\text{membrane}] / [\text{aqueous}]$

5.14 $Re = D\,u\,\rho / \mu$

5.15 $N_{ov} = (K_{ov}\,A / V^r)\,[\,C^d - C^r\,]$

5.16 $V^r\,C/t = K_{ov}\,A\,[\,C^d - C^r\,]$

5.17 $\int_{t=0}^{t=t} K_{ov}\,A / V^r = \int_{c=o}^{c=c} 1 / [C^d - C^r\,]$

5.18 $(K_{ov}\,A / V^r)\,t = \ln [\,C^d - C^r\,]$

Abbreviations

ADH	alcohol dehydrogenase
aq.	Aqueous
b1	bulk phase 1
b2	bulk phase 2
BSA	bovine serum albumin
BSTR	batch stirred tank reactor
CHMO	cyclohexanone monooxygenase
CSTR	continuous stirred tank reactor
DOT	dissolved oxygen tension
E.coli	*Escherichia coli*
FAD	flavin adenine dinucleotide
GC	gas chromatography
GDH	glucose dehydrogenase
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
i1	interface 1
i2	interface 2
IPA	iospropanol (2-propanol)
IPTG	isopropyl β-D-thiogalactopyranoside
ISPR	*In-situ* product removal
K_R	ketone enantiomer R
K_S	ketone enantiomer S
L_s	lactone enantiomer S
MCPBA	*meta*-chloroperoxybenzoic acid
NAD^+	nictotinamide adenine dinucleotide (oxidised form)
NADH	nictotinamide adenine dinucleotide (reduced form)
$NADP^+$	nictotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nictotinamide adenine dinucleotide phosphate (reduced form)
NaOH	sodium hydroxide
NMR	nuclear magnetic reasonance

OD	optical density
STR	stirred tank reactor
TLC	thin layer chromatography
Tris	tris(hydroxymethyl) aminomethane
UV	ultraviolet light

Nomenclature.

A	area (m^2)
bpt	boiling point (°C)
ΔC	change in concentration (g m^{-3})
C^{ao}	bulk concentration of a away from boundary phase (g m^{-3})
C^{ai}	concentration of a at interface (g m^{-3})
C^{b1}	concentration in bulk 1 (g m^{-3})
C^{b2}	concentration in bulk 2 (g m^{-3})
C^{d}	concentration in donating vessel (g m^{-3})
C^{i1}	concentration at interface 1 (g m^{-3})
C^{i2}	concentration at interface 2 (g m^{-3})
C^{r}	concentration in receiving vessel (g m^{-3})
D	diameter (m)
D_{mem}	diffusion coefficient in the membrane (m^2 s^{-1})
δ_{mem}	density of membrane (kg m^{-3})
dcw	dry cell weight (g)
e.e.	enantiomeric excess (%)
E	enantiomeric ratio (——)
i.d.	internal diameter (m)
J_a	mass flux of component a (kg m^{-2} s^{-1})
k	racemisation rate constant (dm^3 mol^{-1} s^{-1}, for second order)
K_a	mass transfer coefficient of component a (ms^{-1})
K_{film1}	mass transfer coefficient across boundary layer 1 (ms^{-1})
K_{film2}	mass transfer coefficient across boundary layer 2 (ms^{-1})
K_{mem}	mass transfer coefficient across membrane (ms^{-1})
K_{ov}	overall mass transfer coefficient (ms^{-1})
N_a	rate of mass transfer of component a (g s^{-1})
N_{film1}	rate of mass transfer across boundary layer 1(g s^{-1})
N_{film2}	rate of mass transfer across boundary layer 2 (g s^{-1})
N_{mem}	rate of mass transfer across the membrane (g s^{-1})
N_{ov}	overall rate of mass transfer (g s^{-1})

P^{mem}_{aq}	partition coefficient between the membrane and the aqueous phase (—)
R_e	Reynolds number (—)
R_m	resistance to mass transfer (—)
ρ	density (kg m^{-3})
T	temperature (°C, unless stated otherwise)
μ	dynamic viscosity (Pa s)
u	linear velocity (m s^{-1})
U	units of enzyme activity (μmol min^{-1})
V	volume (m^3)
V^r	volume of receiving vessel (m^3)
v/v	volume per volume (—)

1. INTRODUCTION.

1.1 INTRODUCTION.

Traditional chemical procedures can be difficult to perform stereoselectively. Many reactions result in racemic products which require resolution, and therefore a maximum of fifty percent product yield, to produce a single enantiomer. One chemical solution to this is via asymmetric synthesis, which has become increasingly important over the last fifteen years, but has many practical limitations. These concern the attachment and removal of the auxillary which can lead to a low yielding process. The temperatures, pressures and reagents involved in many of these reactions makes the scale-up of the process from bench to production scale difficult.

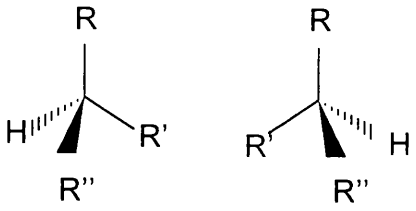
Biotransformations (which can also be called bioconversions) can offer an alternative route to optically pure products from racemic starting materials, although they often suffer from low yields. The combination of a bioconversion and chemical racemisation could produce a step increase in the yield of enantiomerically pure products. This idea shall be explored within the research detailed in this thesis.

A representative system in which a bioconversion and racemisation step can be combined to produce material with high enantiomeric excess and improved yield was chosen. The Baeyer-Villiger reaction involves the conversion of ketones (or cyclic ketones) to esters (or lactones). The reaction can be catalysed by the monooxygenase group of certain enzymes. The cyclohexanone monooxygenase catalysed bioconversion of a 2-substituted cyclic ketone will be used as a model reaction for this combined process.
The following Chapter is designed to give the reader insight into the background of all aspects concerned with this research.

1.2 CHIRAL COMPOUNDS.

Chiral molecules are playing an increasingly more important role within the pharmaceutical and agrochemical industries, as the regulatory authorities' demand for single enantiomer compounds increases. (Juaristi 1997, Stinson 1998.) One of the most important aspect of this research is to develop an integrated process to produce material with high enantiomeric excess and improved yields compared to reported processes.

Stereoisomers are non-identical molecules which differ only in the arrangement of their atoms in three dimensional space. Stereoisomers is a collective term that can describe both enantiomers and diastereoisomers. Enantiomers have chiral centres that are mirror-images of each other (Scheme 1.1.) They have identical physical and chemical properties, except for optical rotation or when placed in a chiral environment (McMurray 1992, Streitweiser *et al.*, 1992.)



Scheme 1.1. Enantiomers

Diastereoisomers are non-mirror image stereoisomers. They have the same configuration at one or more of the chiral centers, but different configuration at other chiral centers.

Stereoisomers play an important role in industry. The majority of synthetic pharmaceutical and agrochemical products on the market are sold as a mixture of enantiomers or diastereoisomers. Juaristi (1997) stated that a recent estimate indicated that only 12% of all chiral synthetic drugs were sold in their

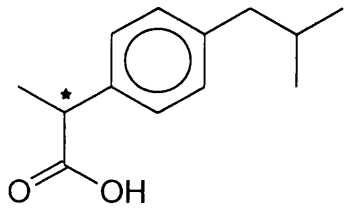
enantiomerically pure form. Stinson (1998) reported that out of the top 100 drugs worldwide, 50 are single enantiomers.

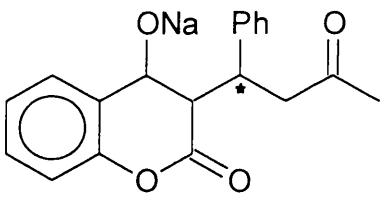
The chiral nature of enzymes will cause both enantiomers of a racemic mixture to have different physiological and/or pharmacological effects on the body and therefore the two enantiomers must be considered as different compounds (Juaristi 1997.)

Hence;

1) Both enantiomers can produce a physiologically desirable effect, for example

- Both enantiomers of ibuprofen (Structure 1.1) are anti inflammatory agents.
- Both enantiomers of warfarin (Structure 1.2) are anticoagulants.





★ denotes a chiral center.

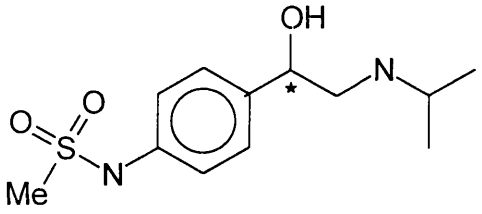
Structure 1.1. Ibuprofen. **Structure 1.2. Warfarin.**

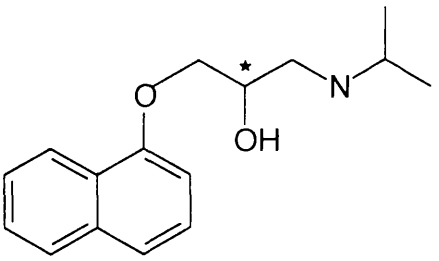
2) The two enantiomers can have completely different pharmacological effects.

- D-sotalol (Structure 1.3) is a type 3 anti-rhythmic and L-sotalol is a β-blocker.

3) One of the enantiomers is pharmacologically active (the eutomer), whilst the other enantiomer is inactive (the distomer).

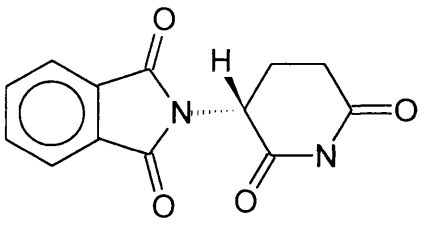
- This is the case for propanolol (Structure 1.4). The L-enantiomer is a β-blocker and the D-enantiomer is inactive.



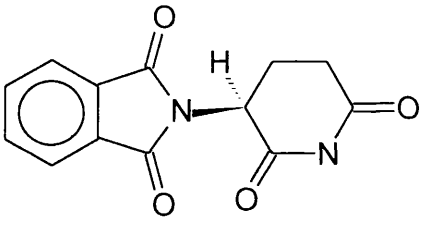


Structure 1.3. Sotalol **Structure 1.4. Propanolol**

Problems arise when one of the pair of enantiomers produce toxic effects. A well known example of this is Thalidomide (Structure 1.5). The R-enantiomer of Thalidomide causes the desired pharmacological effects of sedation. The S-enantiomer is a teratogen causing foetal abnormalities. (Schreier *et al.*, 1995.)



R-(+)-enantiomer



S-(-)-enantiomer

Structure 1.5. Thalidomide.

The ability to produce a single enantiomer of a compound - and therefore a single enantiomer drug - eradicates the need to develop appropriate manufacturing and control procedures to determine the identity, potency, quality and purity of each of the enantiomers. When a racemic pharmaceutical product is manufactured the pharmacokinetic effects of both of the enantiomers are required to be determined individually and when together in a mixture, as

the two enantiomers can affect each others pharmacological effects. The two enantiomers should also, according to FDA guidelines, be evaluated separately and together during phase 1 clinical trials (FDA policy statement 1992.)

The level of testing required on a mixture of enantiomers in a pharmaceutical compound is a great deal higher than the testing required for a compound containing a single enantiomer. It has been suggested that the regulatory authorities will make a more rigid stance towards drugs that contain a mixture of enantiomers and possibly make the decision that only enantiomerically pure drugs should, in future, be approved for release onto the market (Hanson 1995).

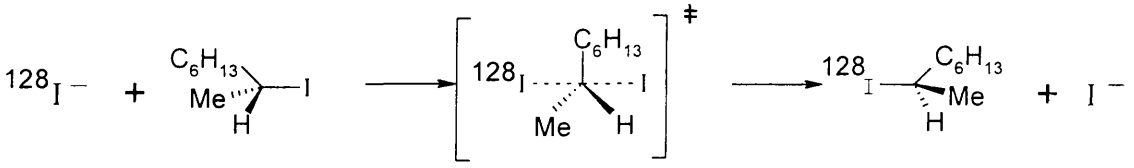
In compounds containing more than one chiral center the easiest, and often only, synthetic route contains at least one bioconversion. The bioconversion can allow for a stereo/regioselective conversion to produce a single enantiomer or regioisomer whilst the chemical reaction would yield a number of different products.

A process that can be used to produce enantiomerically pure compounds has great advantages over traditional methods of synthesis.

1.3 RACEMISATION .

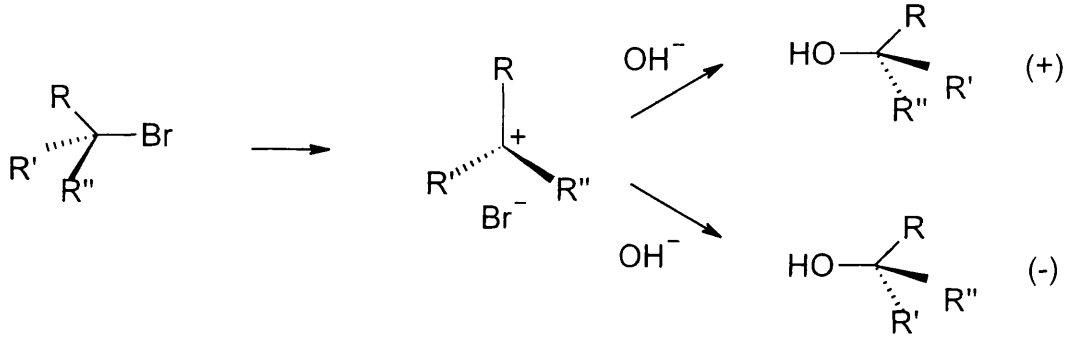
Racemic mixtures contain equal amounts of both of the enantiomers of a compound. They display zero optical rotation, as the rotation from the (+) enantiomer is canceled out by the optical rotation of the (-) enantiomer. The physical properties of a racemate are not always the same as those of the pure enantiomers, due to the different intermolecular interactions between the two enantiomers. Differences can be observed in the density, melting point and also in various spectra (McMurray 1992, Streitweiser et al 1992.)

The process in which a single enantiomer is converted to a 50:50 mixture is called racemisation. This can occur by chemical interconversion, or simply by mixing equal amounts of the two enantiomers (McMurray 1992, Streitweiser *et al.*, 1992.) The chemical interconversion can be performed by a variety of different mechanisms depending on the structure of the enantiomers. Chiral alkyl halides can undergo bimolecular nucleophilic (S_N2) reactions, where the same halide acts as a nucleophile. In Scheme 1.2 radioactive iodine was used as an isotopic label so that the reaction could be monitored.



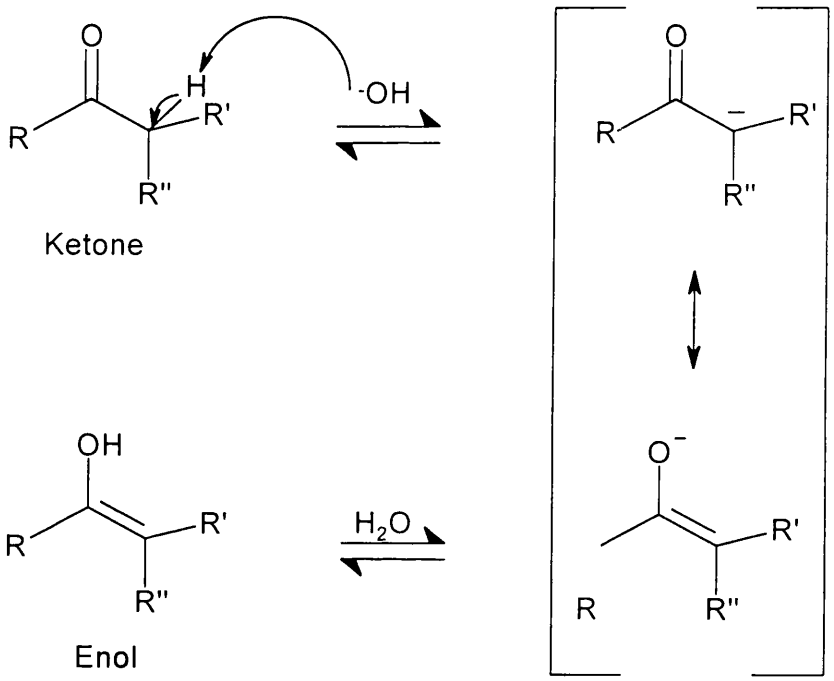
Scheme 1.2 Racemisation via S_N2 reaction.

In some cases, unimolecular (S_N1) reactions occur where the carbocation formed is attacked by a nucleophile such as ^-OH or the solvent (H_2O). Racemisation is only likely to occur in S_N1 reactions when the carbocation is - or can be - stabilised (Scheme 1.3).

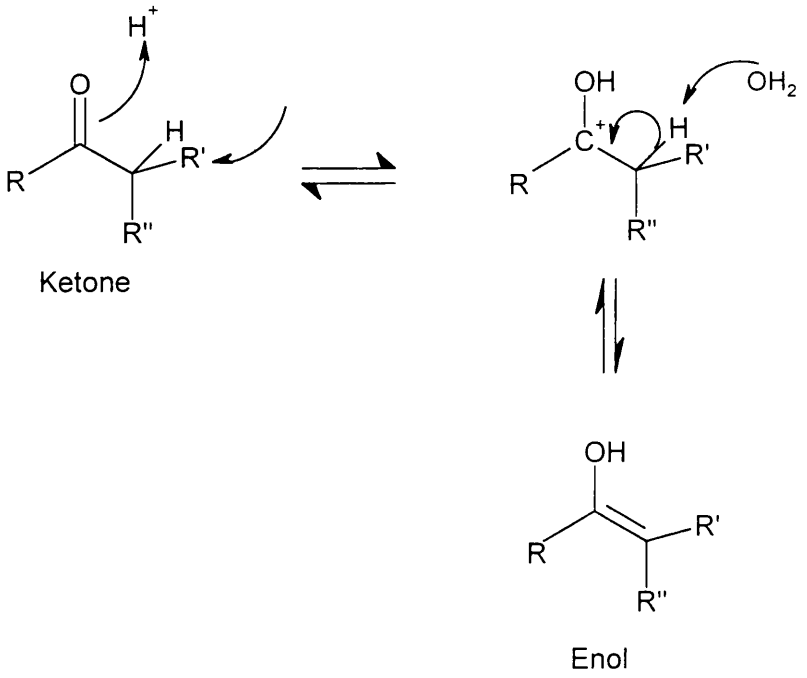


Scheme 1.3 Racemisation via S_N1 reaction.

Racemisation of chiral aldehydes and ketones readily proceeds in the presence of an acid or base catalyst, when the chiral center of the species is α to the carbonyl group. The racemisation involves the formation of an intermediate enol form which contains a planar achiral stereocentre and proceeds via a tautomerism mechanism as seen in Scheme 1.4 and 1.5 (McMurray 1992, Streitweiser *et al.*, 1992, Sykes 1986.)

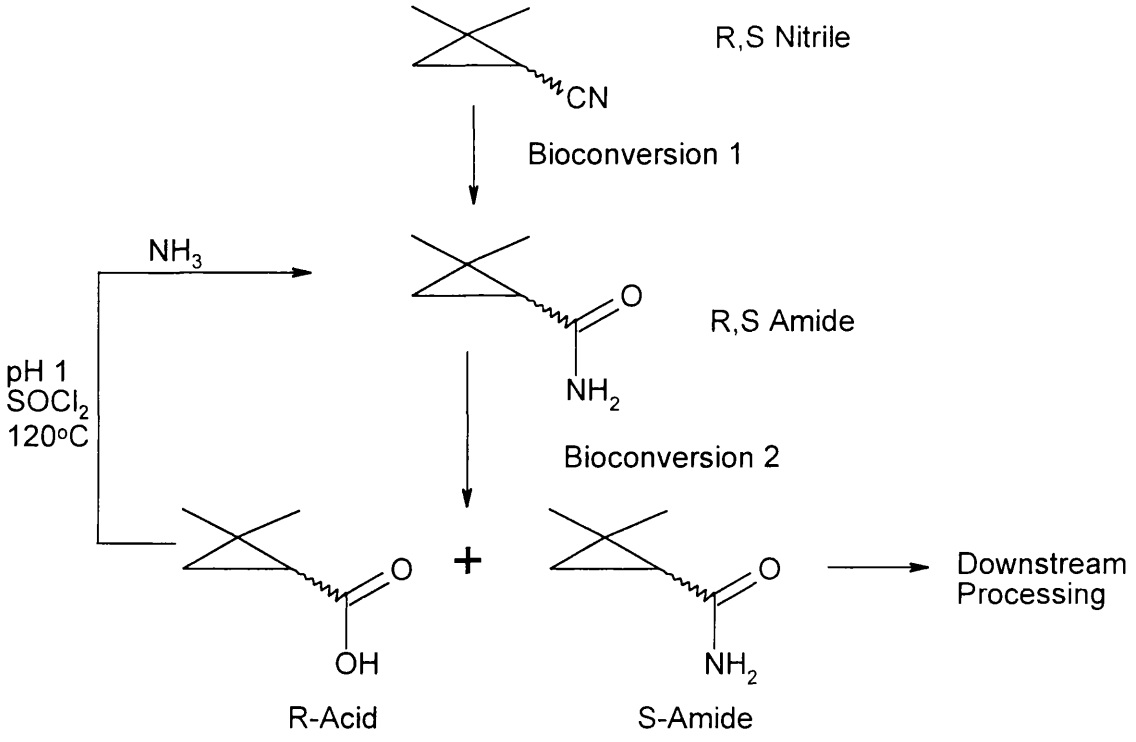


Scheme 1.4 Base catalysed tautomerism mechanism.



Scheme 1.5 Acid catalysed tautomerism mechanism.

An industrially important process including a racemisation step is the production of S-2,2-dimethylcyclopanecaroxamide, an intermediate in the production of cilastatin. Cilastatin is often co-prescribed with certain antibiotics to prevent their degradation in the kidneys. The 'one pot' synthesis as described in Scheme 1.6 was developed by Lonza in 1992, (Tramper 1996, Hoeks *et al.*, 1992).

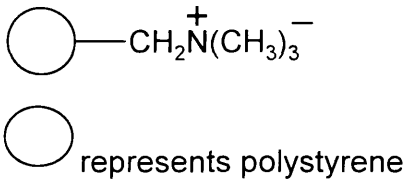


Scheme 1.6 One pot synthesis of S-2,2-dimethyl-cyclopropanecarboxamide.

1.4 ION EXCHANGE RESINS AS SOLID CHEMICAL CATALYSTS.

Ion exchange resins are commonly used industrial heterogeneous catalysts, in laboratory and large scale purifications, as analytical tools and as alternatives to homogeneous catalysts in organic reactions (Zhang *et al.*, 1998, Ferreira and Rodrigues 1993, Lopez *et al.*, 1987). The resins are available as solid, insoluble spherical beads with a diameter of 0.3-1.5mm (Sengupta *et al.*, 1989). They are obtained by polymerisation and can be gel-type or macroreticular type resins. The resins can operate over wide pH ranges and are usually stable up to 60°C, although many resins can be operated at up to 120°C. (Fluka catalogue 1997).

The gel-type resins have functional groups linked to insoluble molecules, often styrene DVB. They do not contain any true porosity but ions to be exchanged must diffuse through the gel structure to access the exchange sites. The apparent porosity is usually below 40 Å. Amberlite IRA 400 is an example of a gel-type ion exchange resin. It consists of trimethylammonium tied to polystyrene, as seen in Structure 1.6. Amberlite IRA 400 is a strongly basic anion exchange, which can be purchased in either the hydroxide or chloride form.



Structure 1.6 Amberlite IRA 400.

The macroreticular resins have large discrete pores with a mean pore diameter often in the region of 1300 Å. As with the gel-type resins the macroreticular resins have functional groups linked to insoluble molecules, usually polystyrene. The presence of the macropores allows an increase in reaction rate between nonpolar molecules, or compounds that have a high molecular weight. (Fluka catalogue 1997, Ferreira and Rodrigues 1993, Roddick and Britz 1997, Zhang *et al.,* 1998)

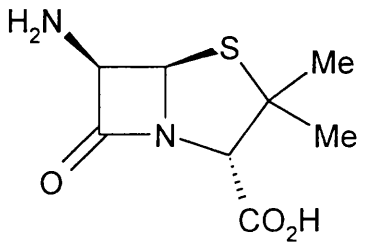
Solid ion exchangers have numerous advantages over homogeneous catalysts. The principal advantages are the ability to remove the catalyst from the reaction and the ability to retain the catalyst in the (bio)reactor. Disadvantages of heterogeneous catalysis are normally associated with mass transfer difficulties or loss of resin capacity with time (Cavender *et al.,* 1992, Lopez *et al.,* 1992)

1.5 BIOTRANSFORMATIONS.

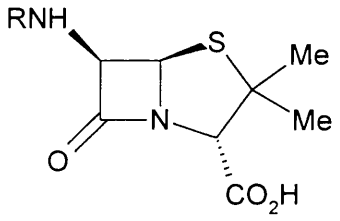
1.5.1 INTRODUCTION.

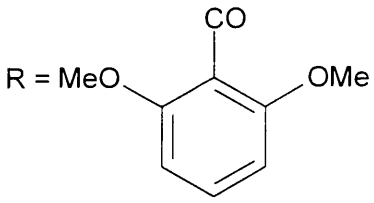
Biotransformations can be defined as the conversion of one compound to another using a biological or natural catalyst or more specifically, the 'selective enzymic modification of defined pure compounds into defined final products' (Kieslich 1984, Meyer *et al.,* 1997.) The starting material may be synthetic or a natural product. The biocatalyst can be an enzyme or a whole cell system. The enzyme system may involve an isolated enzyme, a crude enzyme mixture or an enzyme complex. These different forms of enzymes in turn can be freely suspended or immobilised onto a solid support. Unlike chemical reactions, the products and the reactants of a biotransformation are usually structurally similar (Lilly 1992). Biocatalysts bring a reaction to its equilibrium point at a greater rate than would otherwise be seen. Biotransformations tend to be performed at lower temperatures and pressures than the equivalent chemical reaction, making them more user friendly (Stanbury *et al.,* 1995, Hanson 1995.)

One of the most important early biotransformations was the hydrolysis of penicillin to 6-aminopenicillanic acid (6-APA) (Structure 1.7) which was performed using amidase from *E.coli* or other suitable microorganisms. This process led to the production of semi-synthetic penicillins such as methicillin (Structure 1.8) from 6-APA (Davis *et al.,* 1989)



Structure 1.7 6-Aminopenicillanic acid





Structure 1.8 Methicillin.

Originally biotransformations were applied almost exclusively to systems in which the reactant was highly water soluble. Therefore, the majority of biotransformations were carried out in aqueous media. The exceptions to this were the steroidal bioconversions discovered in the 1950s. Peterson (1952) reported the biotransformation of progesterone to 11-α-Hydroxyprogesterone using *Rhizopus nigricans* (Scheme 1). During the same period two other bioconversions of progesterone were described. Shull (1953) reported the 11-β-hydroxylation by *Curvularia lunata*. Dehydrogenation by *Corynebacterium simplex* was described by Nobile in 1955 (Lilly 1994). Research in the 1980's led to the biological conversion of reactants with low water solubility. Biocatalysts that are suitable for use in two phase (organic/aqueous) systems were reviewed by Woodley and Lilly (1994).

Biotransformations are becoming increasingly valuable tools for the preparation of novel organic molecules (Jones 1986, Faber 1992, Roberts and Turner 1992.) Processes that involve a bioconversion step are especially useful for the production of large molecules which contain a high degree of functionality. Biocatalysts can perform transformations that might not be possible using traditional organic synthetic methods. The high specificity of enzymes can allow the conversion of reactants to products with predicted selectivities, such as regioselectivity, chemoselectivity and enantioselectivity. These products can be used as chiral synthons and combined with traditional organic chemistry for the production of pharmaceuticals and other fine

chemicals. The biotransformation process tends to be more specific than the equivalent chemical reaction. The process can enable the modification, addition or removal of functional groups on specific sites of complex molecules. The chemical reaction to perform the same process often requires cost intensive techniques and the use of protecting groups.

The biotransformation processes can be simplified as in Figure. 1.1

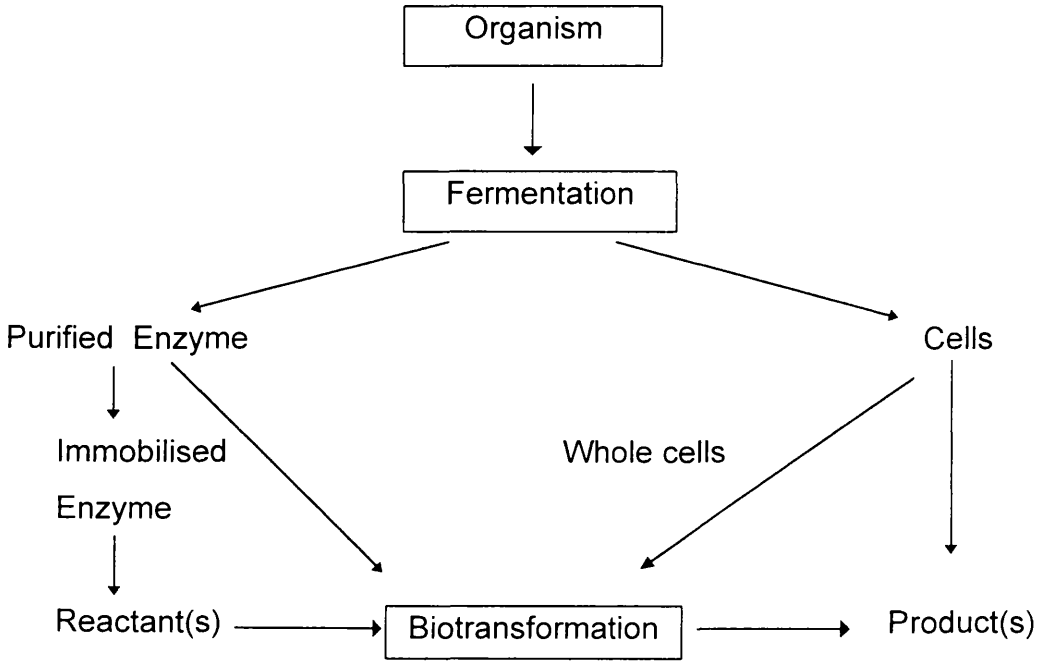


Figure 1.1 Bioconversion Processes (from Lilly 1994.)

1.5.2 BIOTRANSFORMATIONS USING ENZYMES

Enzymes are, due to their nature, highly specific catalysts. They are of great use in the synthesis of chiral synthons (Roberts *et al.*, 1992.) For an enzymic biotransformation the enzyme must be removed from the cell and purified sufficiently to remove all other enzymes that are likely to lower the yield of the

bioconversion product. The product yield can be decreased by other enzymes that will cause side or degradation reactions.

1.5.3 Biotransformations using whole cells.

The most commonly used biocatalysts are whole cells. This is due to the lack of need to purify the cells before use and the robust nature of the cells (Lilly 1977.) There are disadvantages connected with the use of whole cells in biotransformations. The size and densities of the cells make their recovery during downstream processing difficult without the use of separation techniques, such as centrifugation or filtration. Another major disadvantage concerning the use of whole cells is the possibility of metabolism of the product by other enzymes that are present in the cells (Faber 1992.) This can decrease the purity and yield of the final product, as the by-products of the biotransformation require removal during the downstream processing.

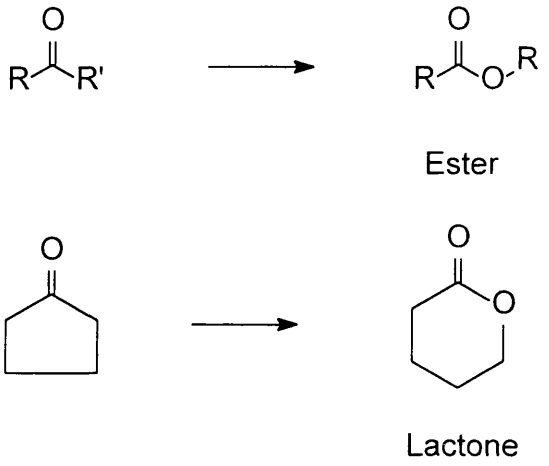
In some cases these problems can be overcome by the immobilisation of the whole cells onto a solid support, although this is not often a successful procedure. Immobilisation can allow easier recovery of the biocatalyst for the reactor, thus allowing reuse of the catalyst and can therefore make the process more economically viable. The immobilised catalyst can be retained in the reactor.

1.6 THE BAEYER-VILLIGER REACTION.

1.6.1 Introduction.

The Baeyer-Villiger reaction involves the insertion of an oxygen atom between the carbonyl carbon and one of the R groups in a ketone or cyclic ketone.

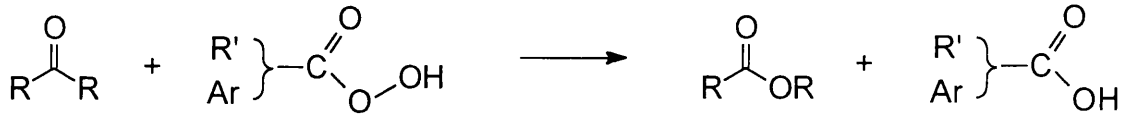
(Baeyer and Villiger, 1899.) This reaction produces an ester or a cyclic ester (a lactone) respectively (See Scheme 1.7)



Scheme 1.7. The Baeyer-Villiger Reaction.

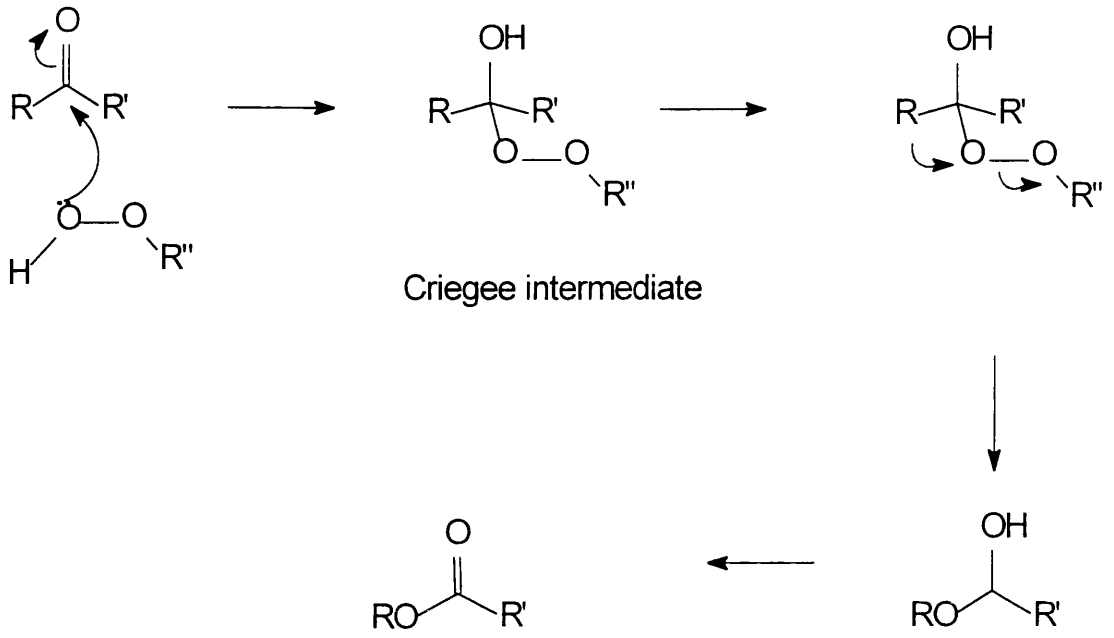
1.6.2 The Chemical Baeyer-Villiger Oxidation

The Baeyer-Villiger oxidation is extensively used in organic synthesis and generally proceeds at room temperature and pressure using a peracid catalyst, see Scheme 1.8. (Roberts and Wan 1998, Gagnon *et al.*, 1994)



Scheme 1.8 Chemical Baeyer-Villiger Reaction

The mechanism of the Baeyer-Villiger oxidation involves the formation of the non rate-limiting Criegee intermediate, which then rearranges to the ester/lactone in a rate limiting manner. (Roberts and Wan 1998, Ottolina *et al.*, 1996, Strukul *et al.*, 1998, see Scheme 1.9)

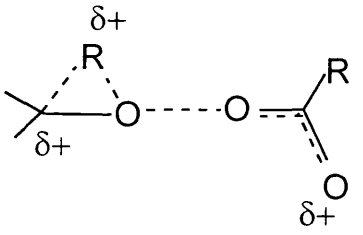


Scheme 1.9. Chemical Baeyer-Villiger Mechanism.

In Scheme 1.9 in can be seen that the lone pair of electrons from the peracid attack the carbonyl carbon in a nucleophilic fashion forming a tetrahedral intermediate (Criegee intermediate). Electronic rearrangement of this intermediate allows the migration of one of the R groups onto the incoming donor atom of oxygen.

In asymmetric ketones the relative ability of the R groups to share the partial positive charge with oxygen in the transition state determines the migratory aptitude. (see Scheme 1.10). In general, a tertiary substituted R group will migrate more readily than a secondary substituted R group (see Scheme 1.11). (Roberts and Wan 1998). The migrating group will migrate with retention of configuration . In Baeyer-Villiger oxidations where the R groups are the same, both of the possible regioisomers will be formed. Wright (1994) stated that no enantioselective chemical Baeyer-Villiger oxidation had been previously reported. In the same year (1994) Bolm and co-workers reported

enantioselective Baeyer-Villiger type oxidations of various 2 subsitited cyclic ketones using molecular oxygen and a metal catalyst. This produced lactones with enantiomeric excesses of over 47% and yields of over 21% in the worst case (2-phenyl cyclohexanone) and and e.e of over 60% with a yield of above 60% in the best case (2-phenyl cycloheptanone).



Scheme 1.10 Transition State.

$$t\text{-}R > Ar \cong 2^{\circ}\,R > 1^{\circ}\,R > CH_3$$

Scheme 1.11 Migratory Aptitude.

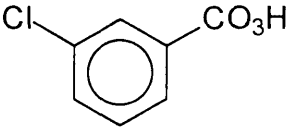
There are numerous peracids that are suitable for this type of oxidation. Typical peracids used are peracetic acid **1.9**, pertrifluoroacetic acid **1.10**, *meta*-chloroperbenzoic acid **1.11**, performic acid **1.12** and hydrogen peroxide **1.13**. The hazardous nature of these peracids must be considered during large scale oxidative reactions.

CH_3CO_3H

1.9

CF_3CO_3H

1.10



1.11

HCO_3H

1.12

HOOH

1.13

Structures 1.9 - 1.13 Peracids.

Roberts and Wan (1998), Strukul (1998), Chisem and co-workers (1998) have all recently reported the use of transition metal complexes as catalysts for Baeyer-Villiger oxidations.

Chisem and co-workers (1998) reported the use of a silica supported nickel complex for the room temperature Baeyer-Villiger oxidation of cyclic and linear ketones using molecular oxygen. All of the cyclic ketones oxidised by the group were symmetric, so no information regarding the migratory aptitude or regio/stereoselectivity of the reaction is available. The yields reported for the reactions were all above 75%, except for the conversion of cyclohexanone, where the yield was only 15%.

Stukul (1998) reviewed transition metal catalysts in Baeyer-Villiger oxidation of ketones using platinum, titanium silicate and methyltrioxohenium as the catalysts. The review also includes details of peracid catalysed Baeyer-Villiger oxidations. Strukul summarises that the number of transition metal catalysts capable of efficient catalysis is very limited, and that the use of chiral transition metal catalysts appears to be the only promising alternative to catalysis by microorganisms. Roberts and Wan (1998) stated that the yields and enantiomeric excesses of the products obtained using chiral metal catalysts are very poor.

1.6.3 The Biological Baeyer-Villiger Oxidation

As early as the 1950's an enzyme-catalysed Baeyer-Villiger reaction was reported by Bradshaw (1959). It is possible to perform this type of biotransformation using either enzymes or whole cell systems. The more user friendly reaction conditions of the biological Baeyer-Villager oxidation - mild pH, lower pressure and temperature - makes the biotransformation more advantageous than the corresponding chemical reaction. Biocatalysed Baeyer-

Villiger oxidations are often the best and easiest route to chiral products, due to the chiral nature of enzymes. Alphand and co-workers (1996) stated that the biotransformation approach appears to be the best way to achieve asymmetric Baeyer-Villiger oxidation. The biooxidation reaction can also produce compounds with different regiochemistry to the traditional organic synthetic reaction. A good review of the synthetic applications of Baeyer-Villiger type reactions is given by Willetts (1997).

The regiochemistry of the biocatalysed Baeyer-Villiger reaction is identical to that of the chemical reaction, where the migratory aptitude of the R groups determines which R group migrates to oxygen. The chemical and biological Baeyer-Villiger oxidations proceed with the same stereochemistry, *i.e.* retention of the configuration of the migrating carbon center. (Schwab 1983, Taschner 1988, Walsh 1988)

Baeyer-Villiger enzymes are enantioselective, with the conversion of one of the enantiomers of a racemic mixture occurring at a much greater rate than the other. In some cases the second enantiomer is not converted at all. Enantioselectivity does not occur during tradition chemical Baeyer-Villiger oxidations.

1.7 MONOOXYGENASES AS BAEYER-VILLIGER CATALYSTS.

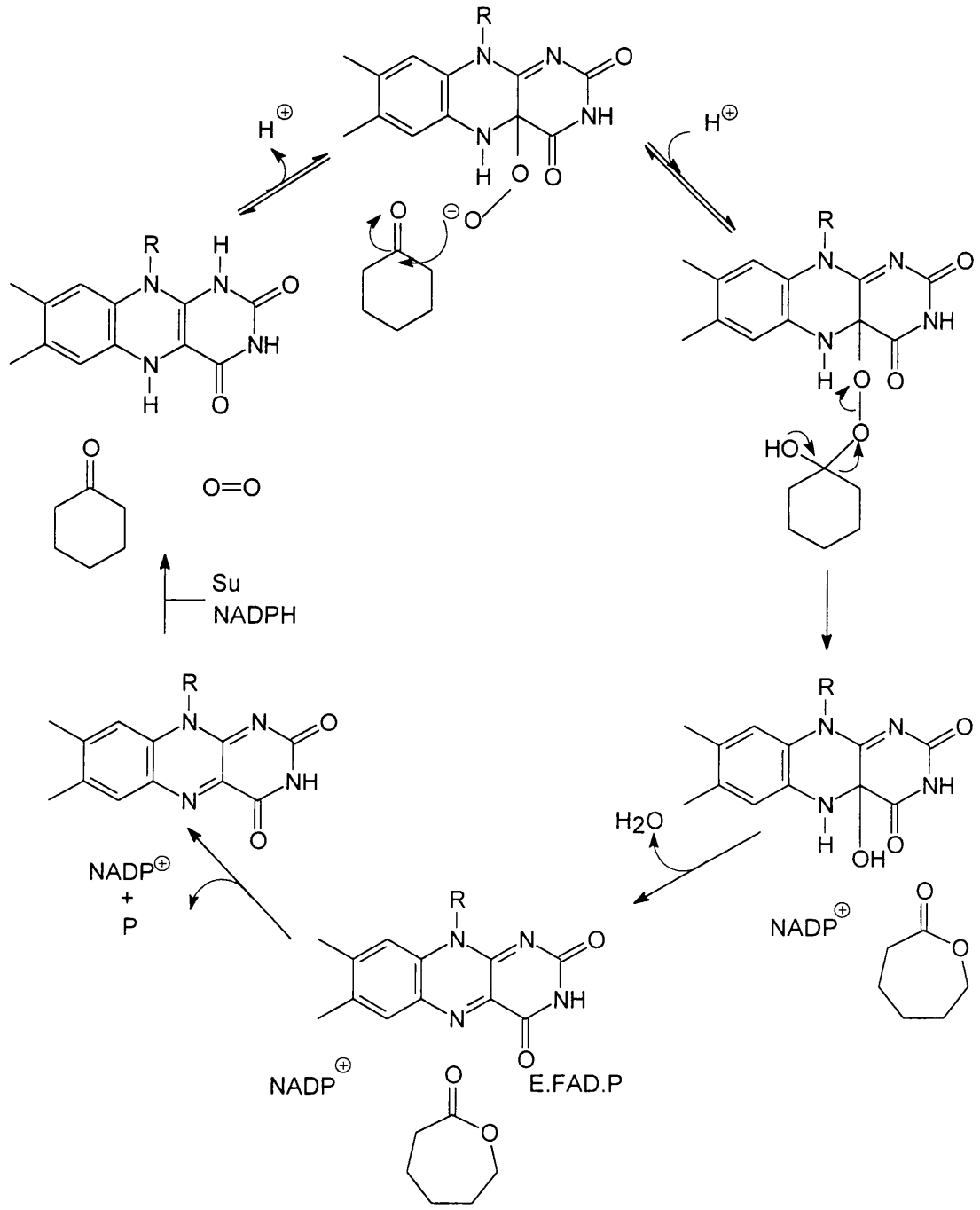
1.7.1 INTRODUCTION

The Baeyer-Villiger biooxidation is catalysed by Flavin dependent monooxygenase enzymes which do not require metal ions for their catalytic activity (Schwab *et al.,* 1983). This group of enzymes has been discovered in over one hundred organisms (Kelly 1996a) ranging from fungi to bacteria.

Stewart and co-workers (1996) reported the use of a 'designer yeast' as a new catalyst for Baeyer-Villiger oxidations.

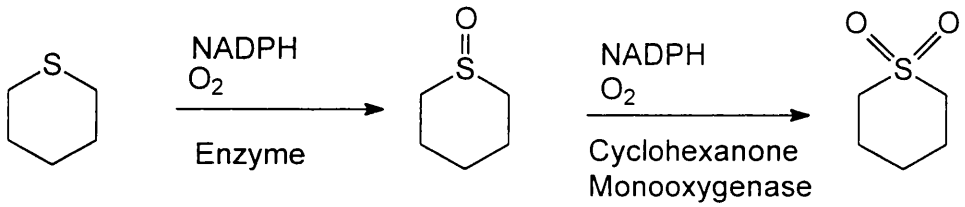
Monooxygenase enzymes provide one atom of oxygen to the substrate during the oxidation process. The remaining atom from the molecule of oxygen is converted to water. This is a redox reaction which requires the presence of either a transition metal (such as Fe of Cu) or a co-factor, *i.e.* Flavin coenzyme. The movement of single electrons to the molecule of oxygen is possible using an oxygenated cofactor intermediate. As mentioned previously the Flavin dependent monooxygenases do not require the presence of a metal. Baeyer-Villiger enzymes are flavoprotein monooxygenases. They contain Flavin for the redox process and either NADH or NADPH (Roberts and Willetts 1993) as the cofactor.

The proposed mechanism of oxygen insertion by cyclohexanone monooxygenase is shown in Scheme 1.12. In this mechanism the oxidation of the reduced Flavin generates E-FAD-4a-OOH, which serves as the oxygen donor. Elimination of water regenerates oxidised Flavin for the next catalytic cycle.



Scheme 1.12 The proposed mechanism of oxygen insertion by cyclohexanone monooxygenase.

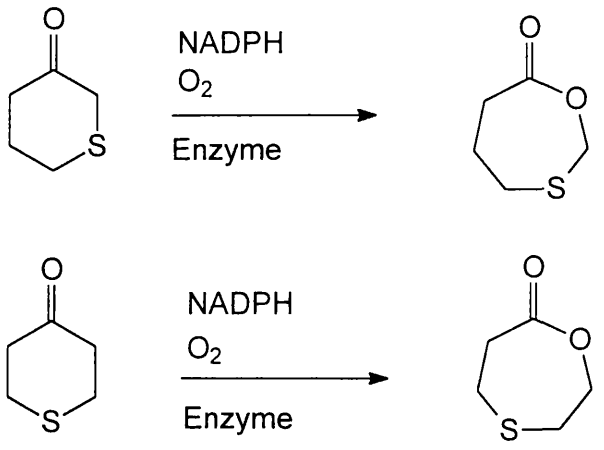
Monooxygenase enzymes are able to catalyse the biodegradation of aliphatic hydrocarbons and steroids (Konigsberger *et al.*, 1990), the biotransformation of aromatic hydrocarbons using phenolic α-hydroxylation, as well as the previously mentioned bioconversion of ketones and cyclic ketones to esters and lactones respectively. Walsh and Chen (1988) detailed seven examples of biotransformation by monooxygenases, they also reported the likelihood of other bioconversions to yield secondary plant metabolites. The enzymatic oxidation of sulphides to sulphones via sulphoxides (Scheme 1.13) has also been shown using the monooxygenase enzyme cyclohexanone monooxygenase (Walsh and Chen 1988, Kelly *et al.*, 1996b).



Scheme 1.13 Enzymatic oxidation of sulphides to sulphones via sulphoxides.

Other examples of sulphoxidation were reported by Alphand and co-workers (1997) and Carrea and co-workers (1992).

The enzyme catalysed oxidation of thiocyclohexanones occurs with regioselectivity. The oxygen atom is predictably inserted α to the carbonyl with no sulphur oxidation (Scheme 1.14).



Scheme 1.14 Enzyme catalysed oxidation of thiocyclohexanones.

The previous example of the oxidation of sulphides has demonstrated the versatility of the enzyme cyclohexanone monooxygenase. The enzyme can deliver nucleophilic oxygen to the carbonyl carbon of ketones and provide electrophilic oxygen to electron rich heteroatoms such as sulphur, selenide and phosphorus.

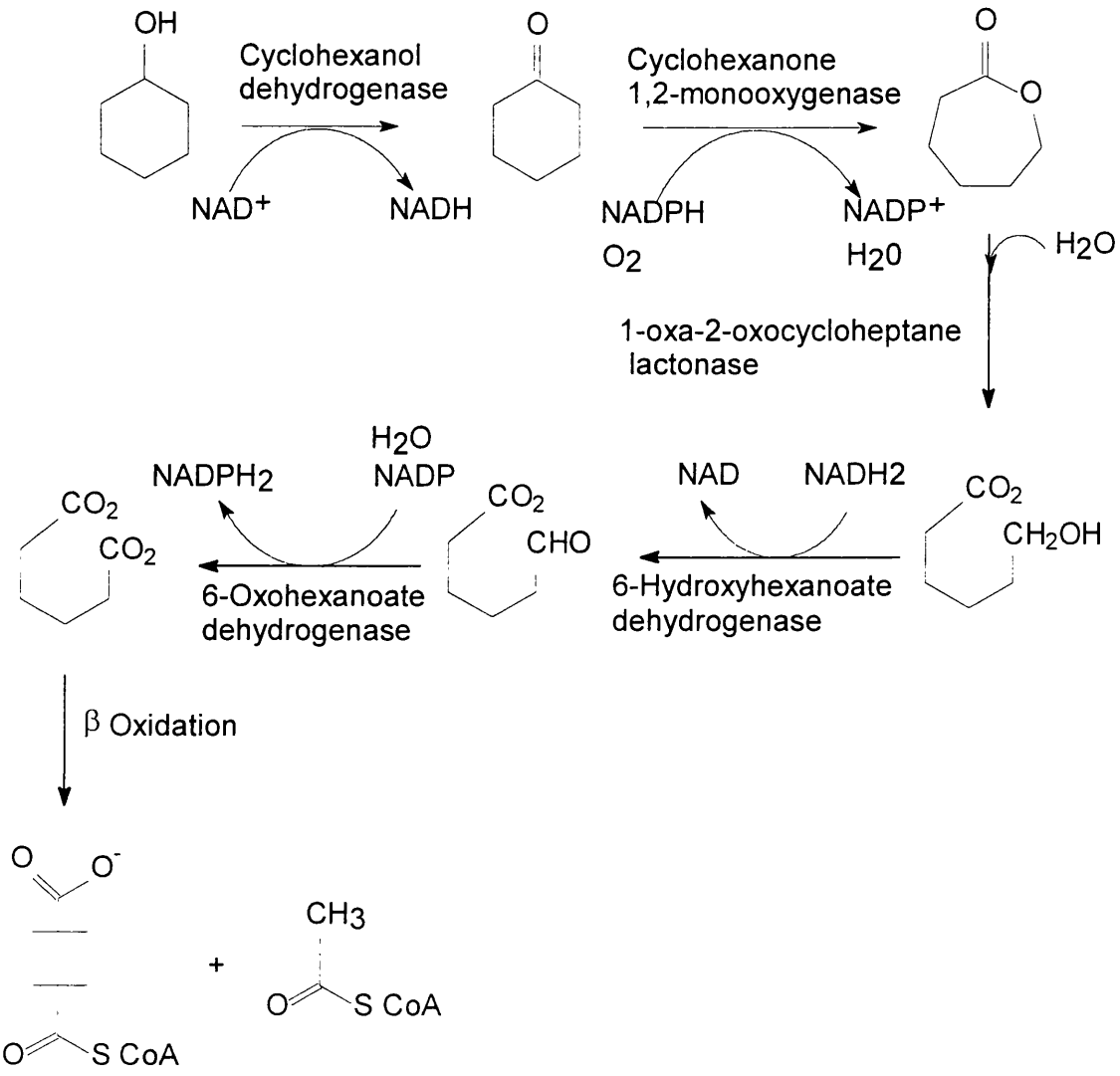
1.7.2 Baeyer-Villiger Oxidations

Many microorganisms have been found to contain monooxygenase enzymes. These include *Acinetobacter, Corynebacterium, Curvularia, Cylindrocarpin, Cunninghamella, Nocardia, Pseudomonas, Rhodococcus,* and *Xanthobacter.* The majority of reported studies concerning microbial Baeyer-Villiger oxidations have used *Acinetobacter calcoaceticus* or *Pseudomonas putida* as the biocatalyst. Baeyer-Villiger oxidations are possible with whole cells or monooxygenase enzyme preparations (crude or purified enzyme) (Gagnon *et al.,* 1994, Bes *et al.,* 1996, Grogan *et al.,* 1993.)

Acinetobacter calcoaceticus contains the monooxygenase enzyme cyclohexanone monooxygenase (CHMO). CHMO was first isolated and purified from *Acinetobacter* by Donogue and Trudgill (1976). They reported the biotransformation of C4-C8 cyclic ketones, some acyclic ketones and various aryl ketones. The enzyme is known to contain tightly held, non covalently bound FAD, 542 amino acids and have a molecular weight of 59 000 Da. *Acinetobacter* has been shown to grow with cyclohexanediol (Alphand *et al.,* 1989, Petit and Furtoss 1993, Alphand *et al.,* 1990 and Konigsberger *et al.,* 1991), cyclohexanol (Donoghue and Trudgill 1975, Alphand *et al.,* 1990, Schwab *et al.,* 1983, Konigsberger *et al.,* 1990, Grogan *et al.,* 1992 and Levitt *et al.,* 1990) and cyclohexanone (Kelly 1996a) as the sole carbon source. Baeyer-Villiger bioconversions were recently reviewed by Roberts and Wan (1998).

There are two disadvantages concerning the use of whole cell *Acinetobacter sp.* for a Baeyer-Villiger type biotransformation. The main disadvantage is that *Acinetobacter* is a class-two pathogen and therefore unsuitable for use on a large scale. The second disadvantage concerns the presence of further enzymes in the metabolic pathway which allow the degradation of the newly formed lactones, and other side reactions

NADPH is required by *Acinetobacter* during the catabolism of cyclohexanol and trans 1,2-cyclohexanediol. The catabolism of cyclohexanol is shown in Scheme 1.15 (Donoghue and Trudgill 1975, Walsh and Chen 1988). Cyclohexanone monooxygenase catalyses the second step in this metabolic pathway.



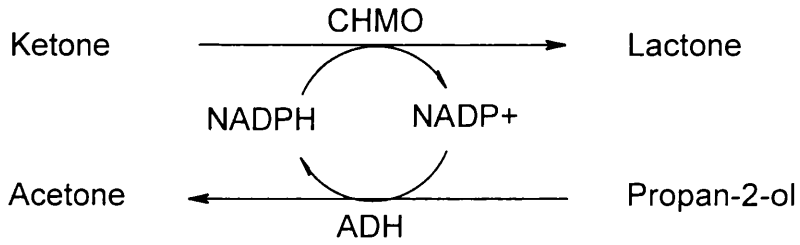
Scheme 1.15 The catabolism of cyclohexanol.

The degradation of the newly formed lactone can be prevented by the addition of a lactone hydrolysis inhibitor. Alphand (1990) demonstrated that the most effective inhibitor of this form of hydrolysis is tetraethyl pyrophosphate (TEPP).

An alternative method of preventing the degradation of the lactone is to perform the bioconversion using the isolated monooxygenase enzyme as the catalyst. The cofactor NADPH is required by CHMO to perform the bioconversion of a

cyclic ketone to it's corresponding lactone. To perform the bioconversion using isolated enzymes requires the addition of NADPH. As NADPH is an expensive co-factor the addition of stoichiometric quantities of NADPH to a bioconversion results in a costly process. One method of reducing the cost of the enzymic bioconversion is to recycle the NADPH using alcohol dehydrogenase (ADH) as shown in Scheme 1.16 (Grogan *et al.*, 1992.)

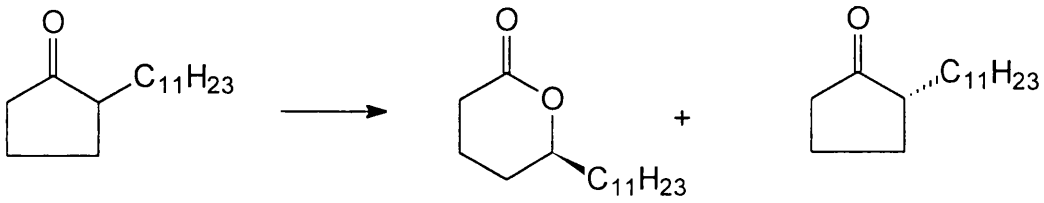
A further alternative method is to clone the enzyme into a host that does not contain any endogenous hydrolase enzymes.



Scheme 1.16 NADPH recycling using Alcohol dehydrogenase.

Whole cell biooxidations using *Acinetobacter* are well documented. Various workers have reported the microbiological Baeyer-Villiger reaction of cyclic and bicyclic ketones. (Alphand 1990 and 1992, Ottolina 1996, Roberts and Wan 1998.)

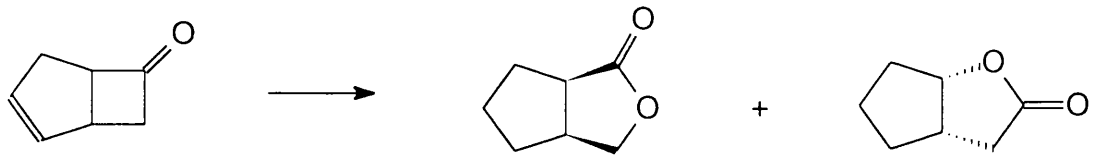
Alphand (1990) reported the use of *Acinetobacter calcoaceticus* NCIMB 9871 to perform the enantioselective biooxidation of racemic 2-undecylcyclopentanone (Scheme 1.17) to produce the 2-S configuration of the lactone with 74% enantiomeric excess. The remaining ketone was isolated and had an optical purity of 95%.



Scheme 1.17. Enantioselective biooxidation of racemic 2-undecylcyclopentanone.

Ottolina (1996) gave a review of biooxidations catalysed by *Acinetobacter* NCIMB 9871 yielding products of high optical purity. The review included data from 20 cyclic ketones, 17 bicyclic ketones and 1 tricyclic ketone.

Alphand (1992) has demonstrated the bioconversion of various bicyclic ketones to the 2 corresponding regioisomeric lactones using *Acinetobacter* NCIMB 9871 and TD 63. The percentage yields produced by each strain of the bacteria are comparable, as are the optical purities of both regioisomers of the lactone.(Scheme 1.14).

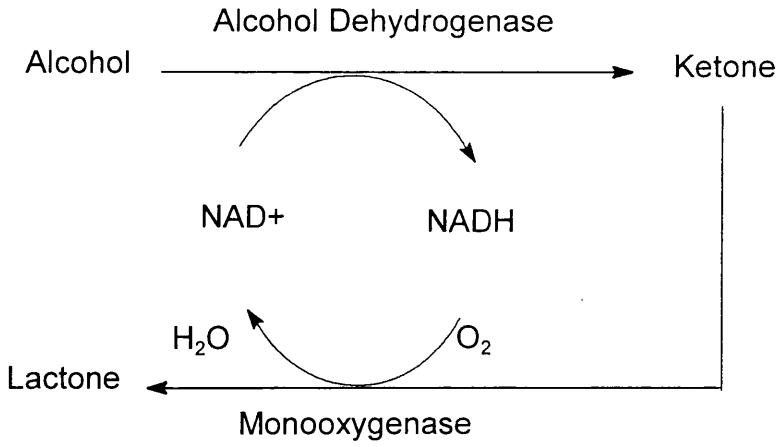


Ketone Lactone Lactone

Scheme 1.18 Bioconversion of bicyclic ketones to 2 regioisomeric lactones.

Extensive studies have been performed using *Pseudomonas putida,* which has been shown to contain two NADH dependent Baeyer-Villiger monooxygenase enzymes both of which have been isolated and characterised. These two enzymes are produced when *P. putida* is grown on either (+) or (-) camphor. The enzymes have been well characterised and it has been proven that the enzymes are actually isozymes, namely 2,5-diketocamphane 1,2-monooxygenase and 3,6-diketocamphane 1,2-monooxygenase. Both enzymes can catalyse the oxidation of bicyclic ketones. (Grogan *et al.,* 1992 and 1993, Gagnon *et al.,* 1994, Jones *et al.,* 1993, Kelly *et al.,* 1996a and 1996b, Alphand *et al.,* 1996, Roberts and Willetts 1993.)

The NADH dependence of these enzymes makes the utilisation of an NADH/NAD+ recycling system, similar to that in Scheme 1.16, an important economic factor when considering the cost of a large scale biotransformation of this type. The recycling of the expensive cofactor NADH means that only a small amount of the cofactor is required during the biotransformation. (Scheme 1.19.) This makes the biotransformation using *Pseudomonas putida* a more economically viable process (Grogan *et al.,* 1992).



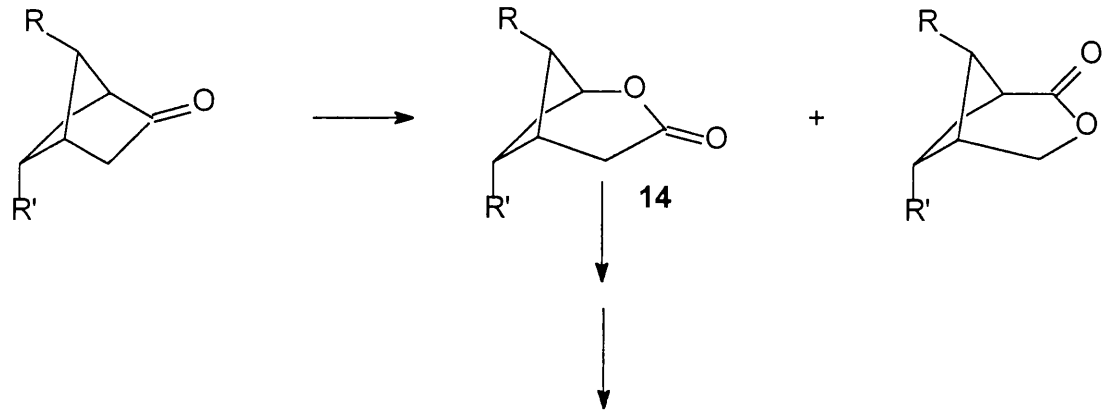
Scheme 1.19. Cofactor recycling of NADH.

Formate Dehydrogenase (FDH) can also be used as the second enzyme in the co-factor recycle system. (Rissom *et al.,* 1997, Seelbach *et al.,* 1996) Seelbach and co-workers describe the use of a cofactor recycle system using FDH with NADH and stated that all advantages of known NAD(H) dependent FDH recycles can be applied to NADP(H) dependent recycle systems. Rissom and co-workers describe a cofactor recycle system using CHMO and FDH with NADP(H) as the cofactor for the bioconversion of 4-methylcyclohexanone.

Personal communication (Doig 1999/2000) indicates that FDH could have many advantages over an ADH/CHMO cofactor recycle system. The disadvantage to such a process is at this time FDH is not commercially available and is under patent to the Institute of Biotechnology, Julich, Germany.

Pseudomonas putida also contains an NADPH dependent enzyme cyclopentanone monooxygenase. Unlike the diketocamphane monooxygenases, the cyclopentanone monooxygenase has a greater affinity for monocyclic ketones than bicyclic ketones. Although both diketocamphane and cyclopentanone monooxygenases have been shown to biotransform mono- and bicyclic ketones to some extent. (Kelly *et al.,* 1996, Bes *et al.,* 1996)

Pseudomonas sp. grown on cyclopentanol (or camphor) are able to bioconvert norbornanone into its corresponding lactones. (Scheme 1.20) One of these lactones (14) is an important chemical synthon for the synthesis of a commercial prostaglandin (Gagnon *et al.,* 1994). This biotransformation is therefore of great importance to the pharmaceutical industry.



Scheme 1.20 Bioconversion of norbornanone into its corresponding lactones

1.8 PROJECT AIMS.

The aims of the project are as follows:

- Develop a chemical procedure to racemise the model substrate.
- Characterise the chemical catalyst to be used for the racemisation procedure.
- Develop a biotransformation to convert the model substrate to its corresponding optically pure lactone.
- Design a process to integrate racemisation and bioconversion procedures.
- Compare the integrated process with other methods of synthesis of the lactone.

2. EXPERIMENTAL METHODOLOGY AND DESIGN.

2.1 INTRODUCTION.

Industrially, biotransformations are usually designed to be process-specific. A systematic approach to process evaluation was suggested by Woodley and Lilly in 1994. They described a structured approach to biotransformation reaction selection and operation.

The development of substrate selection criteria allows the suitability of possible substrates for the biotransformation to be investigated fully. This should be determined with as few experiments as possible. These vital investigations should be performed at the beginning of a project during process selection.

One of the major considerations in this research project is the mode of operation of the biotransformation and racemisation processes. There are many questions that require addressing before the type of reactor can be decided. A structured approach to this bioprocess, although initially time consuming, is most likely to result in optimal conditions and reactor design in the least amount of time.

2.2 OVERALL AIM OF RESEARCH.

The overall aim of this research is to develop a process to produce optically pure lactone via a Baeyer-Villiger oxidation of a cyclic ketone. The procedure is to combine a tradition chemical procedure and a bioconversion to enable a step improvement in the yield and enantiomeric excess of the lactone when compared against traditional chemical routes and the more recently reported biotransformations (see Figure 2.1). The biooxidation will be catalysed by

CHMO and the ketone enantiomer not used in the bioconversion will be chemically racemised to produce more of the desired ketone.

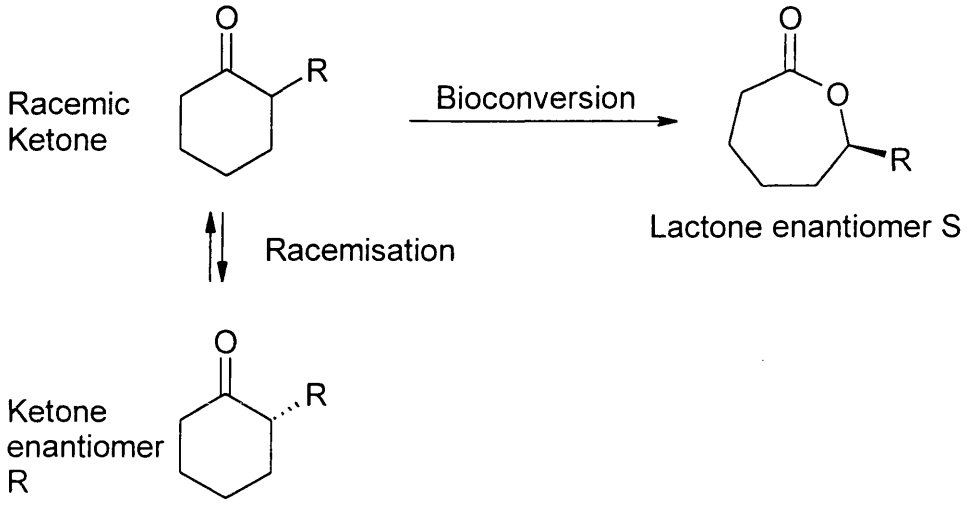


Figure 2.1 Integrated bioconversion and racemisation procedure.

2.3 SUBSTRATE SELECTION CRITERIA.

The availability and the source of the substrate is a key issue in this research. There are many questions that require answering in the selection of the substrate:

- Is a commercially available substrate required?
- If the substrate can be purchased, would it allow the bioconversion to be performed as an economically viable process?
- Can the substrate be synthesised and purified to produce suitable quantities?

The physical and chemical properties of both the substrate and its products require investigation, as they affect every other element of the process.

A viable process is one in which the reactants and products are stable in the given conditions. Therefore the stability of suitable substrates and their products should be investigated in the biotransformation media. The effect of the pH of the biotransformation media on the substrates and products requires investigation. The effect of operational temperature should also be determined.

The bioconversion process must be considered in terms of enzyme and substrate/product interactions. It is imperative that these interactions are examined in terms of an optimum process and also the compromises that can be made. For example a process in which the maximum enzyme activity is high with a poorly water soluble substrate is desired over that in which the maximum enzyme activity is low with a highly water soluble substrate. Important interactions to be considered are:

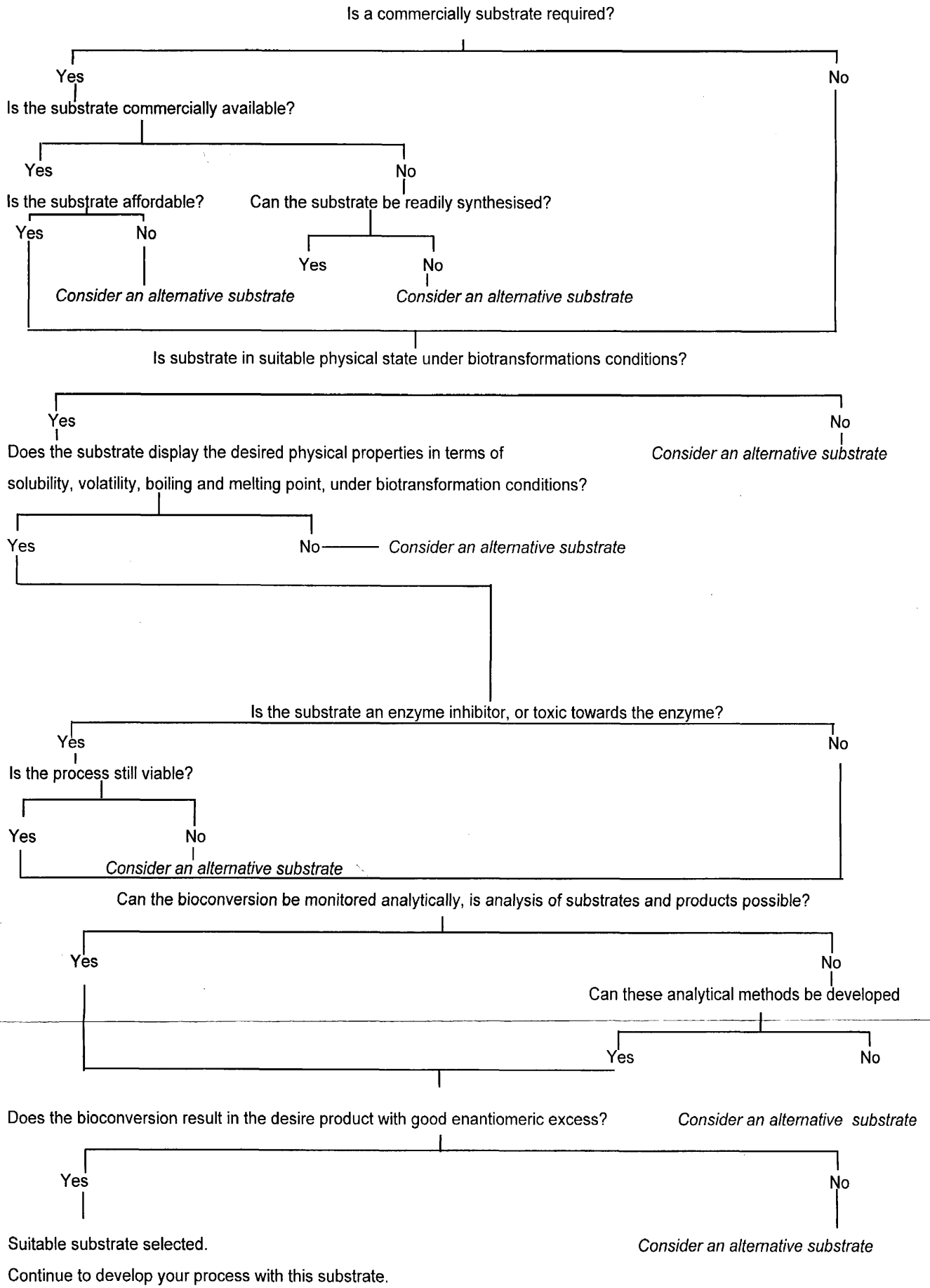
- Maximum enzyme activity in the presence of the substrate.
- Enzyme stability in biotransformation media.
- Substrate and product solubility.
- Extent of substrate and product inhibition.

The final consideration that should be made concerning each of the possible substrates is the ease of analysis.

Questions concerning the analysis such as the following should be addressed:

- Is there a suitable analytical method already in place or is method development required?
- Is the equipment available or can it be purchased?
- What are the analytical limitations due to the nature of the biotransformation media?

A biotransformation process will have to produce a predetermined enantiomeric excess (e.e.) of the product to make the process viable. If the e.e. of the candidate substrates is known, does this meet the process specification? The enantiomeric excess of a product is often not known until one of the later stages of a process. The selection of a substrate that exceeds the required e.e. can save valuable time during the later stages of the process design.


Is a commercially substrate required?
Yes
No
Is the substrate commercially available?
Yes
No
Is the substrate affordable?
Can the substrate be readily synthesised?
Yes
No
Yes
No
Consider an alternative substrate
Consider an alternative substrate
Is substrate in suitable physical state under biotransformations conditions?
Yes
No
Does the substrate display the desired physical properties in terms of solubility, volatility, boiling and melting point, under biotransformation conditions?
Consider an alternative substrate
Yes
No
Consider an alternative substrate
Is the substrate an enzyme inhibitor, or toxic towards the enzyme?
Yes
No
Is the process still viable?
Yes
No
Consider an alternative substrate
Can the bioconversion be monitored analytically, is analysis of substrates and products possible?
Yes
No
Can these analytical methods be developed
Yes
No
Does the bioconversion result in the desire product with good enantiomeric excess?
Consider an alternative substrate
Yes
No
Suitable substrate selected.
Continue to develop your process with this substrate.
Consider an alternative substrate

2.4 CATALYST SELECTION.

The selection of a suitable chemical catalyst for this research was an essential part of the decision making process that occurred before the experimental work for this project was performed. The interactions between the chemical catalyst, the substrate and the aqueous reaction solution are influential in the design of this process. In the case of this catalyst the following properties and interactions were to be considered:

- Can the catalyst perform the racemisation?
- Does the catalyst have any effect on the pH of a solution?
- Does the catalyst interact or bind to the enzyme?
- Does the catalyst have any effect on the lactone products of the bioconversion?
- Is the catalyst mechanically stable? Is the catalyst shear sensitive? Can it be used in a stirred tank or is a packed, expanded or fluidised bed required? Is the catalyst sensitive to pressure changes?

Of these considerations the most important feature of a suitable catalyst is its ability to perform the racemisation process.

Any catalyst that can successfully perform the racemisation will then be studied to determine the other physical properties and interactions as listed in the points above.

In this research the most desired catalyst is one that can perform the racemisation process, does not interact with the enzyme or the lactone products and has no significant effect on the reaction solution.

2.5 RACEMISATION MONITORING.

The chemically catalysed racemisation process is essential to this research. The decision has to be made as to how the racemisation is to be performed, is the substrate required to be enantiomerically pure, or is a racemic substrate suitable? An analytical method is then required to enable the process to be monitored. The most suitable method to monitor this type of reaction will depend on the optical purity of the substrate. Possible methods of monitoring the process and the type of substrate required are as follows:

- Deuteration of the α-proton at the chiral centre of a 2-substituted cyclic ketone (a racemic solution would be suitable) and NMR monitoring of the racemisation process.
- Production of non-racemic material, by collecting fractions of the expelled output after chiral HPLC. The HPLC would separate the two enantiomers of the ketone so they could be collected separately. The racemisation procedure would be monitored by chiral chromatography.
- Racemisation of the non converted ketone from the bioconversion process performed during this research. The non converted ketone would be enantiomerically pure or enriched. The racemisation procedure could be monitored by chiral chromatography.

2.6 BIOREACTOR SELECTION.

2.6.1 BIOREACTOR CONFIGURATIONS.

2.6.1.1 INTRODUCTION.

The reactor options for a bioconversion process are determined by the mode of operation (batch, fed batch or continuous) and the characteristics of the

reaction itself. In this research it is planned to operate the process in batch mode for ease of analysis and the determination of a mass balance.

2.6.1.2 STIRRED TANK REACTOR

A stirred tank reactor (STR) is usually a cylindrical vessel, with impellers for agitation, an air sparger and baffles. These reactors are used widely in fermentation and biotransformation on large scale and laboratory scale. Homogeneity and mass transfer are achieved by mixing. A stirred tank reactor can be run in batch phase (BSTR) or continuous phase (CSTR). The main disadvantage of STR is due to mixing which often results in increased viscosity and grinding of the catalyst. The reactor is therefore limited to low levels of biocatalyst, typically 10% v/v.

2.6.1.3 PACKED BED REACTOR

A packed bed reactor consists of a cylindrical tube packed with biocatalyst particles. This configuration can allow high concentrations of the enzyme to be present in the packed bed. The disadvantage of this system is a poor flow pattern. This can lead to variations dead-legs in the reactor which can result in variations of the concentrations of the substrate and the product throughout the bed.

2.6.1.4 MEMBRANE REACTOR.

Membrane bioreactors are based on the concept of separating enzymes and substrates (or products) using a semi permeable membrane to act as a selective barrier (Prazeres and Carbral, 1994). They are used in preference to traditional stirred tank or plug flow reactors when the cells or enzymes used in a process are too sensitive to their environment, pH, oxygen transfer temperature and media composition (Beeton *et al.*, 1994).

Membrane reactors can offer similar advantages as STR and packed bed reactors, but can also minimise some downstream processing problems. The disadvantages concerning the use of membrane bioreactors are normally due to mass transfer problems or breakthrough where the non wetting solution is able to cross the membrane. The occurrence of breakthrough makes it impossible to use the reactor (Vaidya *et al.,* 1994).

2.6.2 BIOREACTOR SELECTION, DESIGN AND PROCESS CONSTRAINTS.

2.6.2.1 *IN-SITU* BIOCONVERSION AND RACEMISATION.

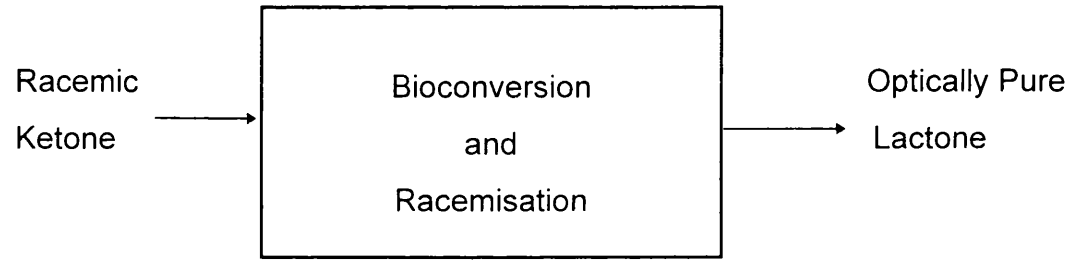


Figure 2.2 *In-situ* bioconversion and racemisation reactor.

The desired process in this research project would feature a 'one pot' type of reaction, where the biotransformation and racemisation would occur simultaneously in the same bioreactor, in this case a stirred tank. This process would involve a racemic substrate, an enantiomerically pure product and two catalysts - a biocatalyst and a solid chemical catalyst. The main considerations in this type of process are the interactions, if any, between the two catalysts, the levels of product and substrate inhibition on the enzyme, and the method of product removal from the system.

The properties of, and the interactions between, the enzyme and the chemical catalysts require investigation to determine the optimal process conditions. For

example, binding between the enzyme and catalyst that resulted in loss of activity of either of the two catalysts would be undesired. In this situation the process would be designed so that the two catalysts cannot come into contact with each other.

There are five possible modes for the two catalysts to operate in the same reactor:

- Free enzyme and free chemical catalyst
- Immobilised enzyme and free chemical catalyst
- Free enzyme and immobilised chemical catalyst
- Immobilised enzyme and immobilised chemical catalyst
- Enzyme immobilised on catalyst.

2.6.2.2 BATCH MODE.

The simplest bioreactor design for a linked biotransformation and racemisation would consist of two vessels as demonstrated in Figure 2.3.

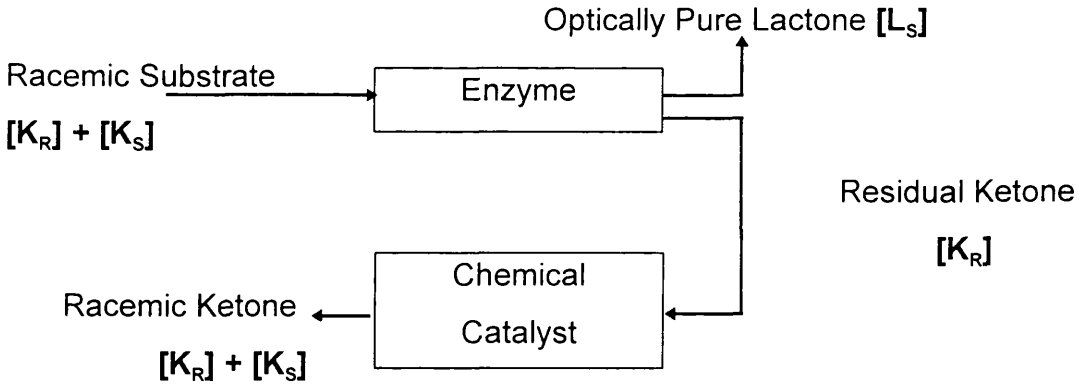


Figure 2.3 Linked biotransformation and racemisation reactor.

This mode of operation is advantageous as it allows the two processes to be carried out in different types of bioreactor if necessary. A shear sensitive

catalyst would require a fluidised bed or packed bed reactor for optimal performance, whereas the enzyme is likely to perform well in a stirred tank. A major advantage of a linked vessel system is the ability for each of the two processes to occur in different conditions. The conditions required for the racemisation may possibly be too harsh for the enzyme, the opposite being that the racemisation may not occur in the pH controlled media required for the bioconversion. In the case that the chemical catalyst interferes with the enantiomerically pure lactone an *in-situ* product removal step (ISPR) may be incorporated into the reactor design. ISPR is an extremely useful tool to increase the yield and productivity of a bioprocess, as well as minimising the interference of the chemical catalyst ISPR can also be effective against product inhibition (Freeman *et al.,* 1993).

The disadvantages of this type of process occur due to the probable difference in reaction rates. This will require either strict control over the flow between the two vessels or the two reactions to be carried out as discrete unit operations in batch mode, where the biotransformation would run to completion before the commencing the racemisation step.

2.7 PROCESS DESIGN.

2.7.1 INTRODUCTION

The most logical method of designing a process such as the combined bioconversion and racemisation is to consider each of the two processes individually, as separate unit operations. Initially the physical conditions of the two processes can be compared to determine whether it is possible to combine the two processes. Compromises, in terms of pH and reactor design should be considered, if necessary, to allow both processes to occur within the same reactor.

2.7.2 SYSTEM REQUIREMENTS

The starting point for the process design was the selection of a suitable substrate, in this case a 2-substituted cyclic ketone, to allow racemisation, via a tautomerism mechanism. The method of monitoring the racemisation procedure was the next important decision to be taking in the design of this process, as this affected the state that the substrate was required in, optically pure or racemic. If an optically pure substrate was desired, this in turn had to be produced and it's enantiomeric purity determined. The choice of catalyst was determined following catalyst characterisation. In our case we were looking for a catalyst that did not bind with, or interfere with, the activity of the enzyme(s) involved in the bioconversion step. The catalyst was desired to have no significant effect on the pH of the solution and to be viable at 30°C. One of the most important considerations is the effect of the catalyst on the lactone produced by the bioconversion. A catalyst that rapidly degrades the lactone should not come into contact with the catalyst. The final consideration was the reactor. The design of the reactor was dependant upon the feasibility of performing an *in-situ* bioconversion and racemisation procedure and the stability of the catalyst in the reactor. A stirred tank reactor would not be suitable for a shear-sensitive catalyst.

2.8 SUMMARY

The selection of catalyst and substrate are to be performed with the aid of the criteria detailed in sections 2.2 and 2.3 of this Chapter.

The initial aims of this research involve the selection of a suitable model substrate and the development of a racemisation monitoring procedure to facilitate the selection of a suitable chemical catalyst.

The overall objective of this research is to design and operate a process which combines traditional chemistry with biocatalysis to produce enantiomerically pure lactone in high yield. The results obtained from the procedure are to be compared and contrasted with the results from non integrated processes to determine whether the integrated procedure offers a process improvement over known methods.

3. CHARACTERISATION.

3.1 INTRODUCTION

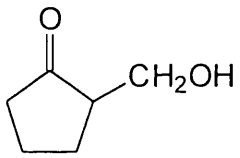
3.1.1 SUBSTRATE SELECTION AND CHARACTERISATION

3.1.1.1 SUBSTRATE SELECTION

3.1.1.1.1 2-HYDROXYMETHYLCYCLOPENTANONE AS A MODEL SUBSTRATE.

The original substrate for this research project was selected before the development of the selection criteria as detailed in Chapter 2.1.

2-hydroxymethylcyclopentanone was initially chosen as the substrate for this work as it was known to be enantioselectively converted to the corresponding lactone by cyclohexanone monooxygenase.



Structure 3.1 2-hydroxymethylcyclopentanone

The substrate was not commercially available, but required in-house synthesis. The ability to monitor the racemisation procedure was as described in Figure 3.1

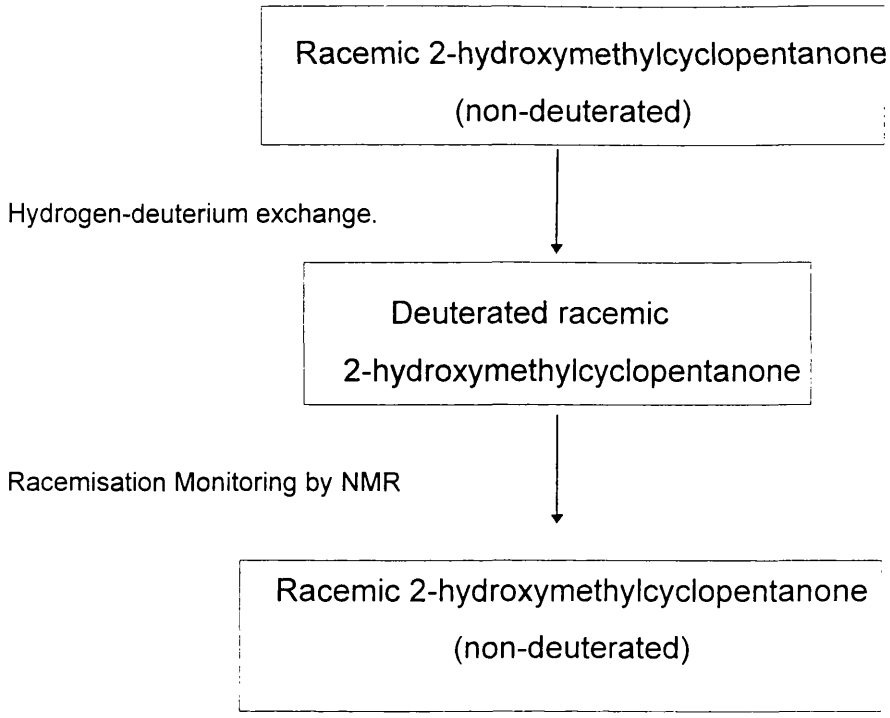


Figure 3.1 Flow diagram of initial racemisation process design.

A number of problems were encountered during the development of this process.

- The deuteration was difficult to perform selectively, as hydrogen-deuterium exchange was observed at many sites other than the chiral centre giving what was thought to be a mixture of products. The monitoring of the racemisation procedure would have been difficult as it would have been unclear which NMR signals were from the deuterium/proton of interest.

- The purification of the 2-hydroxymethylcyclopentanone following synthesis took a number of days and the production of material of high purity was not possible. The product always contained over 4 impurities (by TLC) after extraction and at least one impurity after column chromatography using Keisel gel silica. The yield of the impure ketone was approximately 20%.

- The activity exhibited by the enzyme (CHMO) in the presence of 2-hydroxymethylcyclopentanone was low compared to the activity observed in the presence of other cycloalkanones. Hogan (Ph.D thesis, 1999) investigated the CHMO enzyme activity in the presence of various cycloalkanones during enzyme characterisation studies. His findings are listed in table 3.1.

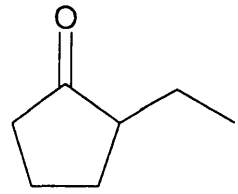
The low activity of 2-hydroxymethylcyclopentanone, combined with the problems associated with the purification of this substrate and the racemisation monitoring led to the decision to select a new substrate.

Reaction substrate	**Boiling point, °C**	**Max Enzyme activity, U/g dcw *E. coli***
Cyclobutanone	99	224
Cyclopentanone	131	193
Cyclohexanone	160	350
2-Methyl Cyclohexanone	163	367
2-Ethyl Cyclopentanone	~200	273
2-Hexyl Cyclopentanone	~200	291
2-Heptyl Cyclopentanone	141/20mmHg	126
Ethyl-2-oxocyclopentyl acetate	~200	263
2-hydroxymethyl cyclopentanone	~200	21
Methyl 2-oxo cyclopentan carboxylate	105/19mmHg	35
Ethyl 2-oxo cyclopentan carboxylate	102/11mmHg	49
4-Methyl cyclohexanone	169	354

Table 3.1 Characterisation of candidate substrates (adapted from Hogan Ph.D thesis, 1999).

3.1.1.2 2-Ethylcyclopentanone as a Model Substrate.

2-Ethylcyclopentanone was selected as an alternative substrate to 2-hydroxymethylcyclopentanone. 2-Ethylcyclopentanone was known to be commercially available and the CHMO enzyme activity was observed to be reasonably high in the presence of 2-ethylcyclopentanone.



Structure 3.2 2-Ethylcyclopentanone

A chiral HPLC column was identified that would facilitate the separation of the two enantiomers of the 2-ethylcyclopentanone. It was decided that optically pure material could be obtained by the collection of the eluent from the chiral analysis. The two enantiomers would be physically separated from each other and therefore they would be in different fractions of the mobile phase that is eluted from the column. This would allow them to be collected separately giving one ketone enantiomer in one fraction and the other ketone enantiomer in a separate fraction. The enantiomerically pure ketone could then be racemised with the aid of a catalyst. An analytical method was developed to produce optically pure ketone.

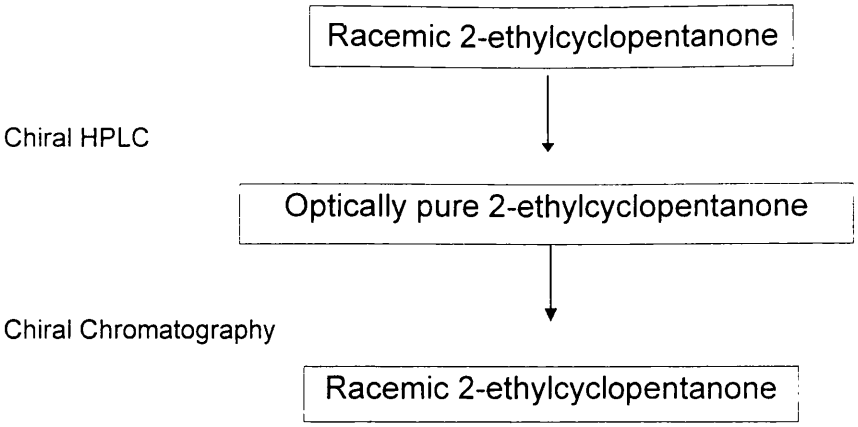
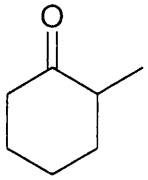


Figure 3.2 Flow diagram of modified racemisation process design.

The practical limitations of the chiral HPLC column were due to the inability to inject large volumes of sample onto the column and the inability to introduce water or water based solutions to the column. This resulted in the need for the removal of water from racemisation samples or the development of an alternative analytical method to monitor the racemisation. The synthesis of 2-ethylcyclopentanone was discontinued by our supplier, resulting in the need to select a new substrate.

3.1.1.3 2-Methylcyclohexanone as a Model Substrate.

2-methylcyclohexanone was chosen as the new substrate as it was known to be commercially available and the CHMO enzyme activity was observed to be reasonably high in its presence.



Structure 3.3 2-Methylcyclohexanone.

The availability of a chiral GC column allowed the separation of the two enantiomers of 2-methylcyclohexanone and the corresponding lactones. The process would be performed according to the diagram in Figure 3.3. The racemisation would be monitored by chiral GC. The presence of the lactone during the racemisation procedure would serve to indicate the possibility of performing an *in-situ* bioconversion and racemisation. Any degradation of the lactone by the chemical catalyst would be observed during the initial racemisation process.

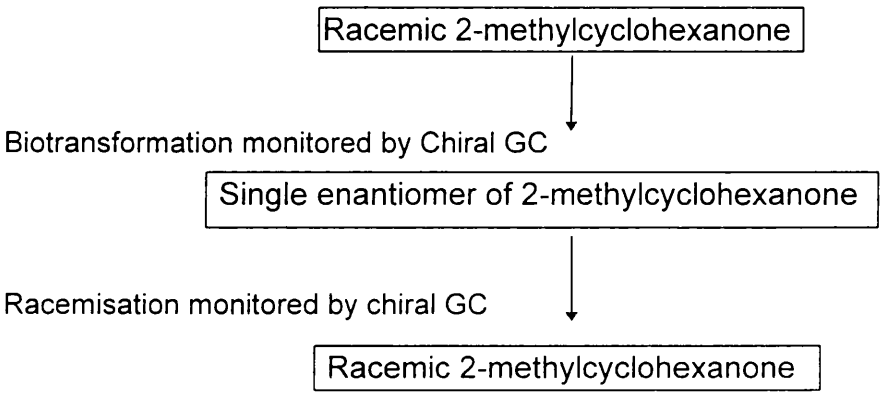


Figure 3.3 Flow diagram of final racemisation process design.

3.1.1.4 SUBSTRATE SELECTION SUMMARY.

Initial investigations as detailed in 3.1.1.1 - 3.1.1.3 have allowed the selection of 2-methylcyclohexanone as a model substrate for the remaining research of this project. All further studies concerning the substrate refer to 2-methylcyclohexanone. No further investigations were performed with either of the other substrates. The remaining part of this Chapter describes the characterisation, racemisation and the bioconversion of 2-methylcyclohexanone.

3.1.2 Catalyst Selection and characterisation

The ideal catalyst for the racemisation was a solid chemical catalyst that would not have an effect on its external environment (the solution it was in) and that was able to catalyse the racemisation of 2-methylcyclohexanone at room temperature.

The catalysts of choice were zeolites, they were known to be good solid chemical catalyst that did not effect the pH of a solution. Zeolite H-Y was kindly donated by The Leverhulme Centre for Catalysis at The University of Liverpool. Zeolite H-Na was kindly donated by Prof. Joaquim Cabral, Instituto Superior Tecnico, Lisbon, Portugal. A limited amount (~10 g) of the two zeolites were donated, therefore the investigation into whether either zeolite was able to catalyst the racemisation of 2-methylcyclohexanone was performed on a small scale (2.5 g of zeolite per litre of buffer solution)

A group of clays were donated by English China Clay (ECC), Cornwall. These were called Starlight C and Starlight K. Four particles sizes of each catalyst were provided in large quantities (1 kg of each). These were investigated to determine whether they could promote the base catalysed racemisation of 2-methylcyclohexanone.

Finally a number of ion-exchange resins were selected from the Fluka catalogue. They were all selected on the basis that they were described as 'basic'. As time was limited it was decided to start with the strongly basic ion-exchangers as they would be more likely to catalyst the reaction than the weakly basic ino exchange resins.

All of the catalysts were investigated for their ability to catalyse the racemisation of 2-methylcyclohexanone using the following method. The catalyst (1 g, except for the zeolites where 0.02 g was used) was added to a 50 ml glass beaker

containing 20 ml of an aqueous solution containing enantiomerically enrich 2-methylcyclohexaone (15 mM). The solutions were stirred at room temperature for 18 hours. Samples were removed at 0.5, 1, 2 and 18 hours and analysed by chiral HPLC.

Over 30 catalysts were investigated for their ability perform the base catalysed tautomerism of an enantiomerically enriched solution of 2-methylcyclohexanone to racemic 2-methylcyclohexaone, for details see Appendix C. Of these catalysts only Amberlite IRA 400 (-OH) and NaOH were able to racemise an enantiomerically enriched solution of 2-methylcyclohexanone. Therefore only Amberlite IRA 400 (-OH) and NaOH were continued to be used during the development of the integrated process.

3.1.3 BIOTRANSFORMATION .

The biotransformations were all performed using intracellular CHMO from *E.coli* JM10/ pQR210. A crude enzyme extract containing CHMO was prepared from the fermentation broth by homogenisation (1000 bar, 3 passes) followed by centrifugation (15 000 rpm, 2 minutes). The resulting supernatant was analysed for cyclohexanone monooxygenase activity assay and then used for the bioconversions.

Initial bioconversions were carried out on a small scale (30 mL) using NADPH as the cofactor. NADPH is expensive, and would therefore have a huge effect on the economics of the integrated process when performed at large scale. The most effective method for decreasing the cost of the cofactor in this type of biotransformation is to using a cofactor recycling system. In the case of CHMO oxidations the NADPH produces NADP+, which can be utilised in many dehydrogenase reactions. Hogan (Ph.D Thesis, 1999) developed a cofactor recycling system employing ADH as a coenzyme and NADP+ as the cofactor,

for use with a CHMO oxidation. These findings were used as an initial starting point for the development of the cofactor recycling system employed with the CHMO oxidation of 2-methylcyclohexanone described in this thesis.

3.2 MATERIALS.

3.2.1 REAGENTS AND CHEMICALS

All chemicals and reagents were purchased from Sigma-Aldrich and were of the highest purity unless otherwise stated. 2-methylcyclohexanone was purchased from Fluorochem Ltd. Amberlite IRA 400 (-OH) was purchased from Fluka.

The recombinant E.coli strain (JM107/pQR210), expressing cyclohexanone was prepared by Dr. J. Ward and Sejal Patel, The department of Biochemistry and Molecular Biology, UCL (unpublished.)

3.2.2 EQUIPMENT.

Glassware was purchased from BDH Laboratory supplies. NMR spectra were recorded on a Bruker DRX500, 500 MHz spectrophotometer unless otherwise stated. All samples were sonicated using a Sonicator MSE Soniprep. All samples were centrifuged using a Heraeus Megafuge 1.0R. 2L fermentations were performed in a 2L LH glass vessel. Gas chromatography was performed using a Perkin Elmer GC, with Turbochrom software. All UV and OD measurements were performed using a Kontron Uvikon 922 UV/Vis spectrophotometer. The orbital shaker used was from New Brunswick Scientific.

3.3 METHODS.

3.3.1 SUBSTRATE CHARACTERISATION

3.3.1.1 STABILITY OF 2-METHYLCYCLOHEXANONE AND 7-METHYLOXEPAN-2-ONE.

The stability of the ketone 2-methylcyclohexanone (and lactone 7-methyloxepan-2-one) was determined by the addition of a known quantity of the ketone (50 μL, 0.4 mmol) (or lactone (50 μL, 0.4 mmol) to a known volume of phosphate buffer (3.95 mL, 50 mM, pH 7.5) in a sealable 25 mL glass bottle. The bottle was left in the light at room temperature. A sample was taken at the initial time point and after 24 hours and analysed by chiral HPLC.

3.3.2 CATALYST CHARACTERISATION.

3.3.2.1 EFFECT OF CATALYST ON SOLUTION PH.

The effect of the catalyst on the pH of a various solutions was determined over a given time period. For Amberlite IRA 400 (-OH) the catalyst (50 g/L) was suspended 20 ml of various (pH 7, pH 8 and pH 9 Tris buffer and water) in a 50 ml glass beaker. The suspensions were stirred with the aid of a magnetic stirrer for 24 hours at room temperature. The pH of each solution was determined at 0, 0.5, 2.5 and 24 hours.

For NaOH (2 M, 10% v/v) the catalyst was added to various solutions (pH 7, pH 8 and pH 9 Tris buffer and water) to make a total volume of 20 ml in a 50 ml glass beaker. The solutions were stirred with the aid of a magnetic stirrer for 24 hours at room temperature. The pH was determined at 0, 0.5, 2.5 and 24 hours.

3.3.2.2 EXTENT OF PROTEIN BINDING TO THE CATALYST.

The extent of protein binding to the catalysts was determined by stirring a suspension of the Amberlite IRA 400 (-OH) catalyst (50 g/L) and BSA (1 mg/mL) in 20 ml Tris-HCl buffer solution at pH 9.0 in a 50 ml glass beaker. Aliquots were removed at hourly intervals for 7 hours. Each aliquot was centrifuged at 10 000 rpm for 2 minutes to remove the suspended catalyst.

A Coomassie Blue protein assay was performed on the supernatant. For this 5 μL of the sample was accurately transferred, in quadruplet, to a microtitre plate. 145 μL of Coomassie dye was added to each well. For the standards, solutions containing 0.04, 0.1, 0.2, 0.4, 0.8 and 1mg/mL BSA were prepared. The volume of standard solution and dye added to each well was identical to that for the samples. The plates were shaken and left for 5 minutes. The samples were then assayed by UV at 595 nm. The absorption obtained from the standards was plotted to produce a calibration curve. The concentration of BSA available in solution was determined by comparison of the absorption gained for the samples against the calibration curve. A representative protein assay calibration curve can be seen in the appendix

3.3.2.3 EFFECT OF CATALYST ON THE LACTONE 7-METHYLOXEPAN-2-ONE

7-methyloxepan-2-one (40 mM) was added to a solution of phosphate buffer (20 mL, pH 7.5, 50 mM) in a 50 ml glass beaker at room temperature and stirred. To this solution various concentrations of catalyst were added (Amberlite IRA 400 -OH was used at a concentration of 100, 50, 10 and 5 g/L. 4, 2, 1 and 0.1M NaOH 10% v/v was used). Samples were removed at 0, 1, 2, 3, 4, 5 and 10 minutes. The concentration of lactone in the aliquots was determined by chiral GC. (In the case of the NaOH studies the aliquots were

neutralised with equimolar HCl immediately after the sample was removed from the reaction vessel. *i.e.* 0.1 M HCl was used to neutralise 0.1 M NaOH).

3.3.2.4 Effect of shear on Amberlite IRA 400 ($^-$OH)

The effect of shear on the catalyst was determined using a Shear device. 100 mL of solution containing 50 g/L of Amberlite IRA 400 (-OH) was prepared in a 200 ml glass beaker. The solution was added to the shear device to fill the shear chamber. The beads were subjected to shear at the speeds of 4 000, 10 000, 15 000 , 20 000, 24 000 and 27 000 rpm. The beads were removed from the chamber and examined under a microscope to determine the extent of damage.

3.3.3 Racemisation and racemisation monitoring

3.3.3.1 Racemisation.

The racemisations were performed on a 15 mL scale in a 20 mL glass beaker at 30°C. Aliquots of the solution were removed at various time points and analysed by chiral GC. The enantiomerically enriched solution used in the racemisation was obtained by biotransformation (see 3.3.9.3) and used without modification. Added to this solution was 50 g/L catalyst.

3.3.3.2 Racemisation monitoring

3.3.3.2.1 Gas Chromatography method development.

The two enantiomers of 2-methylcyclohexanone were separated using a Lipodex E capillary column, from Macherey-Nagel, Postfach 10 13 52, D-52313

Duren, Germany. The two peaks were baseline resolved from each other using the following conditions:

Injection temperature:	120°C
Detection temperature:	220°C
Oven temperature:	65°C to 100°C at 4°C min^{-1} hold for 5 mins
	100°C to 210°C at 10°C min^{-1} hold for 2.25 mins.

The sample injection volume was 1 μL.

3.3.3.2.2 METHOD VALIDATION

3.3.3.2.2.1 METHOD REPRODUCIBILITY

The reproducibility of the method was tested by performing 10 injections from the same vial containing a solution of known concentration. The areas obtained from each enantiomer from the ten injections were compared and the standard deviations of these areas were calculated.

3.3.3.2.2.2 METHOD LINEARITY.

The calibration curve and linear range for this method were determined by performing quadruple injections of standard solutions containing 1, 2, 4, 10, 20, 25, 50 and 100 mM 2-methylcyclohexanone in IPA.

The calibration curve and linear range for 7-methyloxepan-2-one were determined by performing quadruple injections of standard solutions containing 1, 2, 4, 10, 20, 40, 50 and 100 mM of 7-methyloxepan-2-one in IPA.

3.3.3.2.3 CHIRAL GC METHOD TO ASSAY FOR THE TWO ENANTIOMERS OF 2-MTHYLCYCLOHEXANONE AND THE TWO ENANTIOMERS OF 7-METHYLOXEPAN-2-ONE.

Column: Macherey-Nagel, Lipodex E capillary column,

Separation conditions;

Injection temperature:	120°C
Detection temperature:	220°C
Oven temperature:	65°C to 100°C at 4°C min^{-1} hold for 5 mins
	100°C to 210°C at 10°C min^{-1} hold for 2.25 mins.

The sample injection volume was 1 μL, the split was 100:1.

Samples were analysed using the above conditions and compared against standards curves. Calibration injections were included in each sample table to check the reproducibility of the method during each analytical run.

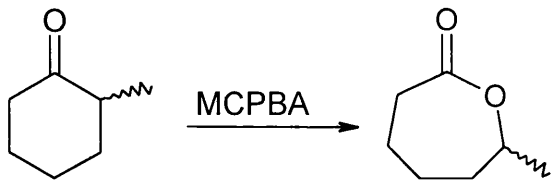
3.3.3.2.4 STANDARDS

3.3.3.2.4.1 2-METHYLCYCLOHEXANONE GC CALIBRATION STANDARDS

2-methylcyclohexanone dissolved in IPA to produce a standard of known concentration. 1 μL injection volumes of the calibration standards were used during each analytical run.

3.3.3.2.4.2 7-METHYLOXEPAN-2-ONE GC CALIBRATION STANDARDS

7-methyloxepan-2-one was synthesised chemically from 2-methylcyclohexanone as follows:



2-methylcyclohexaone (5 g, 40 mmol), sodium bicarbonate (5.05 g, 60 mmol) and MCPBA (13.76 g, 80 mmol) were stirred in dichloromethane (200 mL) in a 500 mL glass round bottomed flask with cooling with ice for 1 hour, followed by stirring at room temperature for a further 23 hours.

The lactone product was purified by filtration to remove the precipitate, and removal of the dichloromethane by vacuum distillation. The product was identifed as 7-methyloxepan-2-one by comparison of chiral GC retention time with a known sample of 7-methyloxepan-2-one. The product was stored at -20°C prior to analysis by GC.

7-methyloxepan-2-one was dissolved in Isopropanol (IPA) to produce a standard of known concentration. 1 μL injection volumes of the calibration standards were used during each analytical run.

3.3.4 Biotransformations

3.3.4.1 Production of biocatalyst

3.3.4.1.1 Spectroscopic methods

3.3.4.1.1.1 Optical Density Measurements.

The optical density (OD) of fermentation broth was determined by measuring the absorption of a suitably diluted sample at 670 nm. For diluted samples the

OD was determined in triplicate, and the mean value was recorded, unless otherwise stated. The dilution was performed using 50 mM phosphate buffer (pH 7.5)

3.3.4.1.1.2 CYCLOHEXANONE MONOOXYGENASE ACTIVITY ASSAY.

The cyclohexanone monooxygenase (CHMO) activity was determined using a time-drive assay on the Kontron Uvikon 922 UV/Vis spectrophotometer. The assay was performed on a crude enzyme extract at 340 nm.

The solutions for this assay were prepared as follows:

Buffer	8.642 g/L Tris EDTA and 7.143 g/L BSA the pH was adjusted to 9
Cyclohexanone	2 mg/mL
NADPH	1.33 mg/mL (to be stored in the dark, at 0°C)
Enzyme extract	The fermentation broth (2 mL) was centrifuged at 15000 rpm for 2 mins, and the resultant pellet was resuspended in buffer (see above. This solution was sonicated using 5 cycles of 10 seconds, and then centrifuged at 15000 rpm for a further 2 minutes. The supernatant was removed and used for the assay. (The crude enzyme extract from a harvested fermentation broth was used without further processing).

The background reading was performed on a solution containing the Buffer (700 μL), NADPH (100 μL), enzyme (100 μL) and water (100 μL). The background reading was measured for 30 seconds and the value recorded. Cyclohexanone (100 μL) was added. The activity of this solution was then

measured. The CHMO activity was determined by subtracting the value of the background reading from that of the final solution.

3.3.4.2 FERMENTATION METHODS

3.3.4.2.1 SHAKE FLASK GROWTH.

E.coli JM107/pQR210 was grown in shake flasks on complex media. The media contained 10 g/L of the following: Gylcerol, Sodium Chloride, Tryptone, Yeast Extract. The media was pH adjusted to pH 6.8 prior to sterilisation at 121°C before inoculation.

2L shake flasks containing 200mL of the complex media were inoculated aseptically with *E.coli* JM107/pQR210 glycerol stock (2 mL). Ampicillin (100 μg/L) was added, by sterile filtration (through a 0.2 μm sterile filter), followed by incubation at 37°C in an Orbital Shaker (New Brunswick Scientific) whilst shaking at 200 rpm. CHMO production was induced by IPTG (isopropyl β-D-thiogalactopyranoside) (238 mg/L, 1 mM) during the exponential growth phase when the optical density reached approximately 1.

3.3.4.2.2 GROWTH IN A 2L BIOREACTOR.

E.coli JM107/pQR210 was grown in a 2L fermenter on defined salts media. The pH was controlled by acid/base addition. Growth was monitored by OD measurements.

Defined Media.

Component	**g/L**
Glycerol	10
KH_2PO_4	3.96
Na_2HPO_4	2.10
NH_4Cl	1.32
K_2SO_4	6.62
PPG	0.2 mL
Yeast Extract	1.0

All of the above to be pH adjusted to pH 6.8 and sterilised at 121°C.

Vessel 1 (to be filter sterilised)

$CaCl_2.2H_2O$	0.33

Vessel 2 Trace elements (to be filter sterilised).

$ZnSO_4.5H_2O$	0.0032
$CuSO_4.5H_2O$	0.00033
$MnSO_4.4H_2O$	0.00057
Thiamine	0.01

Vessel 3 (to be filter sterilised)

Citric acid	0.66
$Fe(NH_4)_2.6H_2O$	0.60

Vessel 4 (to be filter sterilised)

$MgSO_4$	0.66

Ampicillin (to be filter sterilised)	0.05

The previous components are separated into various vessels and filter sterilised to avoid precipitation.

The 2 L fermenter containing 1500 mL of defined media was inoculated aseptically a 10% v/v inoculum from a previously grown defined media *E.coli* JM107/pQR210 shaken flask fermentation. The shake flask was inoculated with a 1% v/v glycerol stock approximately 10 - 15 hours before use as the inoculum for the 2 L fermentation. Ampicillin (50 mg/L) was added to the fermentation broth. The pH of the fermentation broth was controlled at pH 7.0 with NaOH and NH_4OH as required. The temperature of the broth was controlled at 37°C, the stirrer speed and aeration were controlled to maintain a DOT of above 10% were possible.

CHMO production was induced by IPTG (700 mg, 2 mM) during the exponential growth phase when the optical density reached approximately 10.

3.3.4.2.3 PRODUCTION OF GLYCEROL STOCKS.

E.coli JM107/pQR210 stocks were stored in glycerol at -70 °C. These stocks were produced by the addition of a volume of *E. coli* JM107 broth to the same volume of sterile glycerol. The broth was taken during mid-exponential growth. The mixture was then aliquoted into suitable 2 mL sterile containers and stored at -70°C.

3.3.4.2.4 SIMPLE BIOTRANSFORMATIONS.

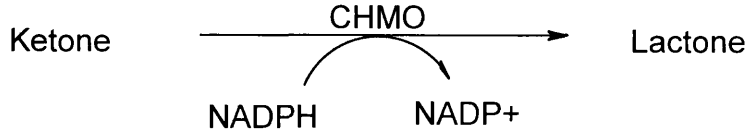


Figure 3.4 Simple biotransformation of a cyclic ketone to an optically pure lactone using CHMO and NADPH.

Simple biotransformations (Figure 3.4) were performed in pH 8 Tris buffer at 30°C on a 30 mL scale in a 250 mL shake flask using equimolar quantities of NADPH (20 M) and substrate 2-methylcyclohexanone (20 mM), with approximately 0.5 U enzyme activity per mL of solution (15 U). Aliquots (0.5 mL) were transferred from the reaction vessel into eppindorfs containing IPA (1 mL). The sample was centrifuged for 2 minutes at 15 000 rpm to allow the extraction of the substrate and any organic matter from the biological matter. The supernatant was removed and analysed by chiral GC.

3.3.4.2.5 BIOTRANSFORMATIONS USING NADP RECYCLE WITH ADH AS A COENZYME

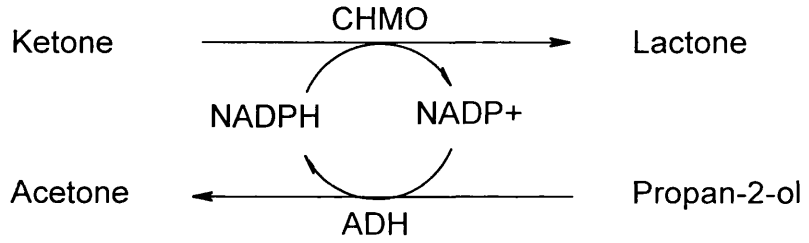


Figure 3.5 Biotransformations of a cyclic ketone to a opitcally pure lactone using CHMO and an NADP+ recycle with ADH as a coenzyme

Biotransformations employing a recycle system (see figure 3.5) developed by Hogan (Ph.D. thesis) were performed in pH 8 Tris buffer at 30°C on a 30 mL scale, in a 250 mL shake flask using 0.3 mg/mL $NADP^+$, equimolar quantities (20 mM) of substrate and propan-2-ol, and equal units (15 U) of ADH and CHMO activity (0.5 U/mL). The biotransformations were performed in open vessels to allow the acetone to evaporate and not interfere with the biotransformation. Hogan (unpublished results) had previously demonstrated that acetone was inhibitory towards CHMO. Aliquots (0.5 mL) were transferred from the reaction vessel into eppindorfs containing IPA (1 mL). The sample was centrifuged for 2 minutes at 15 000 rpm to allow the extraction of the

substrate and any organic matter from the biological matter. The supernatant was removed and analysed by chiral GC.

3.3.4.2.6 BIOTRANSFORMATIONS USING NADP WITH GDH AS A COENZYME.

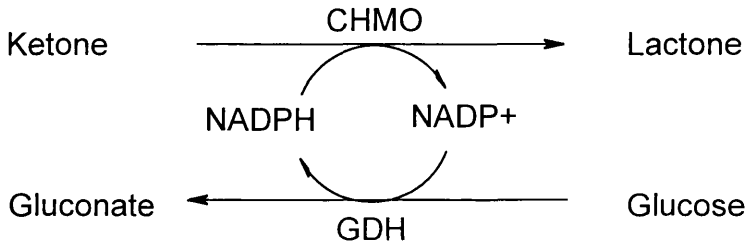


Figure 3.6 Biotransformations of a cyclic ketone to a opitcally pure lactone using CHMO and an NADP+ recycle with GDH as a coenzyme

Biotransformations based on those detailed in 3.2.4.2.5 were modified to use GDH instead of ADH as performed by Hogan (Figure 3.6) (unpublished results). The bioconversions were performed in pH 7.5 phosphate buffer (50 mM) at 30°C on a 30 mL scale in a 250 mL shake flask using 0.5 mg/mL $NADP^+$, 2-methylcyclohexanaone (20 mM) and glucose (50 mM), CHMO (15 U) and GDH (15 U). Aliquots (0.5 mL) were transferred from the reaction vessel into eppindorfs containing IPA (1 mL). The sample was centrifuged for 2 minutes at 15 000 rpm to allow the extraction of the substrate and any organic matter from the biological matter. The supernatant was removed and analysed by chiral GC.

3.3.4.2.7 BIOTRANSFORMATION TO PROVIDE ENANTIOMERICALLY ENRICHED MATERIAL FOR THE RACEMISATION PROCEDURE.

The biotransformation process was performed as detailed in 3.2.4.2.6 (Figure 3.6), but on a 1 L scale, in a 2 L glass stirred tank bioreactor. The amount of biocatalysts were decreased to decrease the rate of bioconversion to allow for

at-line reaction monitoring by chiral GC. The bioconversions were performed in pH 7.5 phosphate buffer (50 mM) at 30 °C using $NADP^+$ (500 mg), 2-methylcyclohexanaone (2.45 mL, 20 mM) and glucose (9 g, 50 mM), CHMO (300 U) and GDH (300 U). Aliquots (0.5 mL) were transferred from the reaction vessel into eppindorfs containing IPA (1 mL). The sample was centrifuged for 2 minutes at 15 000 rpm to allow the extraction of the substrate and any organic matter from the biological matter. The supernatant was removed and analysed by chiral GC.

When the e.e. of 2-methylcyclohexanone as determined by chiral GC reached over 40%, the protein and other biological macromolecules were precipitated out of the solution by the addition of IPA which had previously been stored below 0°C, thus stopping the reaction. The resulting solution was centrifuged in 250 mL aliquots at 4000rpm for 30 minutes. The supernatant of this solution was either used without dilution for the racemisation, or stored at -18°C until required.

3.4 RESULTS

3.4.1 SUBSTRATE CHARATERISATION.

3.4.1.1 STABILITY OF 2-METHYLCYCLOHEXANONE.

No degradation of the ketone was observed to have taken place after 24 hours at the given conditions. It can therefore be said that 2-methylcychexanone is stable at 30°C in pH 7.5 phosphate buffer (50 mM) for 24 hours.

3.4.1.1.1 STABILITY OF 7-METHYLOXEPAN-2-ONE.

Approximately 20% degradation of the lactone was observed to have taken place after 24 hours at the given conditions. It can therefore be said that 7-

methyloxepan-2-one is relatively stable at 30°C in pH 7.5 phosphate buffer (50 mM) for 24 hours.

3.4.1.1.2 PHYSICAL PROPERTIES OF 2-METHYLCYCLOHEXANONE.

The physical and chemical properties of 2-methylcyclohexanone are detailed in Table 3.2. (Fluka 1999)

3.4.1.1.3 PHYSICAL PROPERTIES OF 7-METHYLOXEPAN-2-ONE.

The physical and chemical properties of 7-methyloxepan-2-one are detailed in Table 3.3

Physical and Chemical Properties	Details
Molecular formula	$C_7H_{12}O$
Molecular Weight	112.17
Purity	98%
Appearance	Clear oil, slightly yellow in colour
Boiling point	162-163 °C at 760 mm Hg
Melting point	-14 to 0 °C
Solubility	~25 g/L
Density	0.924 g/cm^3

Table 3.2 Physical and chemical properties of 2-methylcyclohexanone

Physical and Chemical Properties	Details
Molecular formula	$C_7H_{12}O_2$
Molecular Weight	128
Appearance	Clear oil, slightly yellow in colour
Boiling point	162-163 °C at 760 mm Hg
Melting point	-14 to 0 °C
Solubility	~82 g/L
Density	~1 g/cm^3

Table 3.3 The physical and chemical properties of 7-methyloxepan-2-one.

3.4.2 Catalyst Characterisation.

3.4.2.1 Physical properties of Amberlite IRA 400 ($^-$OH)

The physical and chemical properties of Amberlite IRA 400 ($^-$OH) are detailed in Table 3.4 (Fluka 1999)

Name	Amberlite IRA 400
Matrix Structure	Gel type Styrene DVB
Ionic form	Hydroxide
Apparent density wet (g/mL)	0.70
True density wet (g/mL)	1.11
Effective size (mm)	0.4-0.5
Moisture content (%)	42-48
pH range	0-14
Maximum operating temperature (°C)	60
Total exchange capacity (meq/g) dry	3.8
Total exchange capacity (meg/mL) wet	1.4

Table 3.4 Physical properties of Amberlite IRA 400 (-OH)

3.4.2.2 Effect of Catalyst on solution pH.

Amberlite IRA 400(-OH) (50 g/L) had a great effect on the pH of the solutions within 30 minutes. The pH of all of the solutions, including the buffered solutions (pH 7, 8 and 9) was pH 12 +/-0.5 within ten minutes as indicated in table 3.5.

NaOH (10% v/v, 2 M) had a similar effect, as expected on the pH of the solutions. The pH of the solutions were observed to rise to above pH 12 within 30 minutes as indicated in table 3.6.

Time (Hours)	Water	pH 7 Buffer	pH 8 Buffer	pH 9 Buffer
	pH of solution at given time point			
0	6.96	7.01	8.02	8.98
0.5	11.59	12.39	12.60	12.37
2.5	11.73	12.39	12.27	12.58
24	12.01	12.27	12.42	12.63

Table 3.5 Effect of Amberlite IRA 400 (-OH) on the pH of water and buffered solutions.

Time (Hours)	Water	pH 7 Buffer	pH 8 Buffer	pH 9 Buffer
	pH of solution at given time point			
0	7.01	6.98	8.00	9.01
0.5	12.05	12.37	12.46	12.53
2.5	12.43	12.34	12.68	12.49
24	12.78	12.39	12.61	12.64

Table 3.6 Effect of NaOH (2 M, 10%v/v) on the pH of water and buffered solutions.

3.4.2.2.1 EXTENT OF PROTEIN BINDING TO THE CATALYST

No binding of the BSA in solution to the Amberlite was observed. At all time points approximately 1 mg/mL BSA was detectable in the solution (Figure 3.7).

As 1 mg/mL BSA was added to the solution at the beginning of the study it can be said that no binding of BSA to Amberlite IRA 400 (-OH) occurred.

3.4.2.2.2 Effect of catalyst on 7-methyloxepan-2-one.

Both catalysts rapidly degraded 7-methyloxepan-2-one as can be seen in Figures 3.8 and 3.9. The degradation was observed to be rapid over the first 5 minutes, after that the rate decreased.

3.4.2.2.3 Effect of shear on Amberlite IRA 400 (-OH)

Damage to the beads was observed at 15000 rpm and above. At 15000 rpm the beads were seen to fragment into two. No damage was observed below 15000 rpm.

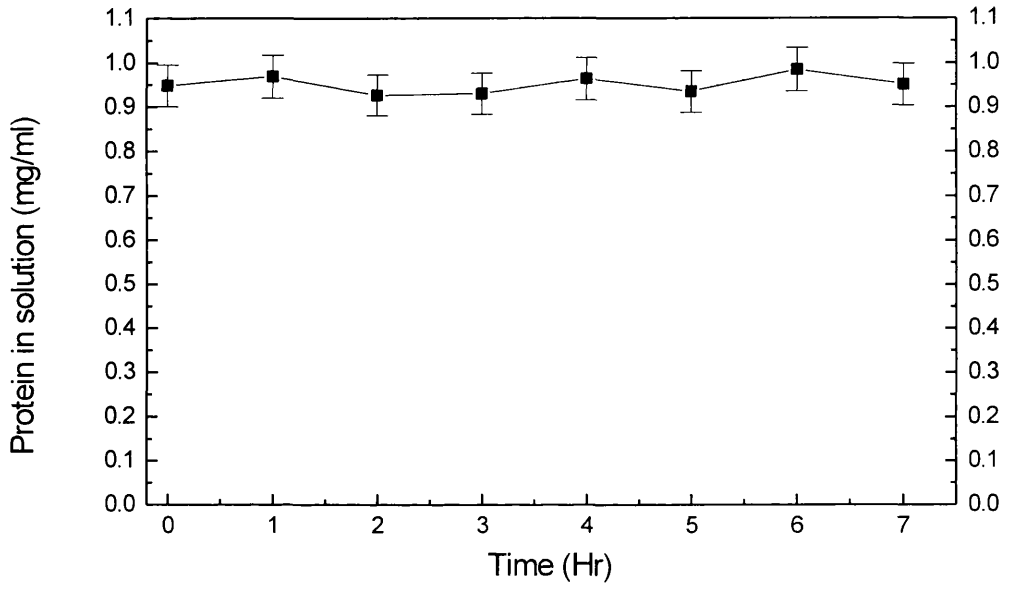


Figure 3.7 Protein available in solution at various time points indicating no binding of BSA to Amberlite IRA 400 (-OH).

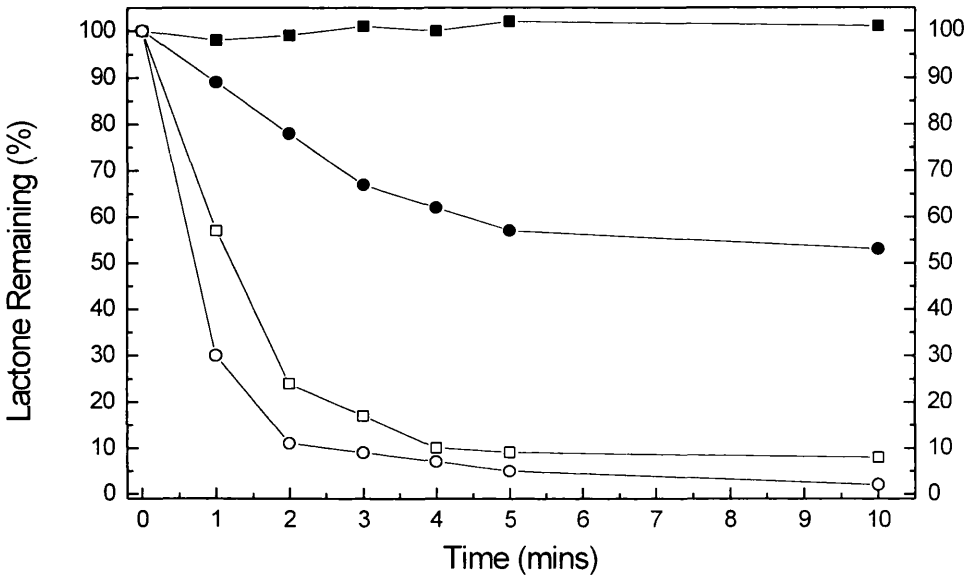


Figure 3.8. Lactone degradation by Amberlite IRA 400 (-OH) where, ■ represents 5g/L Amberlite IRA 400 ($^-$OH), ● represents 10g/L Amberlite IRA 400 ($^-$OH), □ represents 50g/L Amberlite IRA 400 ($^-$OH) and O represents 100g/L Amberlite IRA 400 ($^-$OH) .

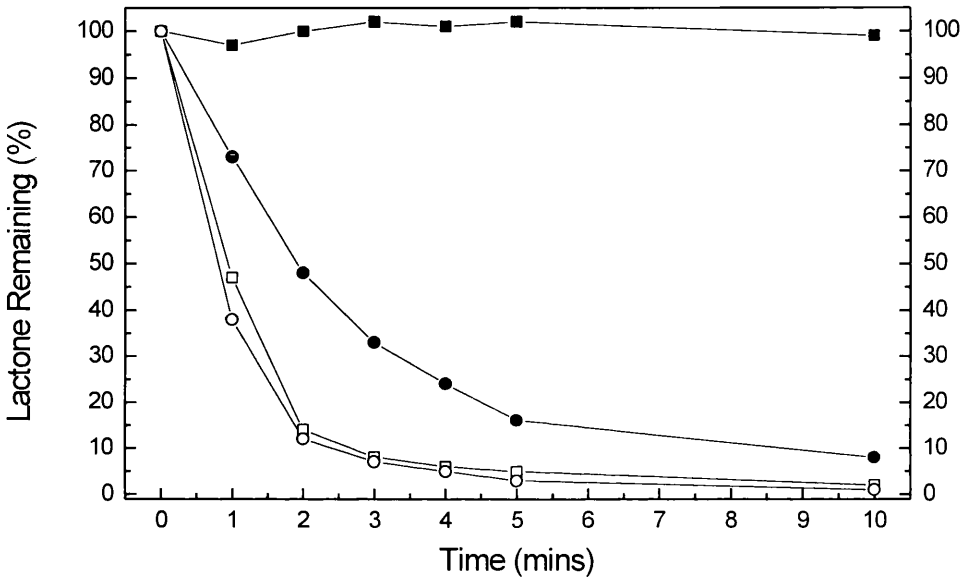


Figure 3.9 Lactone degradation by NaOH where, ■ represents 0.1M NaOH, ● represents 1M NaOH □ represents 2M NaOH and ○ represents 4M NaOH.

3.4.3 Racemisation and Racemisation monitoring.

3.4.3.1 Gas Chromatography Method Validation.

3.4.3.1.1 Reproducibility

Reproducible data can be seen in Table 3.7. From this data the RSD for each peak was calculated. A chromatogram indicating the resolution between the to enantiomers of 2-methylcyclohexanone can be seen in Figure 3.10. Figure 3.11 indicates the separation of 2-methylcyclohexanone and 7-methyloxepan-2-one.

Relative Standard Deviation calculated from the data from peak 1 = 3.93%
Relative Standard Deviation calculated from the data from peak 2 = 3.78%

Injection	Area of Peak 1	Area of Peak 2
1	398060.1	405978.7
2	397660.9	406505.2
3	390425.9	398393.8
4	396364.2	405596.8
5	388014.5	395589.8
6	364730.1	377154.1
7	424027.3	433516.0
8	401568.5	412170.1
9	399227.1	408104.0
10	379011.5	386519.9

Table 3.7 Peak areas obtained to check reproducibility of the chiral GC method.

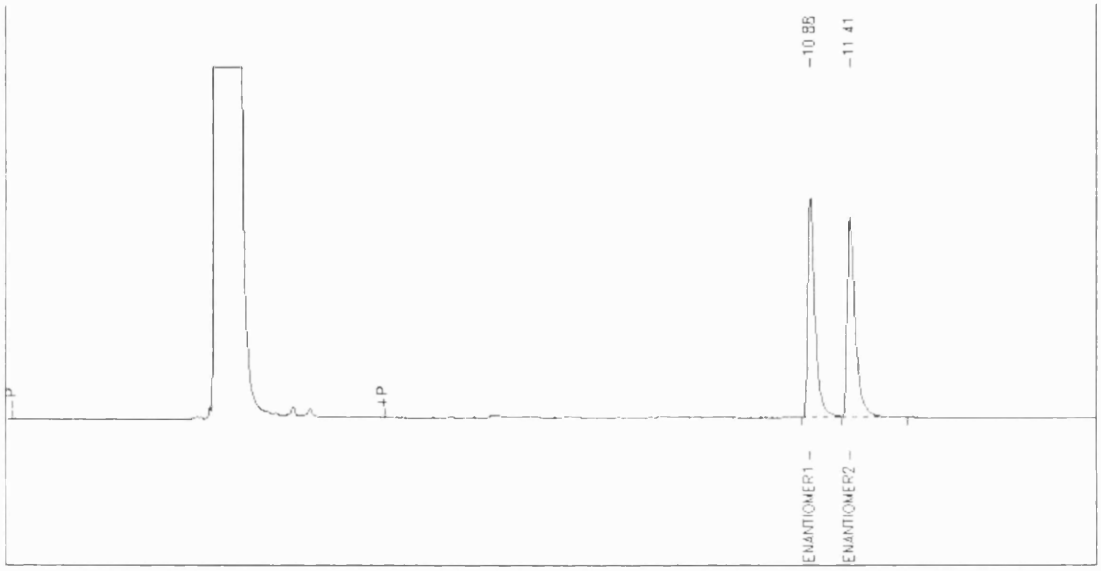


Figure 3.10 Chromatogram demonstrating the separation of the two enantiomers of 2-methylcyclohexanone and propan-2-ol by GC.

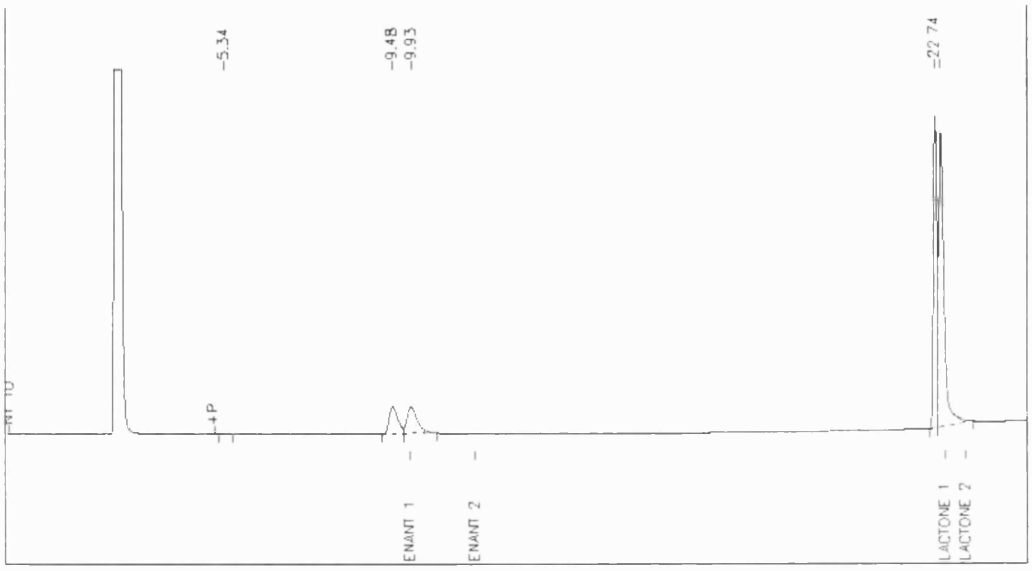


Figure 3.11 Chromatogram indicating the separation of 2-methylcyclohexanone and 7-methyloxepan-2-one in IPA.

3.4.3.1.2 METHOD LINEARITY.

The chiral GC method for the separation of the two enantiomers of 2-methylcyclohexanone and the two enantiomers of 7-methyloxepan-2-one was shown to be linear. The calibration curves can be seen in Appendix B.

3.4.4 BIOTRANSFORMATION.

3.4.4.1 FERMENTATION RESULTS

3.4.4.1.1 SHAKEN FLASK RESULTS.

The optical density data obtained from representative growth of *E.coli* JM107 in a 2 L shaken flask were used to calculate the dry cell weight (dcw) of the fermentation broth. The correlation of OD and dcw can be seen in Figure A.5 (Appendix B). The dry cell weight data for a representative 2L shaken flask fermentation are displayed in Figure 3.12. The optical density reaches 4 units after 8 hours. This represents an approximate dry cell weight of 2 g/L. A representative induction profile can be seen, along with shaken flask growth data in Figure 3.13. In this fermentation the approximate dry cell weight at 9 hours after inoculation is 1.75 g/L. The CHMO activity reaches approximately 300 U/g of dry cell weight 1 hour after induction with IPTG. The activity decreases slightly in the following 30 minutes. After this time the CHMO activity is seen to decrease rapidly. This indicates that the optimal time to harvest the CHMO in these conditions is between 0.75 and 1.5 hours after induction.

3.4.4.1.2 2L BATCH FERMENTATION RESULTS

The dry cell weight data calculated (as described in 3.4.4.1.1) from a typical 2 L batch fermentation is shown in Figure 3.14.

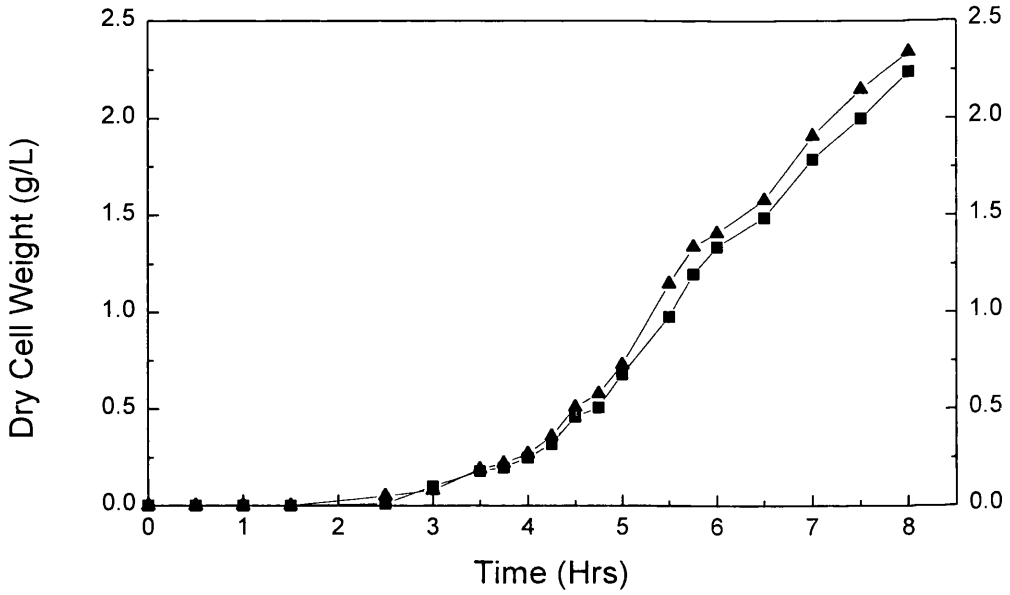


Figure 3.12 Representative 2L shake flask growth of *E.coli* JM107, where ■ represents shaken flask 1 and ▲ represents shaken flask 2.

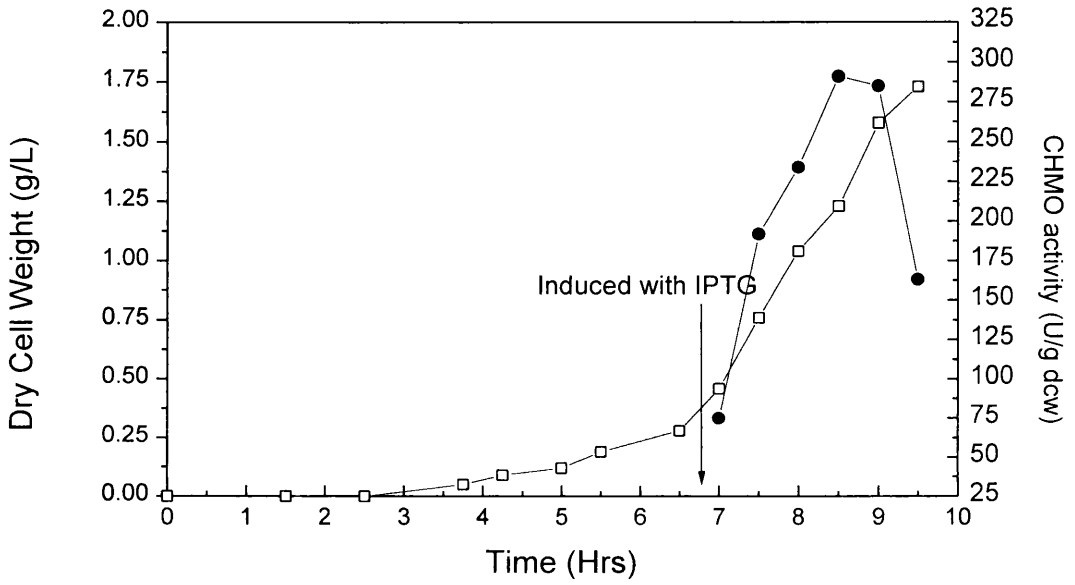


Figure 3.13 Representative Shaken Flask growth, with CHMO production induced by the addition of IPTG. Where □ represents the optical density data and ● represents the CHMO activity in units per gram of dry cell weight.

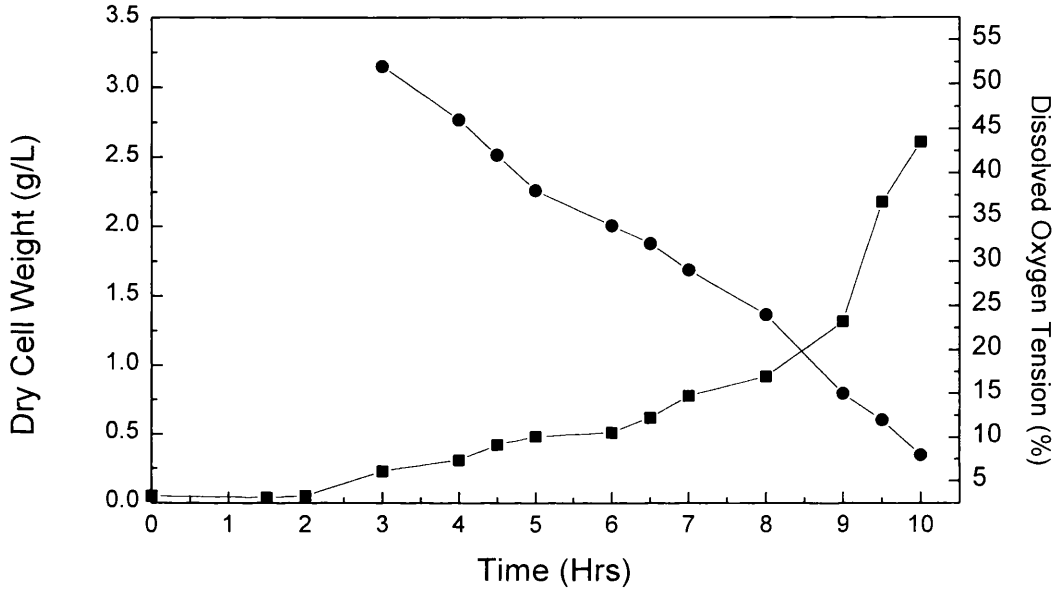


Figure 3.14 OD and DOT data from a 2L batch fermentation of *E. coli* JM107 where ■ represents optical density and ● represents dissolved oxygen tension.

3.4.4.2 Biotransformation results.

3.4.4.2.1 Simple Biotransformations.

The bioconversion of 2-methylcyclohexanone to 7-methyloxepan-2-one by CHMO and the cofactor NADPH (Figure 3.4) resulted in the production of an enantiomerically enriched lactone product and 50% enantiomeric excess of unconverted ketone within 6 hours (Figure 3.15). The high cost of NADPH makes this procedure, on a large scale, uneconomic. Therefore future bioconversions were performed incorporating a cofactor recycling system (as detailed in Figures 3.5 and 3.6).

3.4.4.2.2 Biotransformations using an NADP+ recycle with ADH as a coenzyme.

The bioconversion using NADP and ADH (Figure 3.5) resulted in the disappearance of the peaks corresponding to the two enantiomers of 2-methylcyclohexanone as recorded by chiral GC. No significant lactone peak was observed on the chromatogram. Lamed (1981) reported the reversible enzymatic activity of NADP+ (from *Ta. brockii*) towards a variety of primary and secondary alcohols, as well as linear and cyclic ketones. This suggests that the back reaction occurred and the ketone was reduced to the corresponding alcohol (2-methylcyclohexanol).

3.4.4.2.3 Biotransformations using an NADP+ cofactor recycle with GDH as a coenzyme.

Chiral GC analysis of aliquots removed a various time points from a 20 mM bioconversion of 2-methylcyclohexaone on a 20 mL scale, indicated that two enantiomers of 7-methyloxepan-2-one were produced. The remaining ketone

was known to be the R-enantiomer from previously described data (Alphand 1996) and had an e.e. of approximately 80% after 5 hours. The lactone produced was the S-enantiomer, the e.e. of the lactone was observed to decrease with time. The observed e.e. of the lactone after 5 hours was approximately 60%. 6.4 mM of lactone was produced, the observed yield of the lactone product was 32% (Figure 3.16).

3.4.4.2.4 EFFECT OF CATALYST ACTIVITY ON THE ENANTIOMERIC EXCESS OF THE LACTONE PRODUCT OF THE BIOTRANSFORMATION

Initial bioconversions were carried out using 0.5U/mL CHMO activity and equal units of GDH. Variation of the amount of activity added to the biotransformation was observed to effect the enantiomeric excess of the lactone product. An increase in the number of units of CHMO activity (and GDH activity) was seen to increase the rate of the biotransformation, but decrease the enantiomeric excess of (S)-7-methyloxepan-2-one.

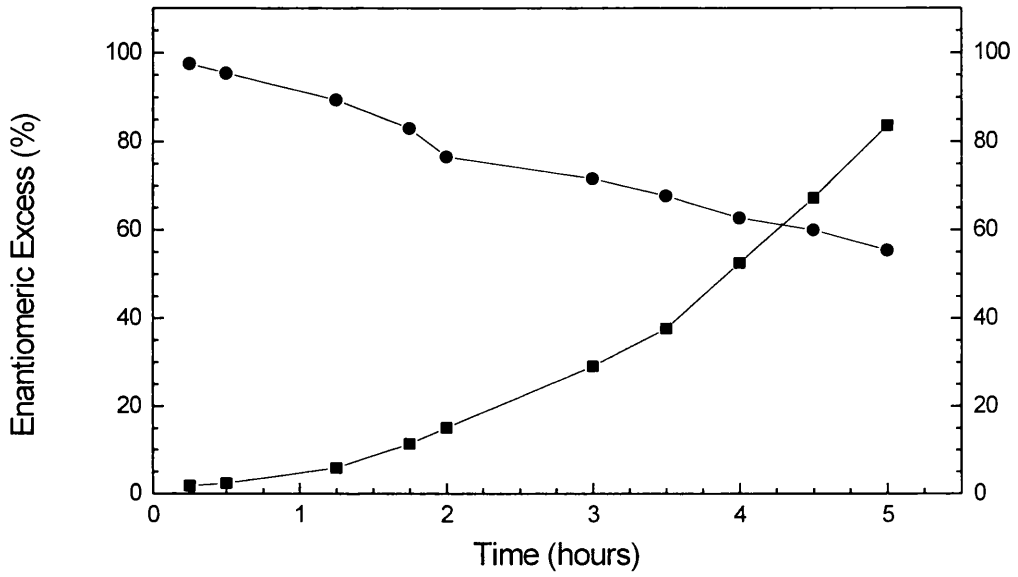


Figure 3.15 Enantiomeric excess (%) of the unconverted ketone ■ and lactone product ● during the bioconversion of racemic 2-methylcyclohexanone to its corresponding lactone using CHMO and NADPH

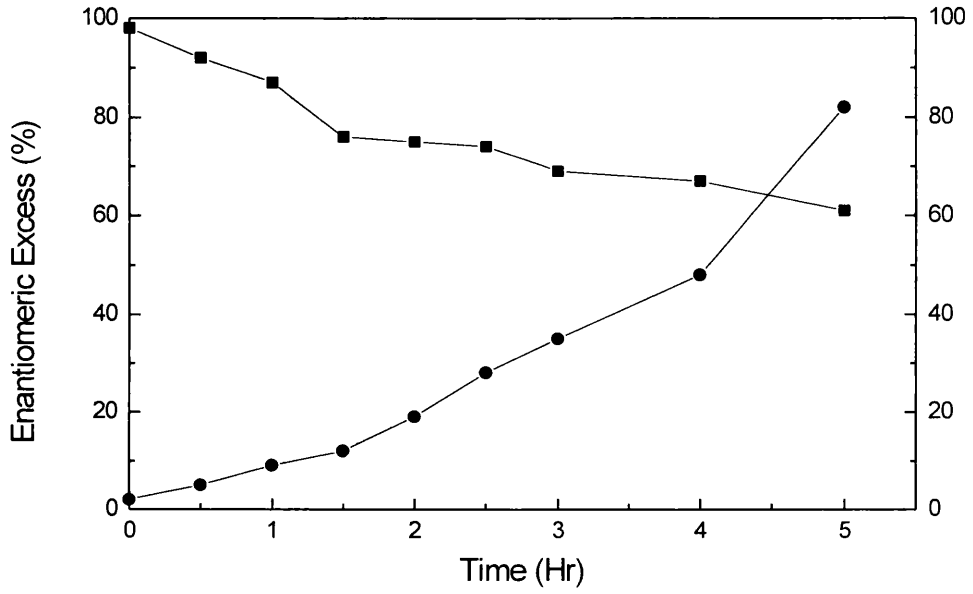


Figure 3.16 Enantiomeric excess (%) of the unconverted ketone (●) and lactone product (■) during the bioconversion of racemic 2-methylcyclohexanone to its corresponding lactone using a CHMO/GDH recycle and NADP+ as the cofactor.

3.5 DISCUSSION

3.5.1 CHOICE OF SUBSTRATE

The initial substrate (2-hydroxymethylcyclopentanone) was selected without the use of the substrate selection criteria detailed in chapter 2. This substrate did not satisfy two of the criterion detailed for the substrate selection. 2-hydroxymethylcyclopentanone was not commercially available and the synthesis of 2-hydroxymethylcyclopentanone was difficult. The activity of CHMO in the presence of 2-hydroxymethylcyclopentanone was poor.

The second substrate used (2-ethylcyclopentanone) was selected using the criteria described in chapter 2. A number of problems were encountered using this substrate. These were mainly chromatographic problems resulting from the substrate being used in aqueous conditions. The chiral HPLC column used was poorly tolerable of water. These problems would have occurred regardless of the substrate. The column was selected on the basis that it was a chiral HPLC column that had been shown to be suitable for the separation of the two enantiomers of cyclic ketones that were substrates for the bioconversion to chiral lactones (Alphand *et al.*, 1996.)

A chiral GC column was purchased (Lipodex E), that had also been shown to separate the two enantiomers of ketones suitable for Baeyer-Villiger type bioconversions. This column had also been demonstrated to separate the two enantiomers of the lactone product of the bioconversions (Alphand *et al.*, 1996). It was not possible to detect 2-ethylcyclopentanone using chiral GC with the Lipodex E column and FID detection. The ketone was thought to co-elute with the injection front and was therefore not retained by the column. The inability to purchase 2-ethylcyclopentanone from a commercial source and the inability to detect the ketone by chiral GC were the reasons why 2-ethylcyclopentanone was not used as the model substrate in this research.

2-Methylcyclohexanone was selected as a model substrate as it was reported to be possible to separate the two enantiomers of the ketone and its corresponding lactone using the Lipodex E chiral GC column (Alphand *et al.*, 1996). 2-methylcyclohexanone was also commercially available from a reliable source. CHMO demonstrated high activity in the presence of 2-Methylcyclohexanone.

The substrate (2-methylcyclohexanone) was shown to be stable in the buffer to be used for the bioconversion. As the substrate was an α-substituted cyclic ketone it was able to undergo base (or acid) catalysed tautomerism resulting in racemisation of non-racemic or enantiomerically enriched ketone.

3.5.2 Choice of Catalyst

Over 30 solid chemical catalysts including zeolites, clays and ion exchange resins were investigated for their ability to racemise an enantiomerically enriched solution of (2R)-methylcyclohexanone. Of these catalysts only Amberlite IRA 400 (-OH) was observed to racemise the enantiomerically enriched solution of 2-methylcyclohexanone.

Amberlite IRA 400 (-OH) was observed to rapidly increase the pH of water and buffered solutions. Amberlite IRA 400 (-OH) was observed to rapidly degrade the lactone product of the bioconversion of 2-methylcyclohexaone. On the positive side, Amberlite IRA 400 (-OH) does not bind protein. An ideal catalyst would not have had these effects, but as time was limited it was decided to proceed with Amberlite IRA 400 (-OH) as the solid chemical catalyst for this research. The ideal catalyst would have no effect on solution pH, not degrade the lactone, not bind protein, but would racemise the ketone.

3.5.3 Selection of Bioconversion Conditions

The bioconversion of 2-methylcyclohexaone by CHMO was performed using an ADH recycle system and a GDH recycle system, based on those described by Hogan 1999, to make the bioconversion more economically viable. Hogan (1999) demonstrated a co-factor recycle system using ADH, where propan-2-ol is used as the sacrificial substrate. Hogan (1999) demonstrated a second co-factor recycling system using GDH and glucose as the sacrificial substrate.

The co-factor recycling system using ADH was deemed unsuitable as no lactone product was observed by chiral GC.

GDH is a suitable coenzyme for cofactor recycling in this system although the use of GDH requires a compromise in the pH of the bioconversion. CHMO activity has been shown by Trudgill (1990) and confirmed by Hogan (unpublished results) to be at its optimum at pH 9, whilst GDH activity is optimum at pH 7 (Smith 1989).

The biotransformation of 2-methylcyclohexanone to 7-methyloxepan-2-one using the GDH/NADPH co-factor recycle system occurs with reasonable yield (32%) and enantiomeric excess of the lactone (55%) if performed over 5 hours. This compares well with the findings of other researchers. Alphand and coworkers (1996) reported the bioconversion of 2-methylcyclohexanone by CHMO from *Acinetobacter* TD63 after 4 hours with a yield of 35% (lactone) and an enantiomeric excess of 61% (S-lactone). Alphand and coworkers also reported a higher yield of S-lactone (50%) and a lower e.e. (38%) with the purified enzyme MO2 after 1 hour.

4. KINETICS OF RACEMISATION

4.1 INTRODUCTION.

The kinetics of the base-catalysed racemisation of 2-methylcyclohexanone were studied to assess the rate of the racemisation under different conditions. This assessment was performed in such a way that the racemisation was not the rate-limiting step. The variables examined are:

- Catalyst concentration
- Substrate concentration
- Temperature.

The kinetics of racemisation were to be determined using the heterogeneous and homogeneous catalysts. The rate of racemisation involves the formation of an enol, therefore the rate of racemisation is equal to the rate of enolisation. Literature states that the overall rate of racemisation is second order (Streitwieser *et al.,* 4th Edition 1992, Sykes) with respect to the concentration of ketone and the concentration of the base catalyst.

4.2 METHODS.

4.2.1 GENERAL METHOD.

A recorded concentration of the enantiomerically enriched ketone solution was diluted with a set volume of phosphate buffer (pH 7.5, 50 mM). A known volume, of known molarity, of NaOH was added to the solution. (In the case of Amberlite IRA 400 (-OH) a known weight of catalyst was added to the solution).

The solution was stirred in a 50 mL glass beaker at a known temperature. Aliquots (100 μL) of the solution were removed every 10 seconds and neutralised with HCl (100 μL) (at equal molarity to the NaOH). The Amberlite catalysed reaction samples were centrifuged at 15 000 rpm for 2 minutes to separate the solid catalyst. The supernatant of these samples was analysed by GC.

Sodium hydroxide was selected as the catalyst for all of the homogeneous catalysis studies.

Amberlite IRA 400 (-OH) was the catalyst used for all of the heterogeneous catalyst studies.

4.2.2 Effect of catalyst on Rate of Racemisation.

4.2.2.1 Effect of Base Concentration on Rate of Racemisation.

The following concentrations of NaOH were used, 4 M, 2 M, 1 M and 0.1 M. The remaining reaction components were ketone solution (15 mL), phosphate buffer (13 mL), NaOH solution (2 mL).

4.2.2.2 Effect of Amberlite IRA 400 (-OH) on Rate of Racemisation.

The following concentrations of Amberlite IRA 400 (-OH) were used, 5, 10, 50 and 100 g/L. The remaining reaction components were ketone solution (15 mL), phosphate buffer (15 mL).

4.2.3 Effect of temperature on rate of racemisation.

4.2.3.1 Effect of temperature on the Rate of Homogeneous Catalysed Racemisation.

The rate of racemisation was determined at the following temperatures; 4°C, 25°C, 45°C and 60°C. The reaction components were ketone solution (15 mL), phosphate buffer (13 mL) and NaOH (2 mL, 2 M)

4.2.3.2 Effect of temperature on the Rate of Heterogeneous Catalysed Racemisation.

The rate of racemisation was determined at the following temperatures; 4°C, 25°C, 45°C and 60°C. The reaction components were ketone solution (15 mL), phosphate buffer (15 mL) and Amberlite IRA 400 (-OH) (100 g/L).

4.2.4 Effect of Ketone Concentration on Reaction Rate

4.2.4.1 Effect of Ketone Concentration on the Rate of Homogeneous Catalysed Racemisation.

The rate of racemisation was determined at 25°C using the following concentrations of ketone solution 1, 10 and 15 mM. The ketone solution was made upto 20mL with phosphate buffer (pH 7.5, 50 mM), 2 mL of 2 M NaOH (10% v/v) was added to the solution to start the reaction. Aliquots were removed, neutralised with 2 M HCl (10% v/v) and analysed by chiral GC.

4.3 RESULTS.

4.3.1 DATA ANALYSIS.

The GC data obtained indicated the concentration of both enantiomers of the ketone. This data was used to plot 1/concentration against time, according to the rate equation for second order kinetics. The rate constant (*k*) for each investigation is equal to the gradient of the second order rate plot (Connors 1990, Logan 1996). Error bars have been omitted from these plots to avoid complication of the data points.

$$\text{Rate} = k\,[\text{substrate}]\,[\text{catalyst}] \qquad (4.1)$$

4.3.2 EFFECT OF CATALYST ON RATE OF RACEMISATION.

4.3.2.1 EFFECT OF BASE CONCENTRATION ON RATE OF RACEMISATION.

The second order rate plot obtained from this investigation can be seen in Figure 4.1. For *k* from each concentration of base see Table 4.1.

Conc of NaOH (M)	4	2	1	0.1
k (10^{-3})	6.61	2.01	0.73	0.57
Rate ($M\ s^{-1}\ 10^{-3}$)	0.2644	0.0402	0.0073	0.0006

Table 4.1 Rate Constant *k* observed with differing concentrations of Sodium Hydroxide.

4.3.2.2 Effect of Amberlite Concentration on Rate of Racemisation.

The second order rate plot obtained from this investigation can be seen in Figure 4.2. For *k* from each concentration of Amberlite see Table 4.2.

Conc of Amberlite (g/L)	100	50	10	5
k (10^{-3})	3.39	0.64	0.29	0.11
Rate (M s^{-1} 10^{-3})	3.390	0.320	0.029	0.006

Table 4.2 Rate Constant *k* observed with differing concentrations of Amberlite IRA 400 (-OH) .

4.3.3 Effect of Temperature on Rate of Racemisation.

4.3.3.1 Effect of Temperature on the Rate of Homogeneous Catalysed Racemisation.

The Arrhenius plot in Figure 4.3 indicates the correlation between reaction temperature and the rate constant. See Table 4.3 for the rate constants for these experiments.

Temp.(°C)	4	25	45	60
k (10^{-3})	1.46	2.01	3.01	4.21
Rate (M s^{-1} 10^{-3})	0.03	0.04	0.06	0.08

Table 4.3 Effect of Temperatures on Rate of Racemisation using NaOH as the catalyst.

4.3.3.2 Effect of Temperature on the Rate of Heterogeneous Catalysed Racemisation.

The Arrhenius plot in Figure 4.4 indicates the correlation between reaction temperature and the rate constant. See Table 4.4 for the rate constants for these experiments.

Temp (°C)	4	25	45	60
k (10^{-3})	0.89	2.39	3.79	5.15
Rate (M s^{-1} 10^{-3})	0.89	2.39	3.79	5.15

Table 4.4 Effect of Temperatures on Rate of Racemisation using Amberlite IRA 400 (-OH) as the catalyst.

4.3.4 Effect of Ketone Concentration on Reaction rate.

4.3.4.1 Effect of Ketone Concentration on the Rate of Homogeneous Catalysed Racemisation.

The second order rate plot obtained from this investigation can be seen in Figure 4.5. For k from each concentration of Ketone with NaOH (2mL, 2M) see Table 4.5.

substrate mM	15	10	1
k (10^{-3})	2.44	2.01	1.68
Rate (M s^{-1} 10^{-3})	0.073	0.040	0.003

Table 4.5 Effect of Ketone Concentration on Rate of Racemisation using sodium Hydroxide as the catalyst.

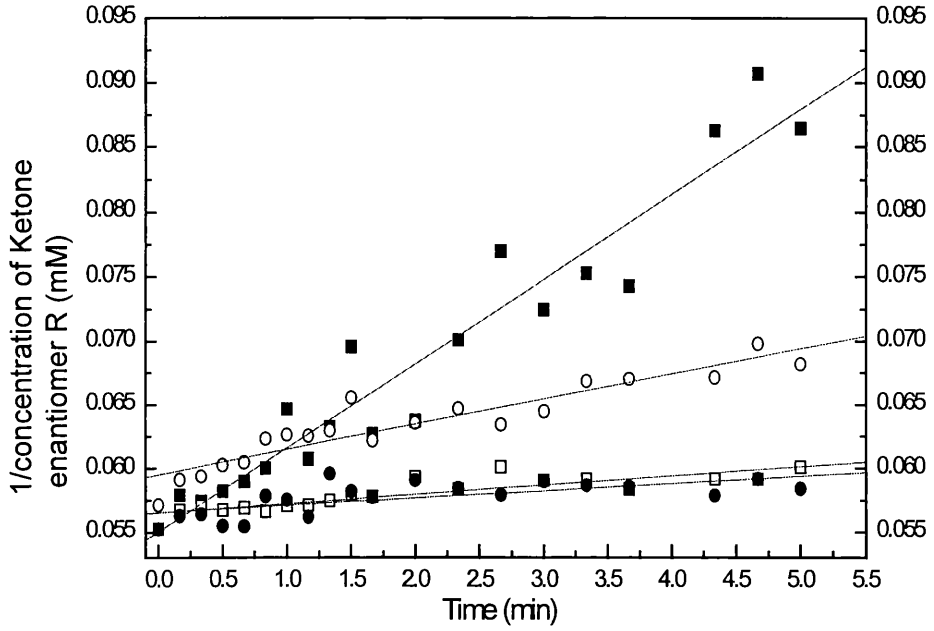


Figure 4.1 The effect of concentration of base catalyst (NaOH) on rate constant *k*, where ■ represents 4M NaOH, O represents 2M NaOH, □ represents 1M NaOH and ● represents 0.1M NaOH.

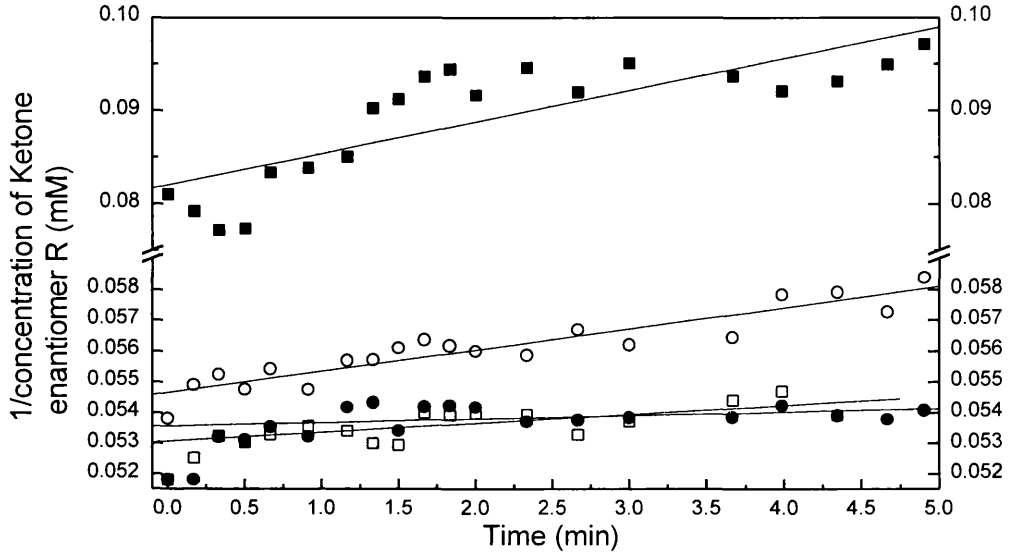


Figure 4.2 Effect of Amberlite concentration on rate constant *k*, where ■ represents 100g/L Amberlite IRA 400 (-OH), O represents 50g/L Amberlite IRA 400 (-OH), ● represents 10g/L Amberlite IRA 400 (-OH), and □ represents 5g/L Amberlite IRA 400 (-OH).

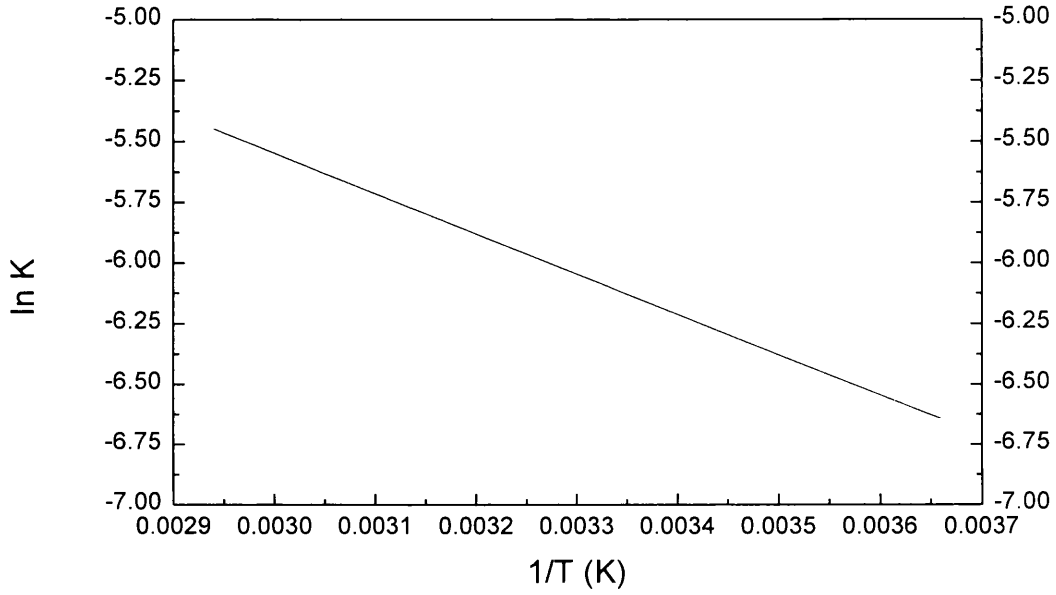


Figure 4.3 Arrhenius Plot indicate the correlation between Temperature and Rate constant *k* when NaOH is the catalyst.

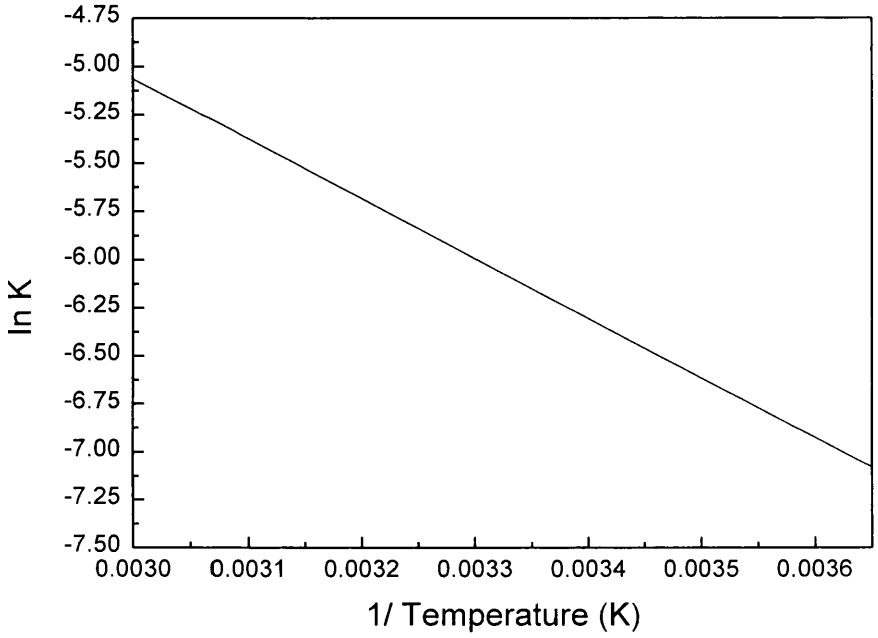


Figure 4.4 Arrhenius plot in indicates the correlation between reaction temperature and the rate constant *k* when Amberlite IRA 400 (-OH) is the catalyst.

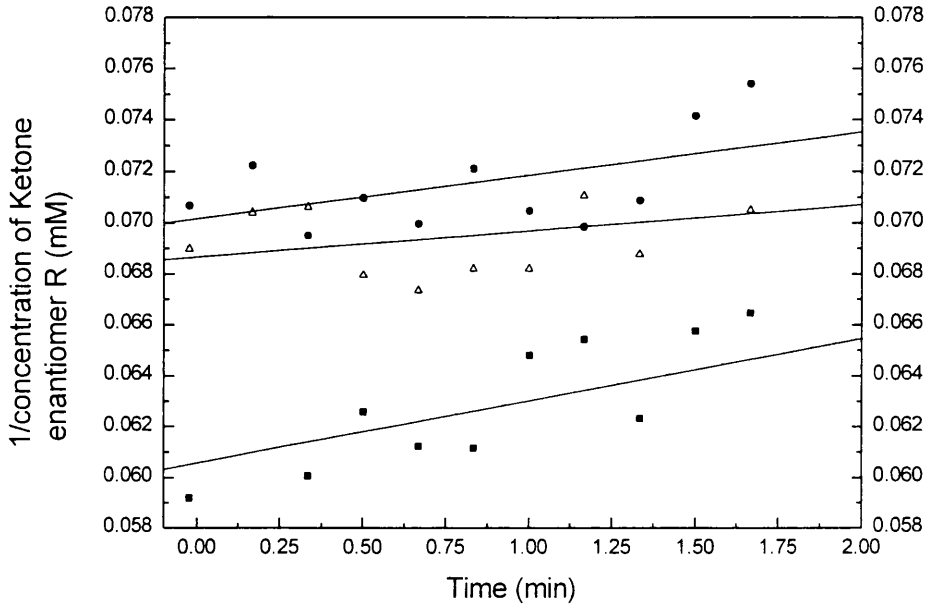


Figure 4.5 Effect of substrate concentration on rate constant *k*. where ● represents 15 mM ketone, △ represents 10 mM ketone, ■ represents 1mM ketone

4.4 DISCUSSION.

As stated at the beginning of this Chapter, the base catalysed racemisation of a 2-substituted cyclic ketone is a well-known second order reaction. The data obtained during the kinetic investigations detailed in this Chapter do fit a second order model, although could, with large errors fit a first order model. Error bars were omitted from the graphs in this Chapter to avoid over-complication of the data points.

There are many errors associated with the data obtained during the kinetics studies detailed in this Chapter. These errors come from multiple sources. The major reason for error in these investigations was the rate of sampling of each reaction. The reactions were sampled every ten seconds for the first two minutes of monitoring, then every 20 seconds up to six minutes. Sampling at such a rate is highly likely to lead to pipetting errors, such as incorrect filling and discharging of the pipette and errors in the timing of aliquot removal from the system.

The neutralisation of the NaOH catalysed samples with HCl is a second source of error, linked to pipetting errors. The samples removed from the reaction media at each time-point were small volumes (100 μL), these were neutralised with an equal volume of HCl. The neutralisation of the aliquot of reaction solution may not have occurred precisely with every sample. As racemisation can be acid- or base-catalysed, a solution that had not been neutralised, or had been 'over'-neutralised to give an acidic solution could have continued to react. Any continuation of the racemisation would occur at a slower rate than the reaction study, but still produce an error in the result from that time point. Errors involved with the neutralisation of the aliquot will also cause incorrect dilution of the sample. This would not have occurred in the Amberlite catalysed reaction, as these samples were not neutralised.

Errors may have occurred in the Amberlite catalysed reactions due to inconsistency in the period of time from removal of the aliquot from the system to the separation of the supernatant from the solid catalyst after centrifugation. The aim of the investigation into the rate of racemisation was to ensure that racemisation would not be the rate-limiting step in the integrated process. As can be seen from the data obtained from these investigations the rate of racemisation is very quick at above 2 M NaOH and above 100 g/L Amberlite IRA 400 (-OH). As the bioconversion step is designed to contain an enzyme concentration that will perform the biotransformation over a number of hours (rather than minutes) the integrated process can be performed with either 1 M NaOH or 10 g/L Amberlite IRA 400 (-OH).

4.5 SUMMARY.

- The data obtained during the racemisation kinetic investigations contained large errors.

- The data obtained for the homogeneous and heterogeneous catalysed racemisation of 2-methycyclohexanone fitted a second order kinetic model.

- Amberlite IRA 400 (-OH) and NaOH catalysed racemisation occurs rapidly.

5. PROCESS DESIGN AND REACTOR CONFIGURATION.

5.1 INTRODUCTION

In this Chapter the integration of the two processes, biotransformation and racemisation, which have previously been examined as separate unit operations will be investigated. The reactor design will be considered according to the system constraints detailed in Chapter 3, and discussed in 5.4.2. The kinetics of the racemisation procedure as detailed in Chapter 4 were determined to allow the investigation of an integrated process where the racemisation procedure is none rate-limiting.

5.2 OPERATING WINDOW AND SYSTEM CONSTRAINTS

One of the main aims of this research is the development of an integrated process where the biotransformation and the racemisation procedure can be performed together. The most simplistic reactor design for such an integrated process is a single vessel or in a series of linked reactors. The characterisation of the catalyst and substrates in Chapter 3 indicates the most suitable conditions for the biotransformation and racemisation procedures. The effects of the various components upon each other were also examined. These interactions are crucial to the reactor and process design. They indicate the physical constraints that the process has due to its components. The two processes will be considered separately first, then their interactions will be discussed to determine the process limitations from a reactor design perspective.

5.2.1 BIOTRANSFORMATION.

The biotransformation as detailed in Figure 3.6 is the most important factor in the determination of system restraints and operating window. The conditions for the biotransformation are determined by the two enzymes present. A change in pH, can drastically affect the performance of an integrated process. The biotransformation will only proceed within a narrow pH range.

The window of operation for the cofactor recycling system relies on a compromise in the activity of both of the enzymes. CHMO activity is at a maximum at pH 9, whilst the maximum GDH activity occurs at pH 7. The activity profiles of the two enzymes at various pH was determined by Hogan (1998) and can be seen in Figure 5.1.

From Figure 5.1 it is possible to determine a pH range for the CHMO/GDH bioconversion. At pH 7 the activity of GDH is approximately 100% of its maximum activity, whilst the activity of CHMO is approximately 20% of its maximum. At a pH to give good CHMO activity, for example pH 9, GDH is not active. For this reason it is necessary to make a compromise, and select a pH that is suitable for both enzymes. Figure 5.2 is a schematic representation of the window of operation for the recycle system where the activity of both enzymes is above 30% of the maximum activity and equal units of each enzyme are added to the system.

If equal weighting is not given to each of the enzymes, and a larger concentration of GDH is added to the system the pH at which the process could take place would increase. In the schematic diagram the area in which the bioconversion would take place would move to the right. The more units of GDH added to compensate for the low activity of GDH at a higher pH the larger the perceived window of operation. As the activity of GDH becomes almost zero at above pH 9.0, an increase in the amount of GDH added to the system would

not allow the bioconversion, with the cofactor recycle, to be performed at the same pH as the racemisation.

A pH at which the activity of GDH is greater than the activity of CHMO would ensure that the rate limiting step of the bioconversion was due to CHMO. This would ensure that there would be adequate NADPH to allow the biotransformation of 2-methylcyclohexanone. For this reason the pH selected for the biotransformation process was pH 7.5. At pH 7.5 the activity of CHMO is approximately 30% of it's maximum activity. GDH is at approximately 75% of it's maximum activity at pH 7.5.

As can be seen from Figure 5.1 the pH profile of ADH is similar to that for CHMO. This would indicate that a cofactor recycle system using ADH instead of GDH would involve less of a compromise towards activity. If the lowest desirable activity was to be 30% (as in the case with GDH) then the window of operation would be large, from pH 7.5 to pH 9.0. The use of ADH is not possible for the biocatalysed Baeyer-Villiger oxidation of 2-methylcyclohexanone, as was mentioned in 3.3.4.2.2 and is discussed in Chapter 7.

Any modification of the pH, temperature, or substrate away from the conditions at which the activity of CHMO is at it's maximum will decrease the observed activity of the enzyme. The inclusion of a recycle system will have a similar effect on the activity of CHMO as the sacrificial substrate (for the ADH or GDH part of the system) and its product will have a negative effect on the activity of CHMO.

The biotransformation procedure is performed at a pH (7.5) which involves a compromise for both enzymes due to the cofactor recycle system. A lower reaction rate than could be achieved using a single enzyme is observed, but the process becomes more economically viable as the system is not reliant on

the stoichiometric amounts of NADPH required to perform this biotransformation without the use of a cofactor recycle system. Again a compromise is made, a decrease in the rate of biotransformation to improve the financial viability of the reaction.

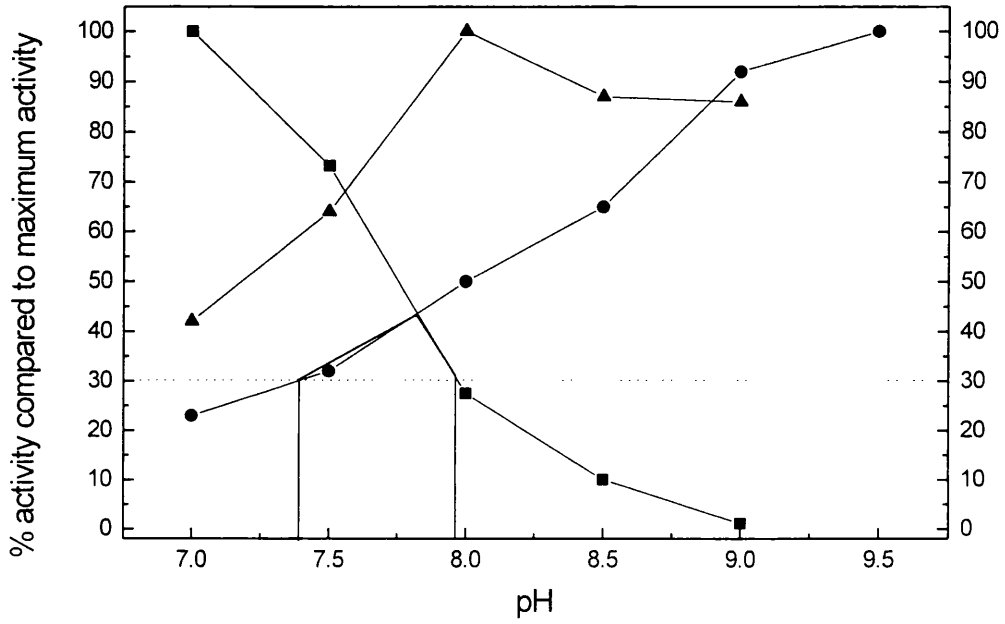


Figure 5.1 A Schematic diagram to indicate the window of operation for an $NADP^+$ cofactor recycling system involving GDH and CHMO, where the enzyme with the lowest activity has an activity of no less that 30% of it's maximum activity. pH profiles of CHMO, ADH and GDH, where ● represents CHMO, ▲ represents ADH and ■ represents GDH. Adapted from Hogan (1998).

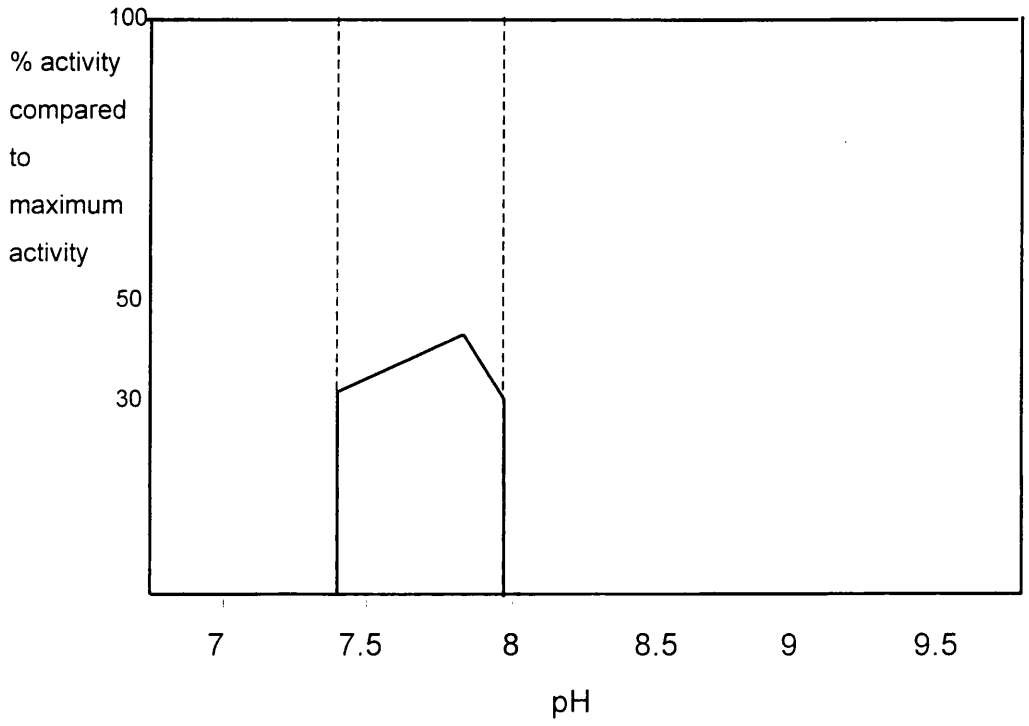


Figure 5.2 Schematic diagram to represent the window of operation for a biotransformation utilising the $NADP^+$ cofactor recycling system, with GDH and CHMO as the linked enzymes as described in this research.

5.2.2 RACEMISATION.

The pH of the racemisation solution is due to the presence of the catalyst. NaOH and Amberlite IRA 400 (-OH) both affect the pH of a solution (buffered or non buffered) within minutes of their addition, as detailed in Chapter 3. The pH of buffered solutions were seen to rise to above pH 12 within thirty minutes of the addition of either catalyst.

The main constraint for the performance of the racemisation procedure was the pH. Racemisation was observed to occur at various temperatures, with varying amounts of substrate and varying catalyst concentrations (see Chapter 4). The racemisation procedure could not be performed at a pH below 9.5, due to the effect that the catalyst had on the solution it was added to. The pH of water and buffered solutions was observed to rise to above pH 12 after the addition of Amberlite IRA 400 (-OH) or NaOH (2 M, 10%v/v) as presented in Tables 3.5 and 3.6. This data can be included in a schematic representation of the window of operation for the racemisation process (Figure 5.3). Racemisation is slow at pH 9.5 due to the small amount of catalyst present. Therefore the lower limits for the window must be above pH 9.5. At pH 12 and above the rate of racemisation is good due to amount of catalyst present. The maximum reaction rate of racemisation can be diagramatically represented as at a pH above 12.

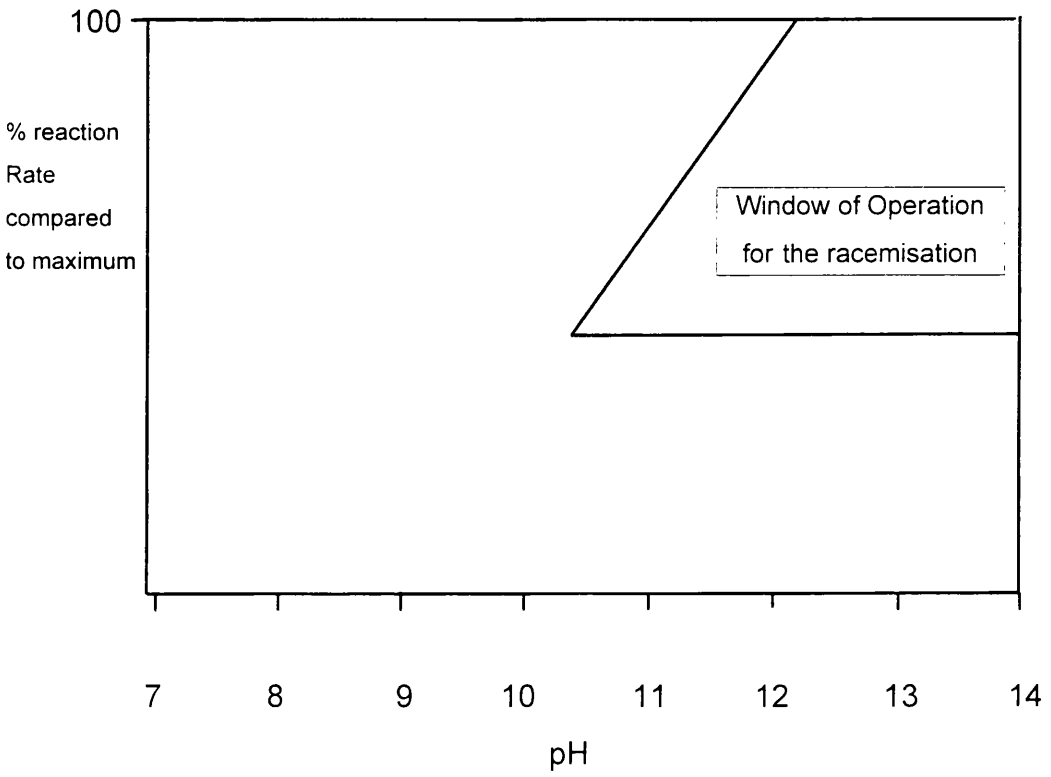


Figure 5.3 Schematic representation of a window of operation for the Racemisation process.

5.3 INTEGRATED PROCESS

An integrated process to be performed in one pot or '*in-situ*' (Figure 5.4) requires the overlap of the windows of operation of each of the individual unit operations and desirable interactions between all components of each of the processes. In this case the window of operation for pH the biotransformation and racemisation do not overlap, as seen in Figure 5.5.

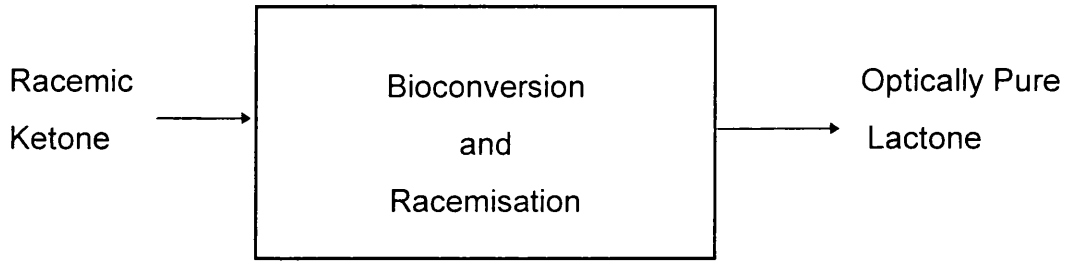


Figure 5.4 *In-situ* bioconversion and racemisation reactor.

It is necessary to consider performing the two unit operations as separate batch processes or determining other methods to 'link' the two unit operations in order to integrate the processes.

To enable the two unit operations to be directly linked, interactions between all of the components of each of the separate processes must, as in the case of an *in-situ* process, still be considered. These interactions, as determined in Chapter 2.5.1 are as follows:

- The catalyst must not bind to or decrease the activity of the two enzymes (CHMO and GDH)
- The catalyst must have no effect on the pH of the bioconversion solution
- The catalyst must be stable below 40°C
- The catalyst must not degrade the lactone

From the results in Chapter 3 it is possible to conclude that neither of the catalysts bind to the enzymes, they are also both stable below 40°C. Both of the catalyst have a significant effect on the pH of the bioconversion solution and they both degrade the lactone 7-methyloxepan-2-one rapidly. The integration of two procedures with such interactions between components is complex. The integrated system is constrained by the necessity to stop the chemical catalysts coming into contact with the biotransformation solution. This is the main consideration in the design of the integrated process and the design of the reactor in which the process is to be carried out.

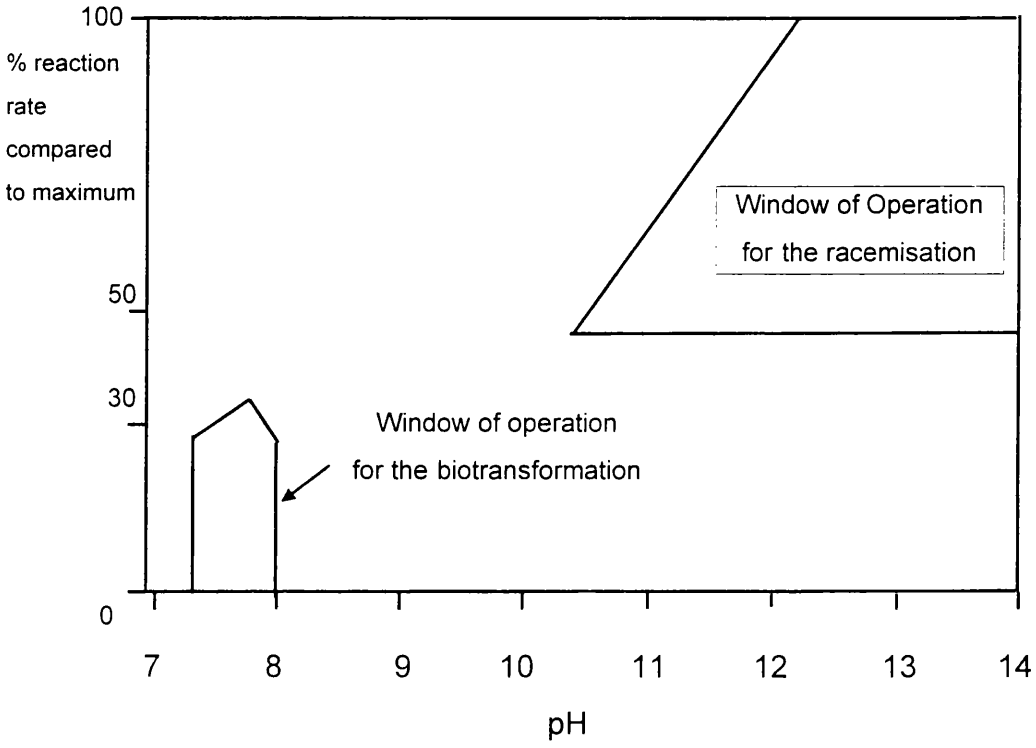


Figure 5.5 Schematic representation of the window of operation of integrated process.

5.4 REACTOR DESIGN

5.4.1 RATIONALE

For an integrated process the reactor must be designed to allow the performance of both a biotransformation and a racemisation procedure. The reactor of choice for the biotransformation is a stirred tank, to allow for mixing and aeration, as CHMO requires molecular oxygen to enable the bioconversion of the ketone to the lactone to be performed. The stirrer speed needs to be kept low to minimise interfacial damage to the enzyme, but allow for adequate mixing and aeration. Initial experiments involving the racemisation were performed in a stirred tank to allow for good mixing and ease of sampling.

5.4.2 PROCESS LIMITATIONS/REQUIREMENTS

The major limitations on the design of the reactor is the need to keep the chemical catalyst away from the bioconversion solution. This will stop the chemical catalyst affecting the pH of the biotransformation solution and degrading the lactone product of the biotransformation. Therefore the reactor should be designed to stop the chemical catalyst from coming into contact with the biotransformation solution.

5.5 REACTOR CONFIGURATION 1(LINKED REACTOR)

Reactor configuration 1 (Figure 5.6) is based on the simplest reactor design, where each individual unit operation is performed in a batch type process. At the end of each of the unit operations an intermediate step is performed to ensure that the solution entering into the next unit operation contains the desired components. This allows the lactone product to be removed at the end

of the bioconversion procedure and the pH to be adjusted back to pH 7.5 (as required by the biotransformation after the end of the racemisation reaction).

5.5.1 Advantages of reactor configuration 1.

The main advantage of this design is the ability to keep the chemical catalyst away from the biotransformation solution. This eradicates the possibility of lactone degradation by the chemical catalyst and stops the chemical catalyst from having an effect on the pH of the bioconversion solution. This reactor complies with the process requirements as detailed in 5.4.2.

5.5.2 Disadvantages of reactor configuration 1

The major disadvantages of this reactor configuration are the necessity of a product removal process and the need to neutralise the racemisation solution before it re-enters the bioconversion vessel.

Product removal of a reaction component that does not form a solid when purified from an aqueous solution can be, and often is, difficult. In the case of the bioconversion of 2-methylcyclohexanone to 7-methyloxepan-2-one, neither the starting material or product are solids at room temperature. The boiling points of the two compounds are approximately 80°C apart and the lactone is more water-soluble than the ketone. Earlier studies as detailed in Chapter 3 indicated that the lactone degrades slowly in phosphate buffer (pH 7.5, 50 mM) as used for the biotransformation. These results mean that the lactone needs to be removed from the aqueous solution as soon as possible to stop the loss of lactone through degradation.

The simplest method to separate the lactone product from the biotransformation solution is distillation under reduced pressure. While not optimal, distillation under vacuum was selected to remove the lactone from the biotransformation solution. As the aims of this research were to investigate the feasibility of the integration of a biotransformation and racemisation processes, not to optimise the removal of a product from a reaction solution it was felt that the use of distillation was justified.

The second disadvantage of the linked system as shown in Figure 5.6 is the need to neutralise the racemisation solution before it re-enters the bioconversion vessel. The pH of the solution will be at approximately 12.5 at the end of the racemisation process. The pH will have to be decreased to pH 7.5 before it can enter the biotransformation vessel. In the case of NaOH the addition of acid to the solution will produce salts which in turn will have an effect on the enzymes within the bioconversion vessel. It is therefore important that the lowest concentration of catalyst that will keep the reaction rate above 50% of the maximum is used.

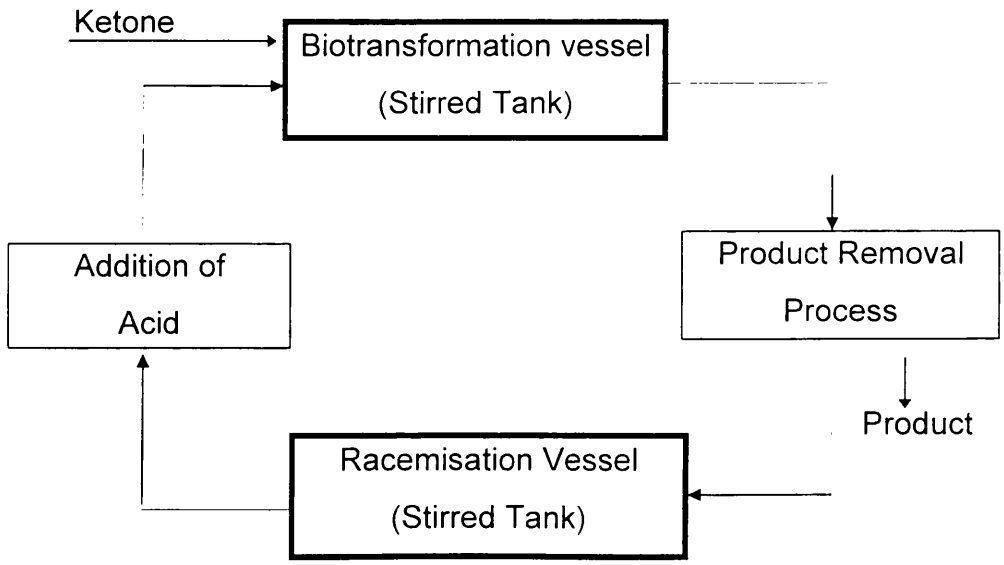


Figure 5.6 Schematic of reactor design configuration 1.

5.6 REACTOR CONFIGURATION 2 (MEMBRANE BIOREACTOR)

5.6.1 INTRODUCTION.

Prazeres and Cabral (1994) describe membrane reactors as reactors that are able to facilitate the separation of enzymes and their products (or substrates) by the means of a selective barrier. These systems have been used to separate phases, for enzyme immobilisation and also as semi-permeable barriers. Membrane bioreactors have been used by many researchers. Vaidya (1994a and 1994b) described the use of a membrane bioreactor in an aqueous/organic reaction. Brindle and co-workers (1996) detailed the use of membrane bioreactors to facilitate bubbleless aeration. Brookes and Livingstone (1995) described the use of membranes to extract organic pollutants through on aqueous phase to a second aqueous phase through silicone rubber membranes. Enzymatic membrane bioreactors and their applications were reviewed by Prazeres and Cabral in 1994.

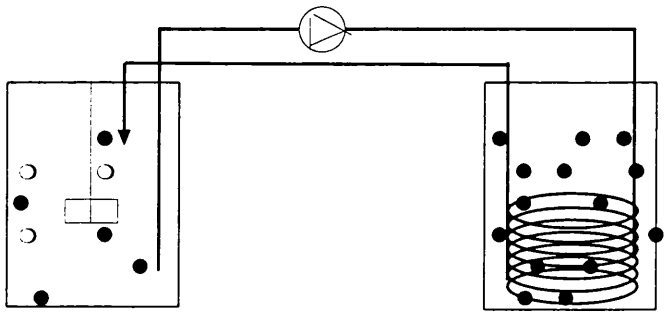
Reactor configuration 2 is a modified membrane bioreactor, containing two vessels, a donating vessel and a receiving vessel (Figure 5.7). The two vessels are linked by PTFE (polytetrafluoroethylene) tubing.

The receiving vessel is a stirred tank containing all of the components for the bioconversion except for the ketone. Two pieces of PTFE tubing are connected to the receiving vessel, one to the outlet, the other to the inlet of the vessel.

The PTFE tubing connected to the outlet of the receiving vessel is connected to a magnetically driven gear pump. A second piece of PTFE tubing is connected to one end of the silicone rubber tubing, which forms the membrane part of the reactor. The other end of the silicone rubber membrane is connected to a third piece of PTFE tubing. The third piece of PTFE tubing is connected to the inlet of the receiving vessel. This allows the contents of the receiving vessel to be

pumped through the silicone rubber membrane and back into the receiving vessel without coming into direct contact with the contents of the donating vessel.

The donating vessel contains the chemical catalyst for the racemisation procedure and the ketone for the bioconversion. The membrane part of the bioreactor, the silicone tubing, is coiled into the donating vessel and kept under the surface of the liquid at all times.



Stirred tank reactor (Receiving vessel)

Membrane module. (Donating vessel)

● Represents substrate ○ represents product.

Figure 5.7 Reactor configuration 2. The membrane bioreactor, where the stirred tank contains the components of the bioconversion. The membrane module contains the silicone tubing membrane, mixing is achieved by a magnetic stirred bar.

The theory behind this procedure is that the ketone will pass through the membrane of the silicone tubing and into the receiving vessel, where it can undergo bioconversion to the lactone. The lactone should not be able to be transported through the membrane and should not therefore enter the donating vessel.

Silicone rubber tubing was used as the membrane material. It has been shown by previous workers (Brookes and Livingstone 1995, Doig *et al.,* 1998) to be effective in providing selectivity to the mass transfer process. It is a dense material and is considered to be a homogenous mixture of PDMS polymer (polydimethylsiloxane) and filler (silica). The structure has no defined pores which molecules can pass through. Instead, mass transfer of the ketone (and lactone) has to occur via diffusion through the membrane. The major consideration in the choice of silicone rubber tubing was its availability.

Tubular silicone rubber membranes were obtained from Silicone Altimex, UK and consisted of 70% PDMS and 30% fumed silica. The membrane was 1mm thick with an inner tube diameter of 3 mm.

The main advantages of reactor configuration 2 are that the chemical catalyst should not come into contact with the lactone product of the bioconversion, and the catalyst should also not be able to have an effect on the pH of the bioconversion solution due to the presence of the membrane. A second major advantage over reactor configuration 1 is that there is only one unit operation. This allows control over the rate of feeding of the ketone into the bioconversion media and minimises losses during the transfer of the reaction media from one unit operation to the next.

The main disadvantage of the membrane bioreactor is the necessity for the reaction to be running over a long period of time and the possibility of the process being mass transfer limited. This can result in a slow process and involve biocatalyst problems. The major problem likely to occur with semi-purified enzymes, as used in this process, is microbial contamination. A second enzyme associated problem is likely to be due to a decrease in CHMO activity over a long period of time as observed by Hogan (1999).

5.6.2 MEMBRANE MODELLING

In single-phase systems, the rate of mass transfer due to molecular diffusion is given by Fick's law. Fick's law of diffusion states that mass flux is directly proportional to the concentration gradient (Doran 1995).

$$J_a = N_a / A \qquad (5.1)$$

Mass flux is defined by the rate of mass transfer per unit area, perpendicular to the direction of travel. The direction of mass transfer is always from high to low concentration.

Whitman two film theory is often used to model the mass transfer of solutes in two liquid phase systems and membrane systems. Two film theory assumes that a stagnant liquid boundary layer exists at the membrane wall (Doran 1995). Mass transfer occurs through this film by diffusion.

Figure 5.8 represents a theoretical concentration profile of a model solute in a biphasic membrane system with a non-porous flat sheet hydrophobic membrane. Using this system we can derive a resistance-in-series model, based on Whitman two film theory.

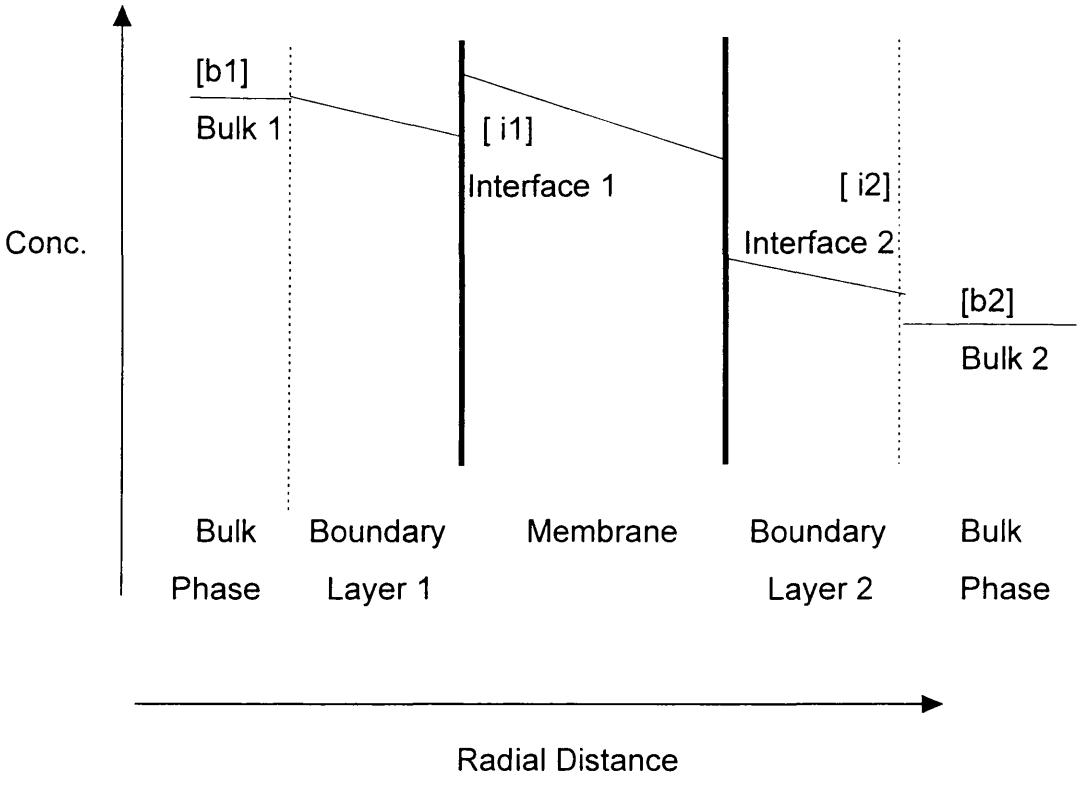


Figure 5.8 represents a theoretical concentration profile of a model solute in a two liquid phase membrane system with a non-porous flat sheet hydrophobic membrane.

The rate of mass transfer is directly proportional to the driving force for transfer. Mass transfer can be described by the equation;

$$N = ka\ A\Delta C = ka\ A\ (C^{ao} - C^{ai}) \qquad (5.2)$$

At steady state;

$$N_{ov} = N_{film1} = N_{mem} = N_{film2} \qquad (5.3)$$

Where;

$$N_{ov} = K_{ov} [C^{b1} - C^{b2}] \quad (5.4)$$

$$N_{film1} = K_{film1} [C^{b1} - C^{i1}] \quad (5.5)$$

$$N_{film2} = K_{film2} [C^{i2} - C^{b2}] \quad (5.6)$$

From solution Diffusion theory; $K_{mem} = P^{mem}_{aq} D_{mem} / \delta_{mem}$ (5.7)

Therefore; $N_{mem} = P^{mem}_{aq} D_{mem} [C^{i1} - C^{i2}] / \delta_{mem}$ (5.8)

The driving forces can be equated such that;

$$[C^{b1} - C^{b2}] = [C^{b1} - C^{i1}] + [C^{i1} - C^{i2}] + [C^{i2} - C^{b2}] \quad (5.9)$$

Therefore;

$$N_{ov} / K_{ov} = (N_{film1} / K_{film1}) + (N_{mem}\delta_{mem} / P^{mem}_{aq}D_{mem}) + (N_{film2} / K_{film2}) \quad (5.10)$$

Where R_m is the resistance to mass transfer;

$$R_m = 1/K_a \quad (5.11)$$

Therefore the overall resistance to mass transfer at steady state can be expressed as;

$$1/K_{ov} = (1/K_{film1}) + (\delta_{mem}/P^{mem}_{aq}D_{mem}) + (1/K_{film2}) \quad (5.12)$$

The rate of mass transfer can be increased by;

- increasing the area available for mass transfer,
- increasing the concentration difference between the bulk solution and the interface (increasing the driving force)
- increasing the magnitude of the mass transfer coefficient.

As a rule the rate of mass transfer and mass transfer coefficient can be improved by reducing the thickness of the boundary layer, or improving the diffusion coefficient in the film (Doran, 1995).

In the system to be investigated in this research both of the phases are aqueous. They could theoretically have different membrane partition coefficients, but for the purpose of this research and to simplify the equations, they will be treated as identical phases. It is important that the rate of lactone mass transfer across the membrane is significantly slower than the rate of ketone mass transfer across the membrane.

From the resistance-in-series model, the membrane contribution to the resistance to mass transfer of each substrate can be predicted by the measurement of P^{mem}_{aq}. In an ideal situation the lactone would not cross the membrane at all.

If the P^{mem}_{aq} is large the mass transfer will not be limited by the membrane. In this case a small resistance to mass transfer across the membrane for the ketone and a large resistance to mass transfer for the lactone is desirable.

At equilibrium the P^{mem}_{aq} is equal to the concentration of the substrate in the membrane divided by the concentration of the substrate in the aqueous phase and can be described equation 5.13;

$$P^{mem}_{aq} = [\text{membrane}] / [\text{aqueous}] \qquad (5.13)$$

When the rate of mass transfer is not limited by the membrane it is limited by the hydrodynamic conditions. To minimise the boundary layer resistance a turbulent flow within the silicone tubing is required. Calculation of the Reynolds

number (Re) can determine whether a given flow rate in the system will result in turbulent flow.

$$Re = D u \rho / \mu \quad \text{(in tubing and pipes)} \qquad (5.14)$$

Before the bioreactor can be used on a 1.5L scale it is important to perform a small number of experiments to ensure that the reaction can be performed efficiently.

Initially the P^{mem}_{aq} of the lactone and the ketone into the silicone membrane should be determined. This would indicate the likelihood of a successful process. A small difference in the partition coefficient would suggest that the membrane would be ineffective at stopping the lactone coming into contact with the chemical catalyst.

The following experiments are required;

- Determine overall rate of mass transfer of the ketone and lactone across the membrane.
- Determine the Reynolds number (Re) for this system to assess whether the flow is turbulent.

5.6.2.1 METHODS.

5.6.2.1.1 MEMBRANE PARTITION COEFFICIENT EXPERIMENTS.

20 cm of silicone tubing was cut into small pieces and added to a 25 mL sealable glass vessel containing 20 mL of an aqueous solution containing a known concentration of the ketone (1 g/L). The vessel was sealed and incubated at 37°C. The vessel was shaken at various time-points to ensure that

the system was well mixed. Aliquots of the aqueous solution were removed after 24 and 48 hours. Analysis was performed by Chiral GC. The method was repeated for the lactone (1 g/L).

5.6.2.1.2 Mass Transfer Experiments.

The K_{ov} was measured in a small scale reactor. These conditions were designed to be a direct mimic of those to be used in the large scale integrated process. Glass beakers (400 mL) were used for the donating and receiving vessels. Flow was achieved using a magnetically driven gear pump.

2-methylcyclohexanone (20 mM), and water (100 mL) was stirred at 30°C in the donating vessel. Water (300 mL) was stirred at 30°C in the receiving vessel. Length of silicone tubing used 29 cm (i.d. 3 mm). Flowrate 450 mL min^{-1}. Aliquots were removed from each vessel at hourly intervals. The samples were analysed by chiral GC.

5.6.2.2 Results

5.6.2.2.1 Membrane partition coefficient experiments.

The GC data obtained indicates the concentration of substrate in each sample, from this we can calculate;

$$P^{mem}_{aq} = \text{[membrane] / [aqueous]} \quad (5.13)$$

P^{mem}_{aq} Ketone = 9.6

P^{mem}_{aq} Lactone = 0.78

5.6.2.2.2 MASS TRANSFER EXPERIMENTS

The samples analysed by GC indicate the concentration of ketone in solution at each time-point.

K_{ov} can be determined graphically using the following equation;

$$N_{ov} = (K_{ov} A / V^r) [C^d - C^r] \quad (5.15)$$

This equation can be integrated;

$$V^r C/t = K_{ov} A [C^d - C^r] \quad (5.16)$$

$$\int_{t=0}^{t=t} K_{ov} A / V^r = \int_{c=o}^{c=c} 1 / [C^d - C^r] \quad (5.17)$$

$$(K_{ov} A /V^r) t = \ln [C^d - C^r] \quad (5.18)$$

By plotting $\ln [C^d - C^r]$ against time (t) it is possible to calculate $K_{ov} A / V^r$. (Figure 5.9).

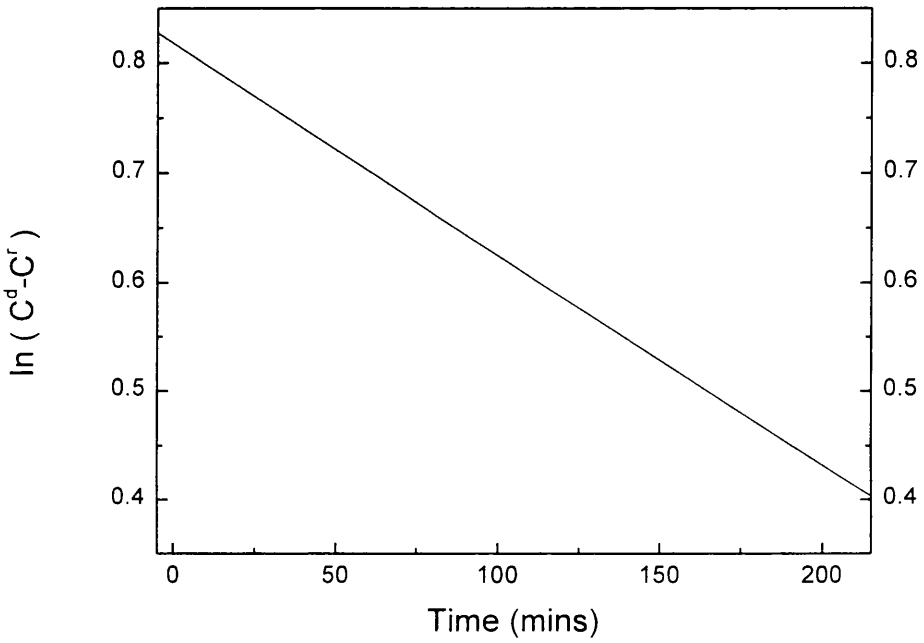


Figure 5.9 Mass transfer of ketone from receiving vessel to donating vessel during initial mass transfer investigation, where — represents the line of best fit, from which the gradient is calculated.

The gradient of the straight line in Figure 5.9 is equal to K_{ov} A / V^r.

0.00193 = K_{ov} A / V^r

It is then possible to determine Kov because the volume of the receiving vessel (V^r) and the area of the membrane (A) are known. A=0.0027 m^2, V^r =0.0003 m^3.

Kov = 3.57 x 10^{-6} m s^{-1}

The overall mass transfer coefficient for the given process is 3.57x10^{-6} m s^{-1}

5.6.2.2.3 DETERMINATION OF REYNOLDS NUMBER IN THE MIMIC SYSTEM.

The Reynolds number can be calculated from equation 5.14,

Re = D u ρ / μ

In this system, D = 0.003 m, u = 1.061 m s^{-1}, ρ = 1000 kg m^{-3}, μ = 1 x 10^{-3} Pa s
Re = 3182.

5.6.2.2.4 DETERMINATION OF AREA OF SILICONE TUBING REQUIRED.

The overall mass transfer coefficient (K_{ov}) for the system was determined to be 3.57 x 10^{-6} m s^{-1}. The concentration of ketone to be converted in the process can be set at 4.5 g/L. The initial rate of mass transfer can be specified, to enable the area of silicone tubing required to be calculated.

The mass transfer process is to be designed so that it is not the rate-limiting step of the overall integrated process. The rate of mass transfer, must therefore be greater than the maximum rate of biotransformation. The receiving vessel will contain the bioconversion components of the process. No more than 500 U of CHMO activity will be added to the receiving vessel at the beginning of the

reaction. At pH 7.5, the pH at which the media in the receiving vessel will be set, the maximum rate for the bioconversion is approximately 35% of the maximum activity.

The maximum activity for the CHMO conversion of its natural substrate (cyclohexanone) is the conversion of 1 μM substrate per minute per unit of activity, *i.e.* at 35 % of the maximum rate 1 U activity will convert 0.35 μM of substrate per minute. As with most enzymes, the rate at which CHMO will convert a substrate other than its natural substrate is reduced. The cofactor recycle process will also reduce the rate of the bioconversion of 2-methylcyclohexanone by CHMO.

Therefore the maximum rate at which the biotransformation will occur is likely to be lower than 0.35 μM of substrate per minute. To simplify the calculations it will be assumed that the rate of biotransformation will be 0.35 μM per minute.

The concentration of ketone available for bioconversion during the process is set at 4.5 g/L (60 mM) with respect to the volume of the receiving vessel (6.7 g/L with respect to the volume of the donating vessel). The minimum time for the bioconversion process is therefore

500 U = 175 mM min^{-1}
500 U will convert 60 000 μM in approximately 6 hours. This is the maximum available rate of the bioconversion.

Therefore the bioconversion process will be performed at no greater that 6.7 g of ketone to be added to the donating vessel in 6 hours

The bioconversion within the large scale integrated process will be oxygen limited. A high air flow rate was shown by Hogan (unpublished results) to increase the air/liquid interface and decrease the life and activity of the enzyme.

The initial rate of mass transfer was set at 3.1×10^{-4} g s^{-1}, to allow the area of silicone tubing to be determined.

$K_{ov} = 3.57 \times 10^{-6}$ m s^{-1}, $\Delta C = 6729$ g m^{-3}, $N_{ov} = 3.27 \times 10^{-4}$ g s^{-1}

$N_{ov} = K_{ov} A \Delta C$

$A = 0.013$ m^2

From this area calculation it is possible to determine the length of silicone tubing required for the given rate of mass transfer.

The length of silicone tubing required in the integrated process to be performed in the membrane bioreactor is 1.38 m.

These calculations are based on a steady driving force of 6729 gm^{-3}. In reality the driving force will decrease as the concentration of ketone in the donating vessel decreases. The length of tubing required to maintain a steady driving force would need to be increased throughout the process. As this is not practical the length of tubing used for the large-scale process should be increased. The length of the silicone tubing used in the membrane model was increased by approximately 15% to 1.6 m to try to compensate for the decrease in driving force.

5.7 DISCUSSION

5.7.1 INTEGRATION OF THE BIOCONVERSION AND RACEMISATION

The schematic representation of the window of operation for the bioconversion and the racemisation indicated that it is not possible to perform the linked process in one vessel using the GDH cofactor recycling system and either

Amberlite IRA 400 (-OH) or NaOH as the chemical catalyst, due to the different pH requirements for each of the processes.

Further reasons that the processes can not be performed in one vessel are that both of the catayst that will be used for the racemisation procedure degrade the lactone product of the bioconversion. It is also known that the biocatalyst can not operate at the pH at which the racemisation occurs. The catalysts used for the racemisation have been shown to have an immediate affect on increasing the pH of the bioconversion media to above a level at which the GDH cofactor bioconversion occurs.

For these reason it was decided to design a bioreactor that would keep the enzyme and the chemical catalyst apart, and keep the lactone and chemical catalyst separated.

5.7.2 Reactor Design

Reactor design 1 was designed so that each of the unit operations could be performed separately, keeping the biocatalyst and chemical catalyst apart. This has the advantage of removing the lactone product from the process so that it is not degraded by the chemical catalyst and the chemical catalyst does not modify the pH of the bioconversion.

The disadvantages with this process are the need to separate the lactone from the bioconversion media by a non-specific product separation technique and the need to neutralise the racemisation solution before it can enter the second cycle of the process.

Reactor configuration 2 was also designed to keep the bioconversion and racemisation processes apart, but maintaining the two processes in one reactor that could be run continuously until all of the ketone is converted. This bioreactor should, as demonstrated by the membrane modelling experiments, be able to allow the ketone, but not the lactone, to pass through the membrane. This would mean that all of the ketone could be added to the vessel containing the chemcial catalyst, from where it should transfer into the vessel that contains the biocatalyst without the lactone product transferring into the vessel containing the chemical catalsyt. This bioreactor should have the advantage of keeping the biocatalyst and the chemical catalyst apart.

The main disadvantages with this process are due to the fact the it may take a long time to perform, due to the constant decreasing rate of mass transfer of the ketone as the driving force (ketone concentration difference between the two vessels) decreases. A timely process may present problems with the enzymes in present in the bioconversion vessel, these are most likely to be microbial contaminations and decreased CHMO activity. Both of these problems can be overcome by the addition of anti-biotics to inhibit bacterial growth and further additions of enzymes and cofactor to maintain good CHMO activity.

It is not possible to predict which of the reactor configurations will produce the best results and what is meant by 'best result'. The best result should be from the reactor producing the highest yield and enantiomeric excess of lactone product in the shortest amount of time, although a reactor that could produce a yield of over 90% and an e.e of over 95% in 10 hours would be preferable to a process that produced a yield of (say) 30% and an e.e of (say) 20% in 1 hour.

6. INTEGRATED BIOCONVERSION AND RACEMISATION.

6.1 INTRODUCTION.

This Chapter details the process runs involving the linked reactor, using NaOH as the chemical catalyst and also the membrane bioreactor process, which was performed using either NaOH or Amberlite IRA 400 (-OH) as the chemical catalyst.

The information collected from each process run enabled comparison between the two types of reactor design and comparison between a homogenous and heterogenous chemical catalyst.

6.2 REACTOR DESIGN.

6.2.1 REACTOR CONFIGURATION 1 (LINKED REACTOR).

The linked reactor was designed to allow all of the individual processes to be performed as separate unit operations (Figure 5.6). The bioconversion step was performed in a stirred tank reactor, to allow for pH and temperature control, and also the addition of air and mixing. The distillation was performed in a quickfit style distillation column with a 'pig' type attachment to allow for easy collection of the fractions of distillate. The distillation was performed in small batches due to the size of the distillation column available. The racemisation procedure took place in the stirred tank reactor to allow neutralisation and further biocoversion occur without the need for further vessels. This allows the process to be simplified.

6.2.2 Reactor Configuration 2 (Membrane Bioreactor)

The reactor was designed to separate the bioconversion and the racemisation procedures whilst retaining them within a single system (Figure 5.7).

The membrane module of the bioreactor was designed to be part of the donating vessel. The ketone is added to the donating vessel. 2-methylcyclohexaone is known to be inhibitory towards the enzyme above 40mM. The ketone enters the receiving vessel (containing the enzyme) at a relatively slow rate, as it has to diffuse into the membrane to leave the donating vessel. It is then pumped into the receiving vessel. This allows a higher concentration of ketone to be added to the system. The concentration of the ketone in the receiving vessel does not go above 40 mM as it is constantly used in the bioconversion.

The receiving vessel was designed to contain all the components required for the bioconversion including the recycle enzymes and co-substrate. The receiving vessel is a stirred tank reactor that has been adapted to allow the flow of biotransformation media out of the vessel, through tubing into the membrane (situated inside the donating vessel) and back into the stirred tank reactor. The pH, and temperature of the bioconversion media are controlled inside the stirred tank reactor during the operation of the process. The media is also aerated and mixed during the process.

6.3 METHODS

6.3.1 LINKED REACTOR.

6.3.1.1 BIOTRANSFORMATION

The components listed in table 6.1 were added to a stirred tank reactor. The biotransformation was performed at 30°C, with a stirrer speed of 200 rpm.

Component	Quantity
CHMO	500 U
GDH	500 U
NADP+	750 mg (0.5 mg/mL)
Glucose (100 mM)	27 g
2-methylcyclohexanone (40 mM)	7 mL
Phosphate buffer (50 mM, pH 7.5)	1500 mL

Table 6.1 Components of the biotransformation.

Samples of 1 mL were removed at various time points. The protein was precipitated from the sample by the addition of ice cold IPA (2 mL). The solution was centrifuged at 13000 rpm for 1 minute and the supernatant was removed. The concentration of ketone and lactone was determined directly by chiral GC of the supernatant.

The biotransformation was quenched by the addition of ice cold IPA in the ratio of 2:1 (IPA : biotransformation solution). The resulting solution was centrifuged for 30 minutes, at 4000 rpm (4°C). The supernatant was then pooled and used in the next stage of the linked process, the distillation.

6.3.1.2 DISTILLATION.

The excess IPA was removed from the system by vacuum condensation using a rotary evaporator. The remaining solution was distilled in batches under reduced pressure, using traditional distillation apparatus and a fractionating column.

6.3.1.3 RACEMISATION.

The pooled products of the distillation procedure were diluted to 1350 mL with phosphate buffer (50 mM, pH 7.5). At the beginning of the racemisation procedure $NaOH_{(aq)}$ (1 M, 150 mL) was added to the vessel to start the racemisation. The mixture was stirred at 30°C. Aliquots were removed at various time points. The samples were neutralised with $HCl_{(aq)}$. The reaction was monitored by direct chiral GC analysis of the neutralised samples. The reaction was quenched by the addition of HCl to the solution, the pH was adjusted back to pH 7.5 ready for the biotransformation procedure in cycle two of the linked process.

6.3.2 MEMBRANE BIOREACTOR.

6.3.2.1 NaOH AS THE BASE CATALYST

The reactor was set up as detailed in 6.2.2. At the start of the process the components detailed in table 6.2, except the enzymes, were added to the receiving vessel. The addition of the enzymes occurred approximately 3 hours after the start of the process, to allow the accumulation of ketone in the receiving vessel. Water (900 mL), $NaOH_{aq.}$ (1 M, 100 mL) and 2-methylcyclohexanone (60 mM, 7 mL) were added to the donating vessel.

The length of silicone tubing used as the membrane part of the reactor was 1.6 m. It was calculated in Chapter 5 that 1.38 m of tubing was required, the extra tubing is to compensate for the decrease in driving force during the process and to ensure that the required area of tubing will be under the surface of the liquid in the donating vessel at all times. This was coiled into the donating vessel, ensuring that all of the tubing was under the level of the liquid.

Component	Quantity
Phosphate buffer (50 mM, pH 7.5)	1500 mL
NADP+	750 mg
Glucose (100 mM)	27 g
CHMO	500 U
GDH	500 U

Table 6.2 Components in receiving vessel of the membrane bioreactor when NaOH was the catalyst..

The pH of the receiving vessel was monitored throughout the process to ensure the conditions remained optimum for the biotransformation. The following antibiotics were also added to the receiving vessel at the beginning and then every 8-10 hours to inhibit contamination by other organisms; ampicillin (50 mg/L), kanomycin (50 mg/L) neomycin (50 mg/L) and tetracycline (100 mg/L).

Additional enzymes and cofactor were added 16 hours after the addition of the initial enzymes and cofactor, when the rate of the bioconversion decreased to almost zero, without the conversion of all of the ketone to lactone, as the life of the enzyme was only approximately 8 hours. After this time the DOT was observed to increase indicating that the oxygen within the air supplied to the vessel was not being utilised. This concluded that the bioconversion was

occurring at a lower, if not decreasing level, and suggested that the CHMO was losing activity.

Samples were taken from each vessel at various time points. The concentration of ketone and lactone in each vessel, at each time point was determined directly by chiral GC.

6.3.3 AMBERLITE IRA 400 (-OH) AS THE BASE CATALYST

The reactor was set up as detailed in 6.3.1. At the start of the process the components detailed in table 6.3 were added to the receiving vessel. Water (1000 mL), Amberlite IRA 400 (-OH) (10 g/L) and 2-methylcyclohexanone (60 mM, 7 mL) were added to the donating vessel.

Component	Quantity
Phosphate buffer (50 mM, pH 7.5)	1500 mL
NADP+	750 mg
Glucose (100 mM)	27 g
CHMO	500 U
GDH	500 U

Table 6.3 Components in receiving vessel.

The length of silicone tubing used as the membrane part of the reactor was 1.6m. This was coiled into the donating vessel, ensuring that all of the tubing was under the level of the liquid.

The pH of the receiving vessel was monitored throughout the process to ensure the conditions remained optimum for the biotransformation. The following antibiotics were also added to the receiving vessel at the beginning and then every 8-10 hours to inhibit contamination by other organisms; ampicillin (50 mg/L), kanomycin (50 mg/L) neomycin (50 mg/L) and tetracycline (100 mg/L).

Additional enzymes and cofactor were added at 22 hours and 27 hours after the start of the process when the rate of the bioconversion decreased to almost zero, without the conversion of all of the ketone to lactone. After this time the DOT was observed to increase indicating that the oxygen within the air supplied to the vessel was not being utilised. As in section 6.3.2.1, this indicated that the CHMO was losing activity.

Samples were taken from each vessel at various time points. The concentration of ketone and lactone in each vessel, at each time point was determined directly by chiral GC.

6.4 RESULTS

6.4.1 LINKED REACTOR.

6.4.1.1 BIOTRANSFORMATION (CYCLE 1)

The reaction was quenched after 7 hours as the dissolved oxygen tension (DOT) of the solution was observed to be rising, indicating that the enzyme activity was dropping and the enzyme was losing its functionality/activity. After the addition of further enzyme the DOT was observed to decrease to zero, indicating that oxygen was being used by the enzyme for the bioconversion.

The first cycle of the biotransformation produced 16.7 mmol of lactone, with an enantiomeric excess of 43%, as can be seen in Figures 6.1 and 6.2. This corresponds to a 29% yield. The overall mass balance of the biotransformation was 96.2%, assuming 30% loss of ketone due to evaporation. This value had been determined from previous batch bioconversions using 2-methylcyclohexanone as the substrate with identical conditions to the biotransformation in this linked process.

6.4.1.2 DISTILLATION. (CYCLE 1)

48% of the ketone present after the biotransformation step was not recovered during the distillation process. This large amount of ketone was found, by GC analysis, to be present in the IPA fraction that was removed during the distillation procedure. The fraction was re-distilled, but the ketone could not be isolated from the IPA.

57% of the lactone was isolated from the biotransformation solution. The remaining lactone was determined, by chiral GC, to be present in the ketone fraction.

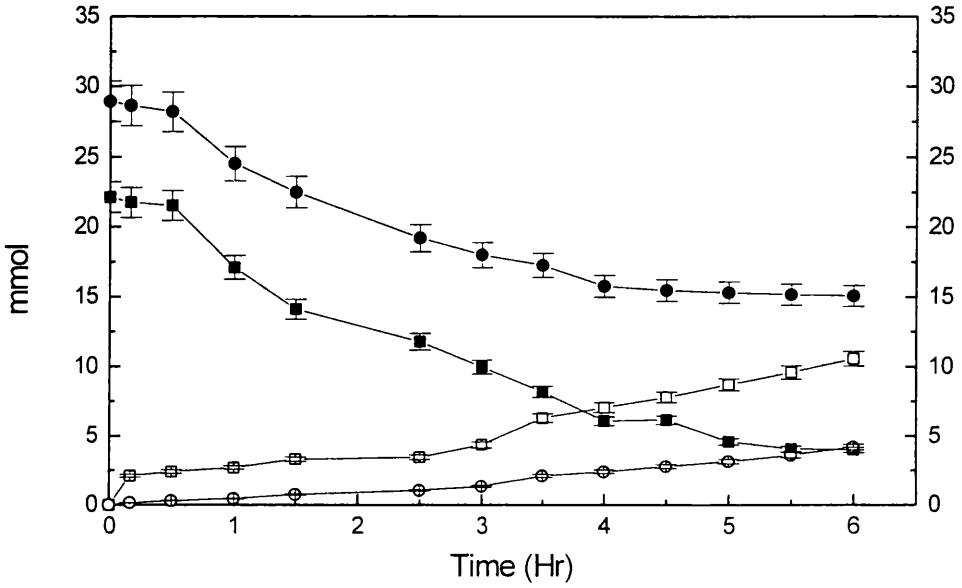


Figure 6.1 Biotransformation data from reactor configuration 1, where ● represents ketone enantiomer R, ■ represents ketone enantiomer S, o represents lactone enantiomer R, and □ represents lactone enantiomer S.

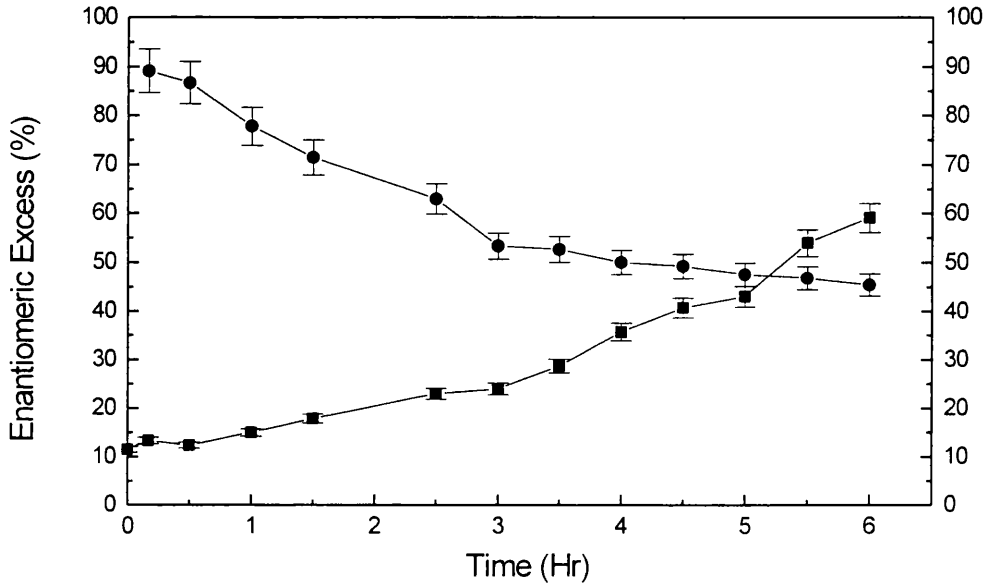


Figure 6.2 Enantiomeric excess data from during the biotransformation step performed using reactor configuration 1, where ■ represents the ketone data and ● represents the lactone data.

6.4.1.3 RACEMISATION (CYCLE 1)

The racemisation process took 2 hours to perform (Figure 6.3). 37% of the ketone was lost during the procedure. These losses are thought to be due to evaporation. Experimental error during the dilution of the product of the distillation process at the beginning of the racemisation procedure is also thought to attribute to the apparent loss of ketone.

6.4.1.4 CYCLE 1.

Figure 6.4 is a schematic representation of the whole of the first cycle. The change in yield and the change in concentration of each enantiomer of both the lactone and the ketone can be observed during each unit operation in Figure 6.4.

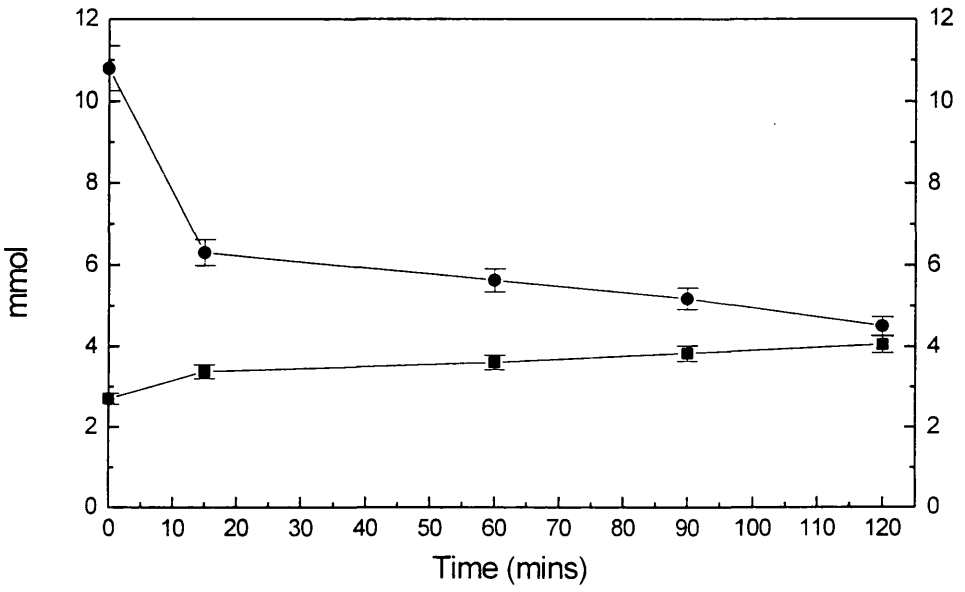


Figure 6.3 Change in concentration of each of the two ketone enantiomers during the racemisation procedure, where ■ represents the R-enantiomer and ● represents the S-enantiomer of 2-methylcyclohexanone.

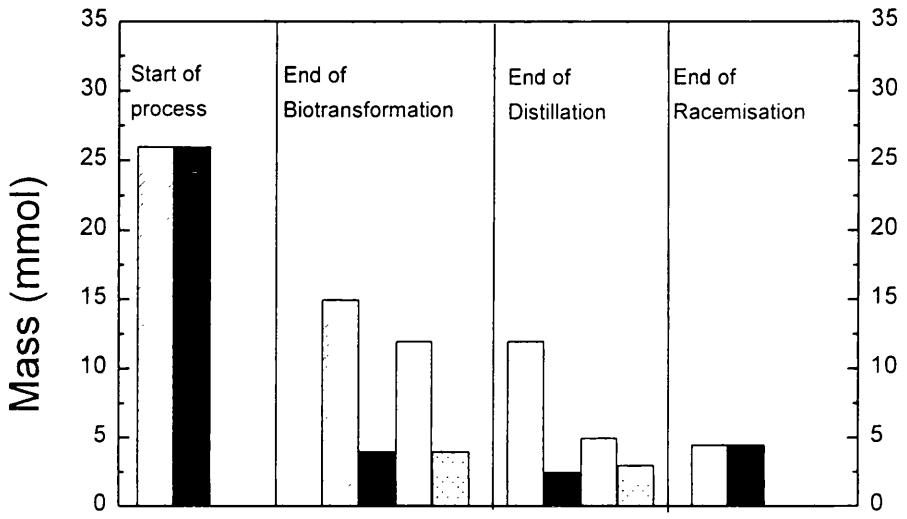


Figure 6.4 Schematic demonstrating cycle 1 of the linked process, using reactor configuration 1. Where ▨ represents ketone enantiomer R, ■ represents ketone enantiomer S, ▩ represents lactone enantiomer R and □ represents lactone enantiomer S.

6.4.2 Membrane Bioreaction Using NaOH as the Catalyst.

The data obtained from analysis of samples removed from the donating vessels during the integrated process in the membrane bioreactor using NaOH as the catalyst can be seen in Figure 6.5. The data obtained from the receiving vessel can be seen in Figures 6.6 - 6.8. Figure 6.6 displays the lactone and ketone concentration data, whilst Figure 6.7 indicates the concentration of each enantiomer of the ketone in the receiving vessel. The concentration of each enantiomer of the lactone in the receiving vessel can be seen in Figure 6.8.

The ketone was seen to start to accumulate in the receiving vessel before the addition of enzymes 3 hours after the start of the process (Figure 6.7).

The overall enantiomeric excess results from the lactone and the ketone during the process can be seen in Figure 6.9.

Figure 6.10 displays the concentration of lactone, ketone and the total concentration in the system as an indication of the mass balance of the system. The data in Figure 6.10 is displayed in mmol to allow a direct comparison of the data from the lactone and ketone. The process was calculated to have a mass balance of 85%.

Figure 6.11 compares the total concentration in the receiving vessel with the total concentration in the donating vessel and the total concentration in the system throughout the process.

The overall yield of lactone obtained from the membrane bioreactor using NaOH as the chemical catalyst for the racemisation procedure was 75%, whilst the enantiomeric excess of this lactone product was 60% (+/- 5%), as can be seen in Figures 6.10 and 6.9 . Comparison with known standards indicated that the S enantiomer of the lactone had been produced, as expected.

The remaining ketone in the receiving vessel was assigned absolute configuration R, with an enantiomeric excess of 3% , and a yield of 7%,as can be seen in Figures 6.9 and 6.10 respectively. The donating vessel contained 4% ketone (w.r.t. the starting concentration) with an e.e. of 7%, the absolute configuration of this ketone was also R, as can be seen in Figures 6.11 and 6.5.

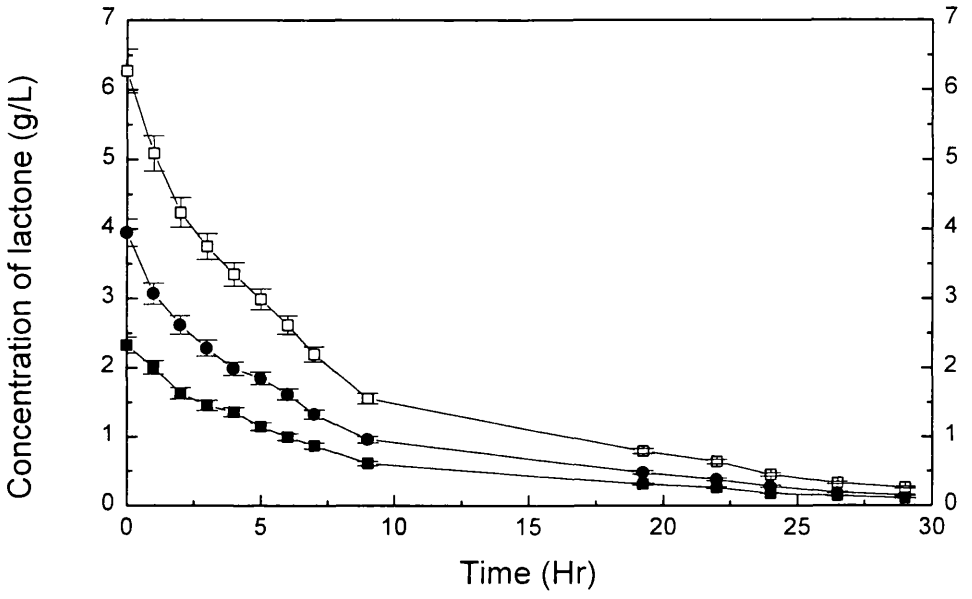


Figure 6.5 Donating vessel during integrated process, using the membrane bioreactor and NaOH as the base catalyst. Where □ represents the total ketone, ● represents ketone enantiomer R and ■ represents ketone enantiomer S.

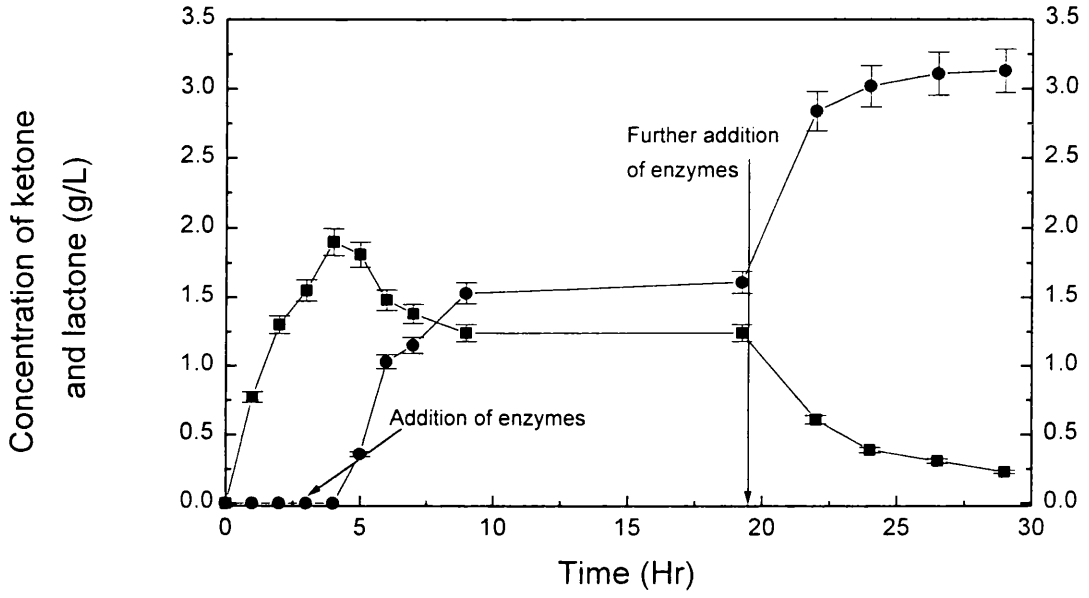


Figure 6.6. Change in total ketone and total lactone concentration in the receiving vessel during the integrated process, where ■ represents the ketone and ● represents the lactone.

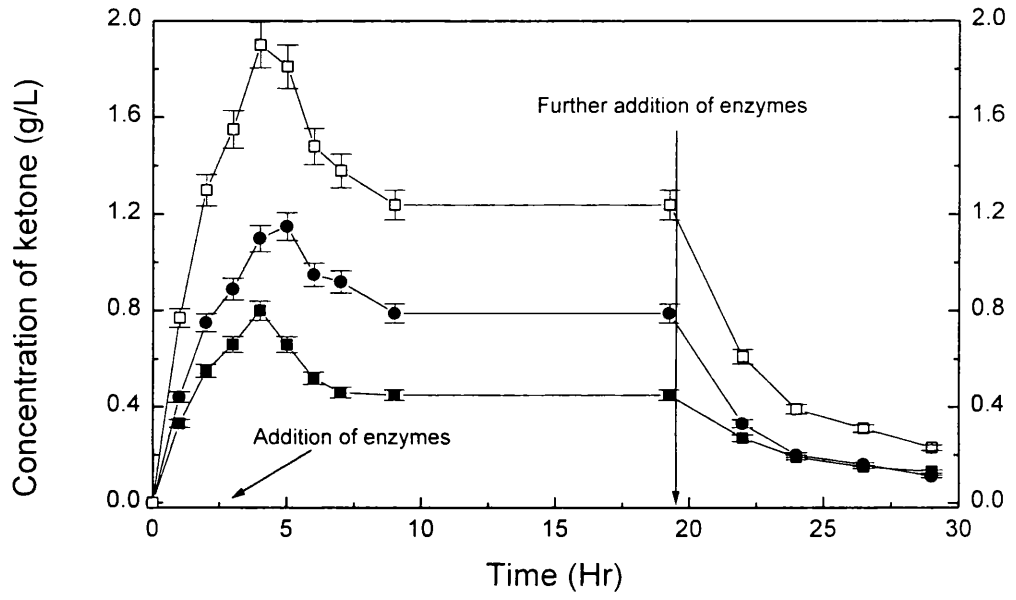


Figure 6.7. Receiving vessel during integrated process where □ represents the total ketone, ● represents ketone enantiomer R and ■ represents ketone enantiomer S.

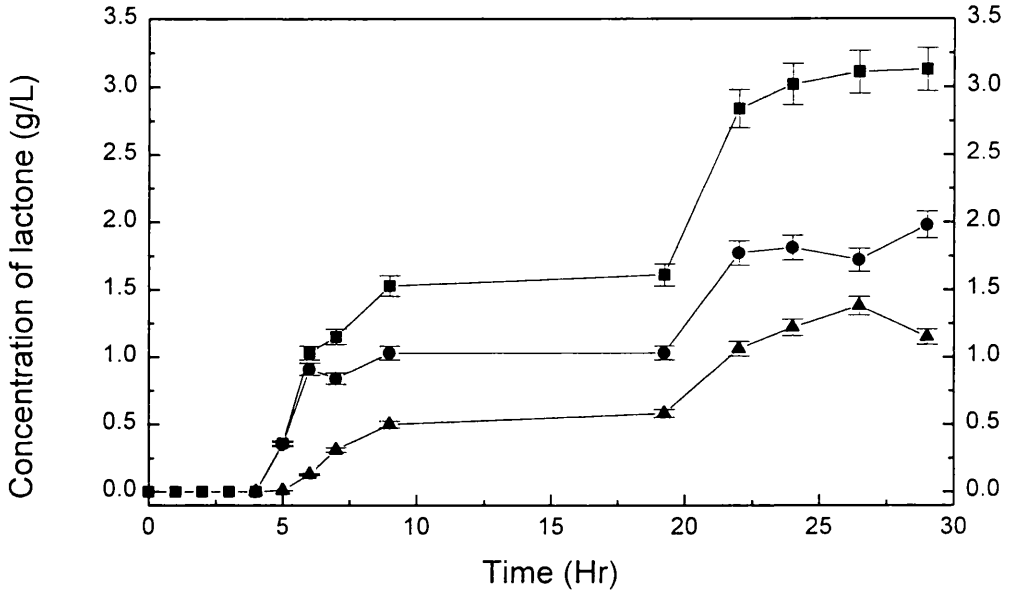


Figure 6.8 Lactone data obtained from the membrane bioreactor receiving vessel during the integrated process. Where ■ represents the total lactone in the vessel, ▲ represents lactone enantiomer R in the vessel and ● represents lactone enantiomer S in the receiving vessel.

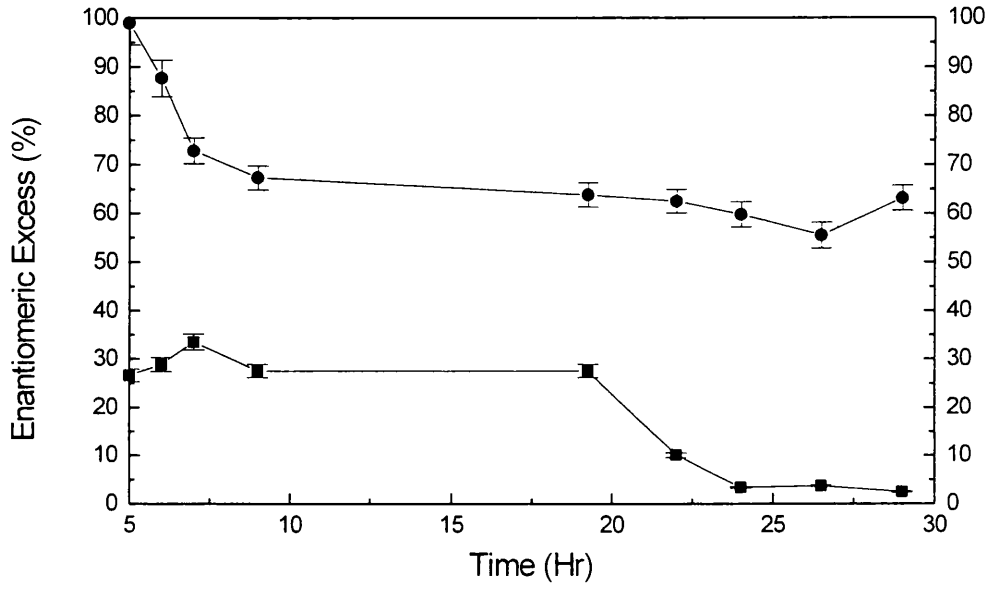


Figure 6.9 The enantiomeric excess of the ketone and lactone within the receiving vessel of the membrane bioreactor, where ■ represents the ketone and ● represents the lactone.

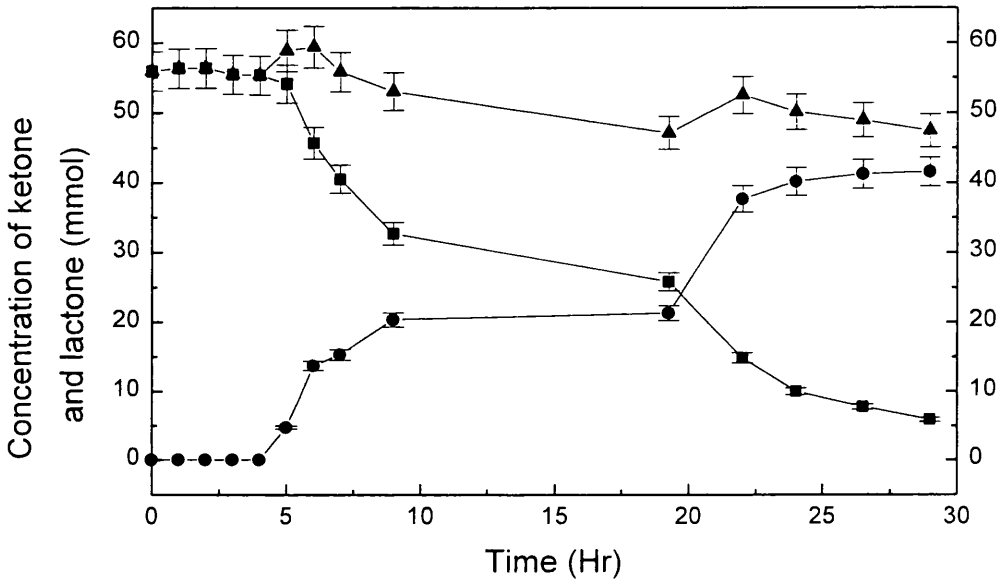


Figure 6.10 Total concentration within the integrated system with NaOH as the catalyst as an indication of the mass balance of the system. Where ■ represents ketone, ● represents lactone and ▲ represents combined ketone and lactone data.

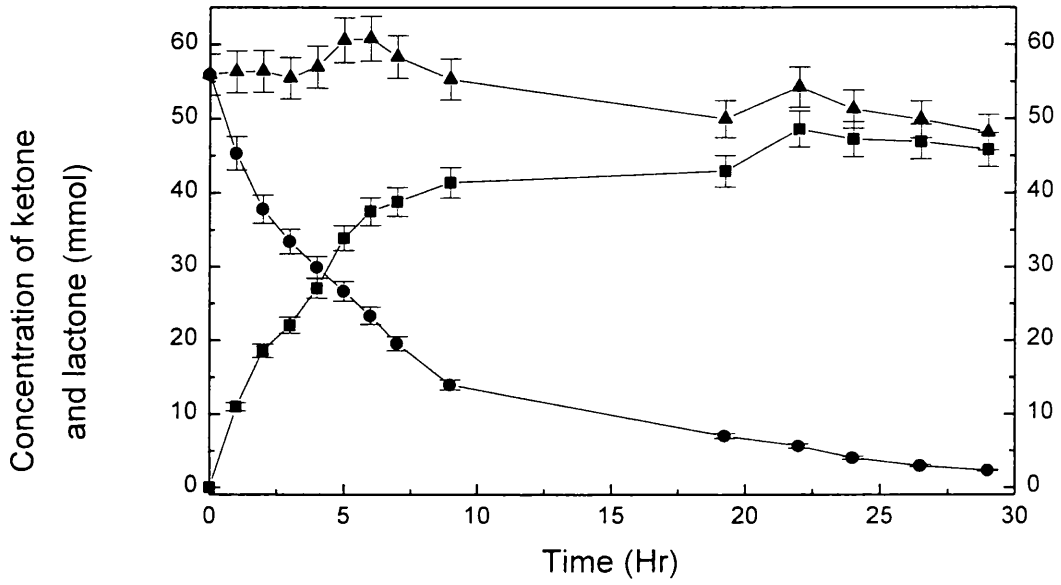


Figure 6.11 Total concentration of lactone and ketone within the integrated system with NaOH as the catalyst. Where ● represents donating vessel , ■ represents receiving vessel and ▲ represents combined donating and receiving vessel data.

6.4.3 Membrane Bioreactor using Amberlite IRA 400 (-OH) as the catalyst.

The data obtained from analysis of samples removed from the donating vessels during the integrated process in the membrane bioreactor using Amberlite IRA 400 (-OH) as the catalyst can be seen in Figure 6.12. The data obtained from the receiving vessel can be seen in Figures 6.13 - 6.15. Figure 6.13 displays the lactone and ketone concentration data, whilst Figure 6.14 indicates the concentration of each enantiomer of the ketone in the receiving vessel. The concentration of each enantiomer of the lactone in the receiving vessel can be seen in Figure 6.15.

The overall enantiomeric excess results from the lactone and the ketone during the process can be seen in Figure 6.16.

Figure 6.17 displays the concentration of lactone, ketone and the total concentration in the system as an indication of the mass balance of the system. The data in Figure 6.17 is displayed in mmol to allow a direct comparison of the data from the lactone and ketone. The process was mass balanced and found toaccount for approximately 100% of the material used.

The overall yield of lactone obtained from the membrane bioreactor using Amberlite IRA 400 (-OH) as the chemical catalyst for the racemisation procedure was 73%, whilst the enantiomeric excess of this lactone product was 58% (+/- 5%), as can be seen in Figures 6.17 and 6.16 . Comparison with known standards indicated that the S-enantiomer of the lactone had been produced, as expected.

The remaining ketone in the receiving vessel was assigned absolute configuration R, with an enantiomeric excess of approximately 30% , and a yield of 23 %, as can be seen in Figure 6.14. The donating vessel contained

6% ketone (w.r.t. the starting concentration) at the end of the process with an e.e. of 29% (Figure 6.12). The absolute configuration of this ketone was also R.

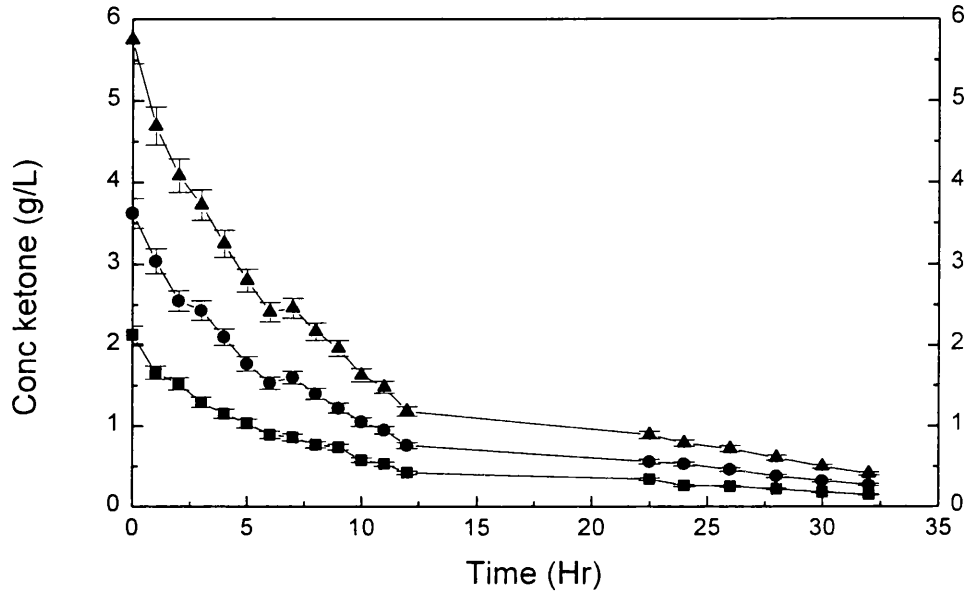


Figure 6.12 Data obtained from the donating vessel of the membrane bioreactor using Amberlite IRS 400 (-OH) as the catalyst. Where ▲ represents the total ketone, ● represents ketone enantiomer R and ■ represents ketone enantiomer S.

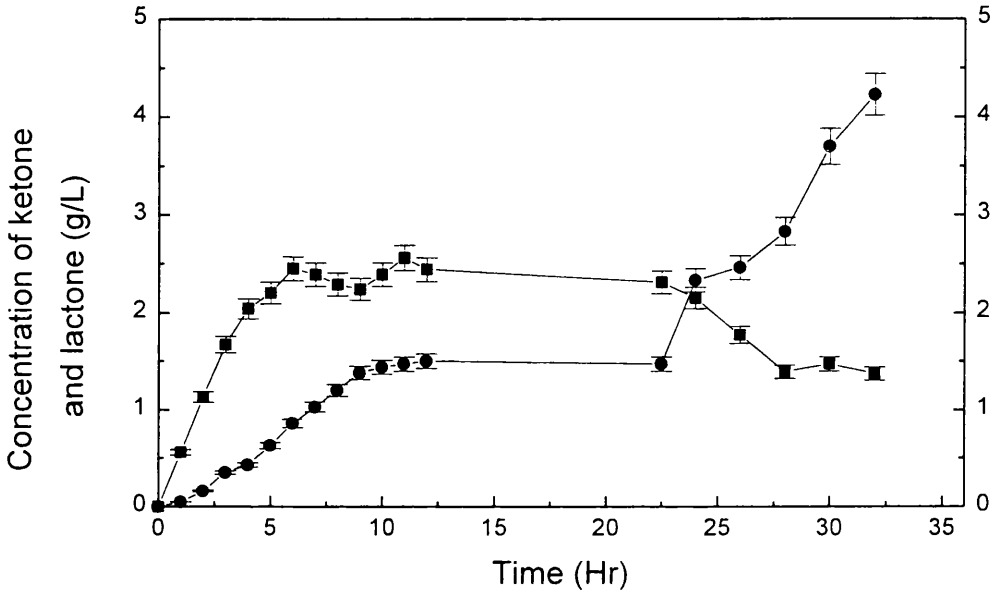


Figure 6.13 Change in concentration of ketone and lactone in the receiving vessel of the membrane bioreactor when Amberlite IRA 400 (-OH) is the catalyst. Where ■ represents the ketone and ● represents the lactone.

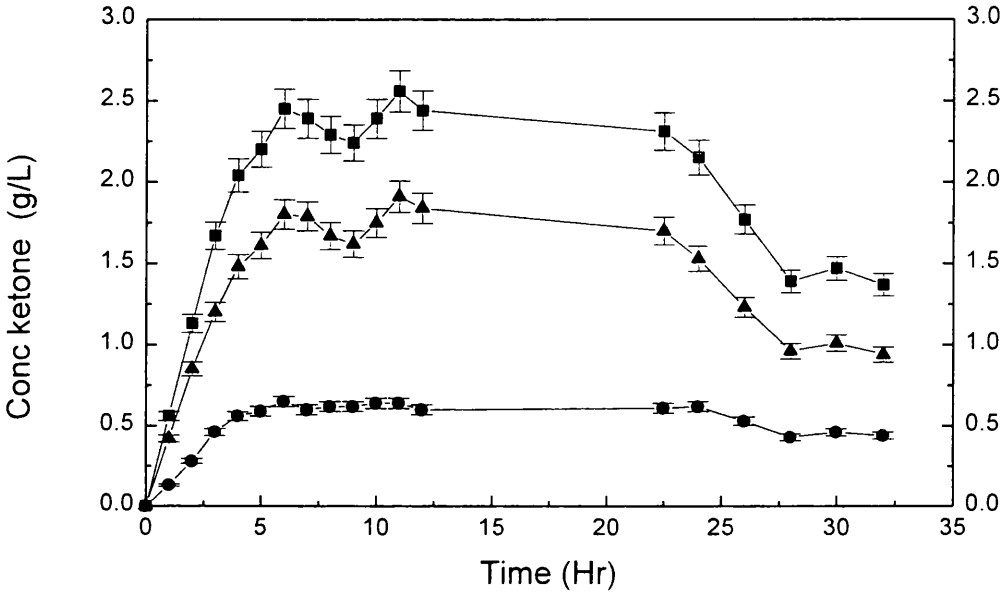


Figure 6.14 Ketone data obtained from the receiving vessel of the membrane bioreactor during the integrated process when Amberlite IRA 400 (-OH) was the catalyst. Where ■ represents the total ketone, ▲ represents ketone enantiomer R and ● represents ketone enantiomer S.

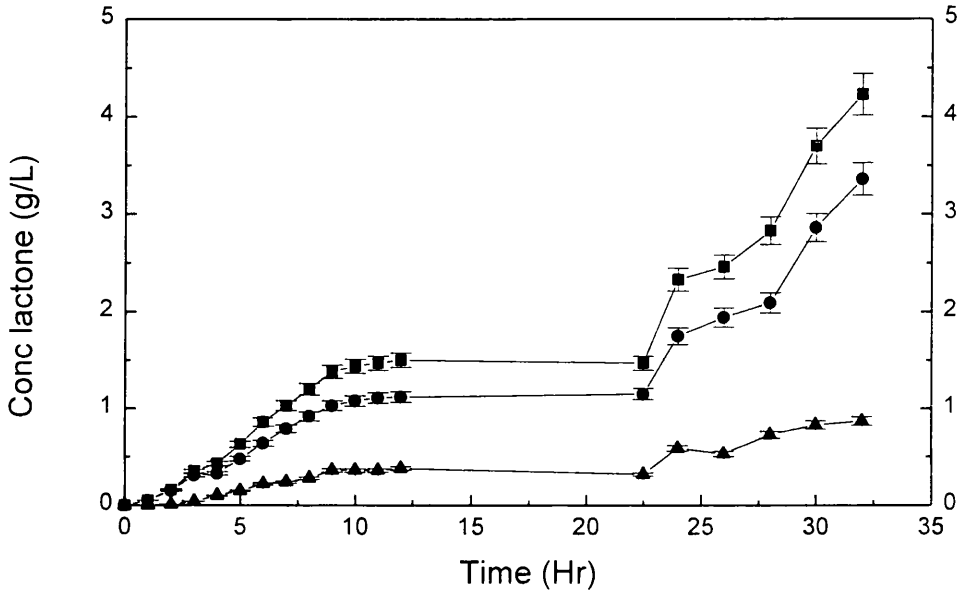


Figure 6.15 Lactone data obtained from the receiving vessel of the membrane bioreactor during the integrated process when Amberlite IRA 400 (-OH) was the catalyst. Where ■ represents the total lactone, ▲ represents lactone enantiomer R and ● represents lactone enantiomer S.

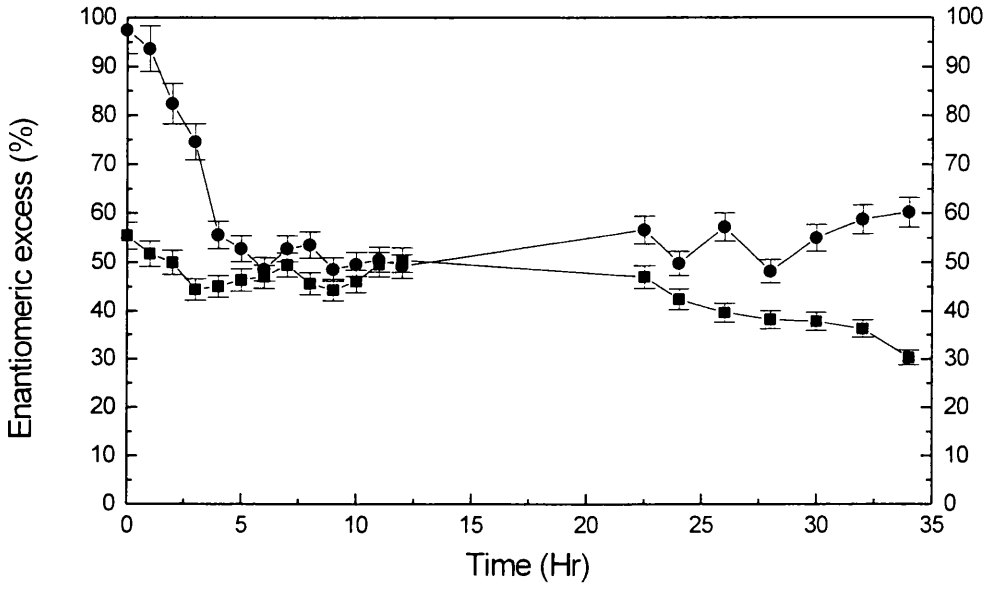


Figure 6.16 The enantiomer excess of the ketone (■) and lactone (●) in the membrane bioreactor with Amberlite IRA 400 (-OH) as the catalyst.

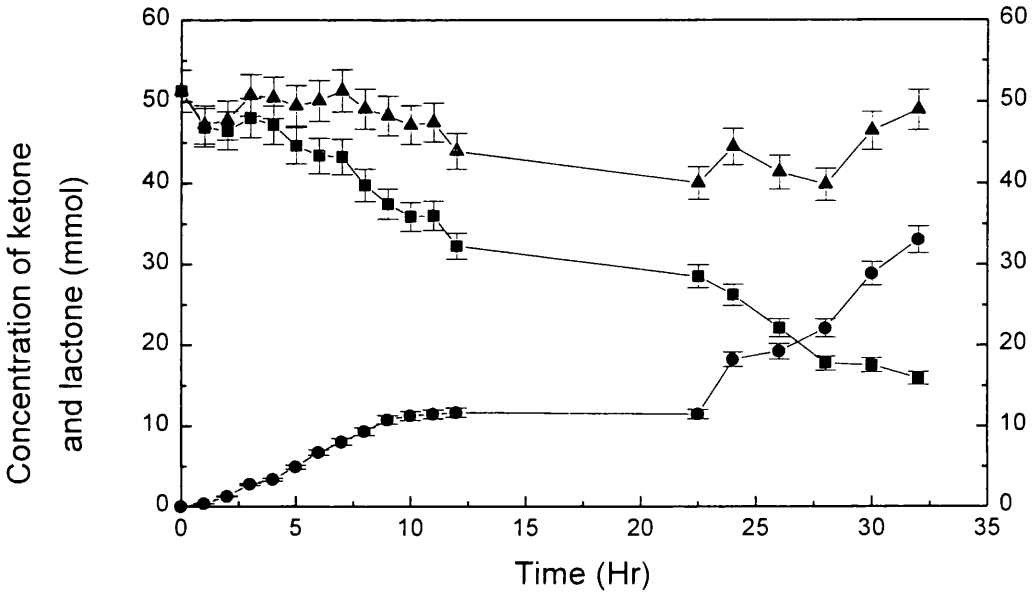


Figure 6.17 Total concentration within the membrane bioreactor with Amberlite IRA 400 (-OH) as the catalyst, as an indication of the mass balance of the system. Where ■ represents the ketone, ● represents the lactone and ▲ represents the combined ketone and lactone.

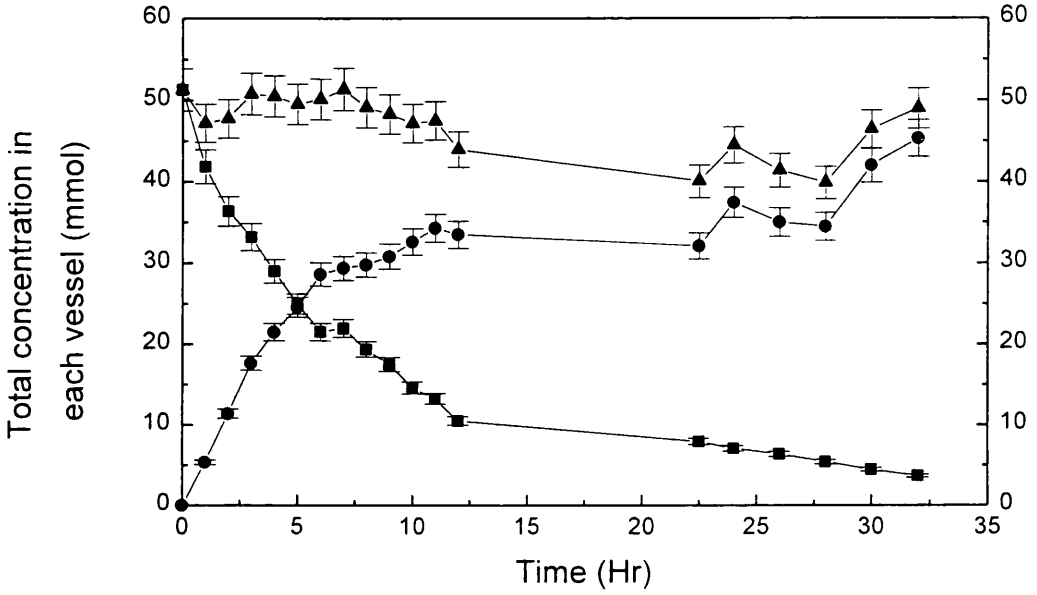


Figure 6.18 The total concentration of ketone and lactone in the membrane bioreactor with Amberlite IRA 400 (-OH) as the catalyst. Where represents the ■ donating vessel, ● represents the receiving vessel and ▲ represents the whole system.

6.5 DISCUSSION

6.5.1 LINKED REACTOR.

From the data obtained from one cycle of the linked reactor it was possible to say that the process was not successful in terms of lactone production and from a mass balance perspective. There was loss of ketone during every unit operation resulting in only 15% of the starting material being available for the second cycle.

One of the most unexpected outcomes of the procedure was the inability to account for all of the ketone during the bioconversion stage. This could have been caused by sampling/dilution error during the analysis of the samples removed during the process. It may also have been due to degradation or ring opening of the lactone.

A large amount of ketone was unrecovered from the IPA after the quenching of the bioconversion. It was not possible to remove the ketone from the IPA by fractional distillation. This was never going to be the optimal process to remove the lactone from the bioconversion media, but the recovery of lactone from the media and ketone from the IPA was more disappointing than expected. Had time allowed this process could have been repeated, omitting the distillation process to determine the overall success of the procedure. In this instance the lactone produced would be rapidly degraded in the racemisation procedure, but the total ketone remaining after the bioconversion could have been racemised and then entered into the next cycle.

The main advantage of this process was the ease of process monitoring and sampling. As the procedure was run as separate unit operations these were

performed during working hours. This allowed samples to be removed at regular intervals and provided data without 'gaps' missing.

6.5.2 Membrane Bioreactor using NaOH as the catalyst.

In this process the enzymes and co-factors were added three hours after the process was started to allow an amount of ketone to build up in the receiving vessel ready for bioconversion.

The process produced lactones in a yield of approximately 75%, with an enantiomeric excess of 60%, which was considered to be good when using 2-methylcyclohexanone as the racemic ketone. The overall procedure had a mass balance of approximately 85%.

One of the major problems with the process was that it took nearly 30 hours to perform. This caused the expected problems in terms of the observed decrease in enzyme activity. Enzymes and cofactor (CHMO, GDH and NADP+) were added to the receiving vessel 16 hours after the initial addition of enzymes to rectify this problem, as the bioconversion had seemed to stop. The DOT was observed to drop back to zero after the addition of the enzymes, indicating that the reaction was once again oxygen limited and the biotransformation process was occurring. A further addition of enzymes was required at 19 hours, again the rate of bioconversion increased after the addition of the enzymes.

6.5.3 Membrane Bioreactor using Amberlite IRA 400 (-OH) as the catalyst.

In this process the enzymes and co-factors were added to the receiving vessel at the start of the procedure.

The process produced lactones in a yield of approximately 73%, with an enantiomeric excess of 58%. The overall procedure had a mass balance of approximately 100%.

Again, as with the process using NaOH as the catalyst in the membrane bioreactor, the process using Amberlite IRA 400 (-OH) as the catalyst took a long time (35 hours) to perform. Enzymes and cofactor (CHMO, GDH, NADP+) were added to the receiving vessel 22 and 27 hours after the start of the process. After the addition of enzymes at 22 and 27 hours the bioconversion resumed, as observed when using NaOH as the catalyst.

A major disadvantage when using the membrane bioreactor and either chemical catalyst is the time to perform the process, but not just in terms of further additions of enzymes and co-factors. The long running time of the process meant that it wasn't possible for one person to be present throughout the procedure, and therefore there were 'gaps' in the data obtained. Luckily these gaps seem to be as expected and no surprises were observed. It is possible that the addition of further enzymes and co-factor should have been performed during the gaps, which would have decreased the overall time for the process. This was a problem that could not easily be overcome by one researcher.

7. GENERAL DISCUSSION.

7.1 WINDOW OF OPERATION.

The main constraint on the process design of the procedure was the inability to operate with all components in one vessel. As discussed in Chapter 5, this was due to the need of the two unit operations to be performed at different pHs. In an ideal situation the pH of the major processes would be compatible and the process would be performed in one pot. All the components of the racemisation and bioconversion would be added to the one vessel and the produce would be removed by a simple process at the end.

For this scenario to be possible, the pH of one of the unit operations must be modified so it will be compatible with the pH of the other process. The racemisation can be base catalysed, or acid catalysed. The pH of the bioconversion, with the given enzymes (CHMO and GDH) must be approximately pH 7 -8. The only way that the racemisation could be modified to occur at pH 7-8 is by utilising a heterogeneous catalyst that does not effect the pH of its external environment. This was the main objective of the catalyst screening detailed in Chapter 3. Over 40 catalysts were investigated initially for their ability to perform the racemisation. Of the catalysts studied, only Amberlite IRA 400 (-OH) and NaOH were able to catalyse the desired reaction. They both had a profound effect on the pH of their external environment. The modification of the racemisation procedure does not seem to be possible without the discovery of a catalyst that will not change the pH of its external environment. This was not possible within the time constraints of this research. Further studies into this area may possibly identify such a catalyst.

The alternative to changing the pH at which the racemisation process could occur would be to modify the pH at which the bioconversion would occur. In the

process described in Chapters 5 and 6 the bioconversion occurs at pH 7.5 with a combination of CHMO and GDH for the enzyme recycle system.

To modify the bioconversion process there are 3 possibilities;
1) Change the co-enzyme
2) Change the substrate
3) Modify the enzymes in the existing recycle system.

In the given process, with 2-methylcyclohexanone the most suitable enzyme for the recycle system is GDH. The optimum pH for GDH is pH 7. Whilst the optimum pH for ADH is pH 8. The optimum pH for CHMO is approximately pH 9. A combination of CHMO and ADH would, on paper seem to be a better combination for this system than CHMO and GDH. Unfortunately the CHMO/ADH system does not result in the production of 7-methyoxepan-2-one as required. A unknown compound, thought to be 2-methylcyclohexanol, was produced. It was not possible to identify this unknown with GC-MS due to the modification of the GC conditions and the low concentration of the reaction components.

A modification of the existing enzyme recycle system to allow the process to occur at pH values above 10, with 2-methylcyclohexanone as the substrate, could only be possible with modification to the enzymes within the system. Protein engineering may be possible to modify the enzymes so that they are not denatured at pH 10 or above.

Overall a modification to either process is likely to be time consuming. Although it may enable a one vessel process to be performed, which from a process view is desirable, it would present the researcher with a further major problem. The lactone product of the integrated process degrades rapidly in acidic or basic conditions and results in significant degradation at neutral pH, in aqueous conditions within 24 hours. A selective *in-situ* product removal (ISPR) step

would have to be designed and included into one vessel bioconversion and racemisation process. Although there has been a significant amount of research into ISPR it would seem that the development of such a process, to remove only the lactone product from the reaction vessel, would prove difficult. The main difficulty is likely to be the development of a procedure that is selective only towards the lactone, and not the ketone substrate that is very similar in size and structure.

There are no simplistic steps to be taken to allow the overall process to be performed as a one vessel procedure, without the use of a selective barrier. There are however many varied options that could be explored by other researchers within the scope of process optimisation.

7.2 STEREOSELECTIVITY OF THE BIOCONVERSION OF 2-METHYLCYCLOHEXANONE TO 7-METHYLOXEPAN-2-ONE BY CHMO.

There were three main requirements for the model substrate at the beginning of this research;

- The model substrate for this research had to be a cyclic ketone to enable the bioconversion to a lactone to be performed,
- The ketone had to be substituted, but not symmetrical or asymmetric to allow a stereoselective bioconversion to be performed,
- The ketone had to be 2-subsituted to allow the racemisation to be performed.

The selection of 2-methyl cyclohexanone was due mainly to the inability to purchase other 2-substituted cyclohexanones or 2-substituted cyclopentanones in sufficient quantity and at a low enough price to allow the research detailed within the thesis to be performed. One of the major problems encounter within this research was the lack of commercially available 2-substituted cyclic ketones. Other researchers (often organic chemists) have overcome this

difficulty by synthesising the compounds required. As the major aim of this research was to perform the integrated process on a preparative scale it was not viable to synthesis the ketone to be used as the substrate in the bioconversion. It was necessary to select a substrate that was commercially available. This limited the choice of ketone available to be considered as a model substrate for the bioconversion.

The ketones that were commercially available and economically viable had to be suitable for chromatographic separation of their two enantiomers within a suitable time period. On this basis 2-methylcyclohexanone was selected as the model substrate for the integrated bioconversion and racemisation.

2-methylcyclohexanone was known not to be transformed by CHMO as stereoselectively as many other 2-substituted cyclohexanones. Alphand and coworkers (1996) report bioconversions of seven 2-substituted cyclohexanones by CHMO from *Acinetobacter* TD63, which does not contain the lactone hydrolase enzyme that metabolises the lactone as soon as it is formed. Of these seven, only 2-ethylcyclohexanone is reported to produce lactone with a lower enantiomeric excess than 2-methylcyclohexanone, 38% e.e. with the 2-ethylcyclohexanone compared to 61% e.e. with the 2-methylcyclohexanone. The enantiomeric ratio (E) of the 2-ethylcyclohexanone conversion was greater than that of 2-methylcyclohexanone (58 for the 2-ethylcyclohexanone compared with 6 for the 2-methylcyclohexanone). These bioconversions took approximately 5 hours to complete. A trend can be seen between the length of the side chain and the enantiomeric excess of the lactone product, with the exception of 2-ethylcyclohexanone (38% e.e.) and 2-nonylcyclohexanone (85% e.e.). As a rule, as the length of the side chain increases so does the enantiomeric excess of the lactone product.

Alphand and coworkers (1996) also detail results for the bioconversion of the same substrates by MO2 from *P. putida* NCIMB 10007, with the reactions in

this case taking 1 hour to complete. In this case both the enantiomeric excess and enantiomeric ratio for 2-ethylcyclohexanone (25% e.e. and 2) were lower than for 2-methylcyclohexanone (38% e.e. and 4). Interestingly the yield of lactone produced from 2-ethylcyclohexanone (60%) in the case of the bioconversion by CHMO is higher that the yield produced from any of the other six substrates, with the yield for the conversion of 2-methylcyclohexanone being 35%.

Kayser and coworkers (1998) report the bioconversion of 2-substituted cyclopentanones by engineered Bakers' Yeast, again as the size of the alkyl side chain increases so does the enantiomeric excess of the lactone produced. In the case of n-butyl, n-hexane and n-octane the e.e. of the lactone product is over 98%. It would seem that an increase in steric hindrance leads to a high % e.e. of lactone product. For 2-methylcyclohexanone the side chain is small, so the steric hindrance is limited, leading to a lower enantioselectivity of the bioconversion. In the conversion of 3-subsititued cyclopentanones by engineered Bakers' Yeast the size of the alkyl side chain did not appear to affect the enantiomeric excess of the lactones produced (Kayser *et al.*, 1998). Kayser and coworkers conclude that the high enantioselectivities observed must result from the favourable interactions between long side chains and the active site of the enzyme. The group also add that a similar effect is observed with cyclohexanones.

2-methylcyclohexanone was therefore not the best 2-substituted cyclohexanone to produce a lactone with high enantiomeric excess. In this case the ketone was used as a model substrate to investigate the ability to integrate a bioconversion and racemisation. Future work could include using a long chain 2-alkyl substituted cyclic ketone to demonstrate the ability of the integrated process to produce lactones with high enantiomeric excess and high yield.

7.3 REACTOR DESIGN

As can be seen from the results presented in Chapter 6 the reactor design can play a vitally important role in the outcome of the process.

Reactor design 1 (the linked reactor), where all processes were performed as individual unit operations was not successful. It resulted in a lactone yield of 29% with an e.e. of 43%. This process offered no advantage over the traditional chemical route or the bioconversion procedure to produce a single enantiomer lactone. The process was more time-consuming and less economically viable than the two alternative routes (bioconversion and traditional chemical oxidation). As detailed in Chapter 3 the bioconversion of 2-methylcyclohexanone to 7-methyloxepan-2-one by CHMO takes approximately 5 hours to produce lactone with an e.e. of 55% and a yield of 32%. The chemical oxidation of 2-methylcyclohexanone by MCPBA takes approximately 24 hours to produce lactone with an e.e. of 2 % and a yield of 73% after purification.

Reactor design 2. The results obtained using the heterogeneous and homogeneous catalyst in the membrane bioreactor had a significant increase in yield to those quoted in the literature using the biotransformation route. The results obtained using the membrane bioreactor had a comparable yield to that gained from the chemical route (73% chemical route, 74% membrane bireactor with NaOH as the catalyst and 73% membrane bioreactor with Amberlite IRA 400 (-OH) as the catalyst). The enantiomeric excess of the lactone produced using the membrane bioreactor (60% e.e. with NaOH as the catalyst and 58% e.e. with Amberlite IRA 400 (-OH) as the catalyst) were significantly higher than the e.e. obtained from the chemical route (2% e.e.).

The major improvement of the membrane bioreactor over the linked reactor was the ability to perform the integrated process in what was essentially

'one- pot'. The yield obtained from the linked process was so low due to the number of individual steps that were performed. There were significant losses of ketone and lactone during each stage. There was no obvious possible way to perform the racemisation procedure without removing the lactone produced by the bioconversion. Any remaining lactone within the reaction solution would have been completely degraded by the conditions in the racemisation vessel.

The major advantage to the process that was performed in the membrane bioreactor was the ability to keep the lactone product and the enzyme away from the catalyst for the racemisation procedure. This enabled the lactone product to increase in concentration without being degraded by the high pH required for the racemisation.

7.4 LINKED PROCESS COMPARED TO BIOTRANSFORMATION AND TRADITIONAL CHEMICAL PROCEDURE.

At this time, the linked process performed using reactor configuration 1 cannot compete with the traditional chemical procedure or the biotransformation process to produce an enantiomerically pure (or enriched) lactone from a racemic cyclic ketone.

There are numerous ways in which this process could be performed in the future with similar or improved results to the chemical or biotransformation process. The first modification that must be performed is the incorporation of an efficient *in-situ* product removal (ISPR) step, to remove the lactone product. The other modifications involve the development of a method to immobilise the enzyme so that it could be retained in one part of the reactor, whilst the racemisation was performed in an adjacent part of the reactor. The production of an enzyme (or whole cell catalyst) that could withstand the high pH required for the racemisation procedure could enable the racemisation and

bioconversion procedures to be performed in the same vessel. In this situation the ISPR step would be required to be extremely efficient as any lactone produced would be rapidly degraded by the high pH of the solution.

7.5 INTEGRATED PROCESS COMPARED TO BIOTRANSFROMATION AND TRADITIONAL CHEMICAL PROCESS.

The integrated process performed in the membrane bioreactor offers an improvement in terms of lactone yield and enantiomeric excess compared to the established routes to enantiomerically pure (or enriched) lactone from a racemic cyclic ketone. The integrated process also gives a decrease in process hazards compared to the chemical oxidation as the temperature, pH and components of the bioconversion are less much less hazardous than those used in the traditional chemical route. The chemical oxidation commonly uses MCPBA (metachloroperbenzoic acid) as an oxidising agent. The large-scale use of MCPBA should be avoided, if possible, due to its hazardous nature.

The enantiomeric excess obtained for the bioconversion of 2-methylcyclohexanone to 7-methyloxepan-2-one was approximately 60%, whilst the yield was over 70% (over 73% when using Amberlite IRA 400 (-OH) as the catalyst and 74% when using NaOH as the catalyst). This is a significant improvement over the e.e. and yield reported for the bioconversion of 2-methylcyclohexanone by Acinetobacter TD63 (61% e.e., 35% yield) and MO2 (38% e.e., 50% yield) (Alphand *et al.,* 1996.) The enantiomeric excess obtained for the enantioselective oxidation of 2-methylcyclohexanone using CHMO is expected to be lower than the e.e. obtained for the same procedure but using a 2-substituted cyclohexanone with a longer side chain.

7.6 SCALE UP.

The process involving the membrane bioreactor was performed on a 1.5L scale. This scale is larger than the scale at which the major of the enzymatic CHMO oxidation of cyclic ketones have been performed. To scale-up the process to the next level there are two major issues to be considered:

- The size of the membrane module. In this case we used coiled silicone tubing, inside a large conical flask. To increase the scale of the reaction the area of membrane must be scaled accordingly, so the rate of mass transfer of the ketone through membrane does not become rate limiting.

- The production of the large volume of enzyme required for the large scale integrated process using the membrane bioreactor. During the 1.5L scale process using Amberlite IRA 400 (-OH) as the catalyst, approximately 1500U of CHMO were added to the vessel to convert 6.7g of ketone. A larger scale process would require more enzyme. The production of sufficient enzyme to enable the process to be performed on a larger scale could make the process economically unviable.

Immobilisation of the enzyme onto a solid support is often performed for use within a bioreactor during a large scale process. The immobilised enzymes tend to become stabilised by the immobilisation procedure and retain their activity for longer than semi-purified free enzyme. They can therefore be used for longer. Development of a suitable method for the immobilisation of CHMO and GDH onto a solid support is likely to be time-consuming, but could result in supported enzymes with good retained activity. The use of immobilised enzymes with good retained activity could make the integrated process an economically attractive option for the production of optically pure lactones.

From a scientific perspective the change from a traditional chemical oxidation (that could only produce a maximum of 50% yield) to an integrated process (that could product towards a 100% yield of an enantiomerically pure lactone from a racemic starting material) would be automatic.

Industrially, the change from a chemical Baeyer-Villiger oxidation to an integrated process would be difficult. The difficulties are due to the regulatory requirements of validation. If a validated process is to be modified (in any way) the regulatory bodies (such as the FDA and MCA) require the industrial company to perform what is called a change validation. This results in the company having to convince the regulatory bodies that the change in the manufacturing process does not affect the final product in anyway. I do not feel, at this time, that the majority of industrial companies would implement a change in an established process to include a bioconversion step.

7.7 FURTHER RESEARCH

1. Investigate the yield and enantiomeric excess of optically pure lactone from a number of 2-substituted cyclic ketones. Perform the integrated process using both Amberlite IRA 400 (-OH) and sodium hydroxide as the catalyst for the racemisation. Compare the results from the homogeneous and hetereogeneous catalyst together and against the results from bioconversions using the same substrates.

2. Investigate the effect of using a CHMO/ADH recycle during the bioconversion of 2-substituted cyclic ketones (other than 2-methylcyclohexanone) in the integrated process. Compare the yield and enantiomeric excess of the lactones produced to those produced in the same process using CHMO/GDH to determine the effect of the recycle enzyme on the process.

3. Investigate the link between the size of the side chain and the enantiomeric excess of the lactone produce. Determine if the link is effected by rate of bioconversion.

4. Investigate the feasibility of scale-up of the integrated process. Determine the effect of scale-up on the membrane module of the integrated process.

5. Immobilise CHMO and GDH onto a solid support. Determine the effect of immobilised enzymes on the integrated process, can the process be performed without the need of addition of further enzymes? Determine how long the immobilised enzymes can be used in the integrated process without a significant loss of activity.

6. Investigate an *in-situ* product removal step (ISPR) to facilitate the removal of the lactone product from the bioreactor during the integrated process. This would stop lactone degradation during the process.

7. Investigate the feasibility of protein engineering to improve the integrated process. Determine the effect of a recombinant protein containing the two recycle enzymes (CHMO/GDH or CHMO/ADH) on the integrated process. This would allow for a whole cell catalyst to be used and eradicate the need for the addition of NADPH to the bioconversion, making the process less expensive.

8. Investigate the feasibility of utilising FDH in the co-factor recycle instead of ADH or GDH. This would ensure that the product of the FDH part of the recycle was carbon dioxide. The CO_2 could be expelled from the system, thus shifting the equilibrium and ensuring the recycle of NAD would not be the rate limiting stage of the recycle system. As mentioned in the introduction to this thesis FDH is NAD(H) dependent which is less expensive than NADPH. (Seelbach *et al.*, 1996).

8. CONCLUSIONS

1. The overlap in pH profile of CHMO and GDH is between pH 7.5 and pH 8.0. A CHMO/GDH recycle system using NADP+ as the cofactor can be performed effectively between pH 7.5 and 8.0.

2. The CHMO/GDH recycle system involves an activity compromise for CHMO and GDH. The maximum CHMO activity occurs at pH 9.5. At pH 7.5 the activity of CHMO is approximately 30% of its maximum. The activity of GDH at pH 7.5 is approximately 70% of its maximum.
3. The bioconversion of 2-methylcyclohexanone to 7-methyloxepan-2-one using a CHMO/GDH recycle must be performed at neutral pH, pH 7.5 offers the best compromise in terms of enzyme activity.

4. The racemisation can be either acid or base catalysed, but cannot be performed at neutral pH.

5. The conditions required for the bioconversion were not compatible with the conditions required for the racemisation. At the pH required for the racemisation the enzymes would not be active and lactone degradation would occur. The catalysts used for the racemisation (Amberlite IRA 400 (-OH) and NaOH) would increase the pH from 7.5 to approximately pH 12 within 30 minutes.

6. The yield and enantiomeric excess of lactone produced in the linked reaction (29%, 43% e.e.) does not offer any improvement on the yield and e.e. of lactone produced by the chemical (73%, 2% e.e.) or bioconversion route (32%, 55% e.e.). The linked process is not suitable to produce optically pure lactones at this time.

7. The yield and enantiomeric excess of the lactone produced during the integrated process using either of the catalysts was greater than the yield and the e.e. gained from the biotransformation. The yield and enantiomeric excess observed using NaOH as the catalyst was 74%, 60% e.e. (+/- 5%), using Amberlite IRA 400 (-OH) was 73%, 58% e.e (+/- 5%). The yield and e.e. from the bioconversion route was 32% and 55% e.e.

8. The enantiomeric excess of the lactone produced during the linked process and integrated process is dependent on the enantiomeric excess during the bioconversion procedure, which appears to be linked to the size of the ketone side chain.
9. The e.e. for the lactones obtained during the biotransformation route and the integrated process are comparable (biotransformation 55%, integrated process ~ 60%). These e.e's. are also comparable with results obtained by other researchers. Alphand and coworkers reported enantiomeric excess of 61% for 7-methyloxepan-2-one (1996).

10. The rate of mass transfer of the ketone across the membrane in the integrated process was not the rate limiting step, as the concentration of ketone in the receiving vessel was observed to slowly increase throughout the integrated process regardless of the catalyst (NaOH or Amberlite IRA 400 (-OH)).

11. The mass balance obtained using either catalyst in the membrane bioreactor was good, 100% with Amberlite IRA 400 (-OH) and 85% with NaOH.

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APPENDICES

APPENDIX A. CHEMICAL AND EQUIPMENT SUPPLIERS NAMES AND ADDRESSES.

BDH Laboratory Supplies
Merck Ltd,
Hunter Boulevard,
Magna Park,
Lutterworth,
Leicestershire,
LE17 4XN,
UK.

Beckmann Instruments,
2500 Harbour Boulevard,
Fullerton,
CA 92634-3100,
USA.

Fluka (See Sigma-Aldrich)

Fluorochem Ltd,
Wesley Street,
Old Glossop,
Derbyshire,
SK13 9RY,
UK.

Heraeus Instruments Ltd,
9 Wates way,
Brentwood,
Essex,
CM15 9TB,
UK.

Kontron Instruments,
Blackmoor Lane,
Coxley Business Park,
Watford,
WD1 8XG,
UK.

MACHEREY-NAGEL GmbH & Co.KG
Postfach 10 13 52
D-52313 Düren
Germany

MSE Ltd,
Crawley,
Sussex,
UK.

New Brunswick Scientific,
Edison,
New Jersey,
USA.

Perkin-Elmer Corporation,
761 Main Avenue,
Norwalk,
Ct 06859-0010,
USA.

Sigma-Aldrich-Fluka,
The Old Brickyard,
New Road,
Gillingham,
Dorset,
SP8 4BR
UK.

APPENDIX B. SPECTROSCOPIC AND CHROMATOGRAPHIC STANDARDS.

Spectroscopic standards.

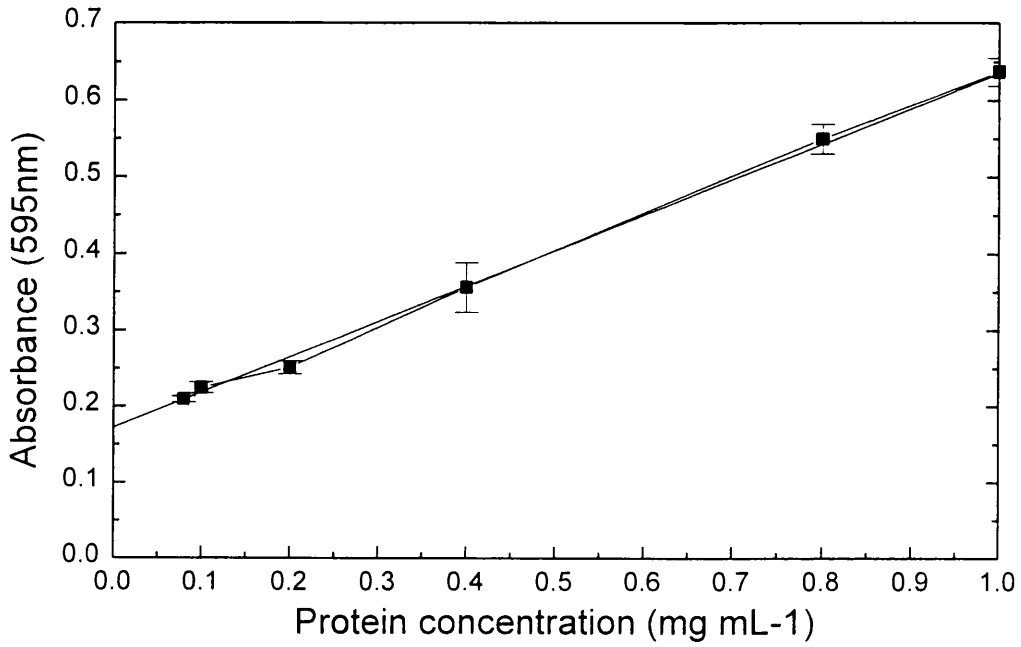

Figure A.1 Coomaise Blue Protein standard curve.

Chromatographic standards.

Chiral HPLC standards

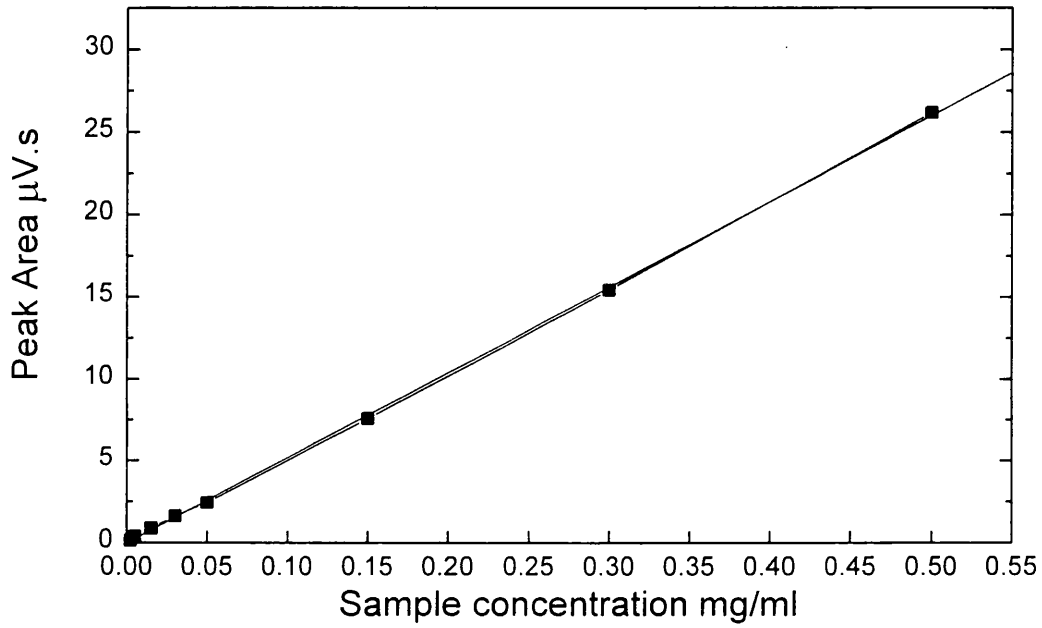

Figure A.2 Calibration curve and linear range for separation of the two enantiomers of 2-methylcyclohexanone by chiral HPLC.

Chiral GC standards.

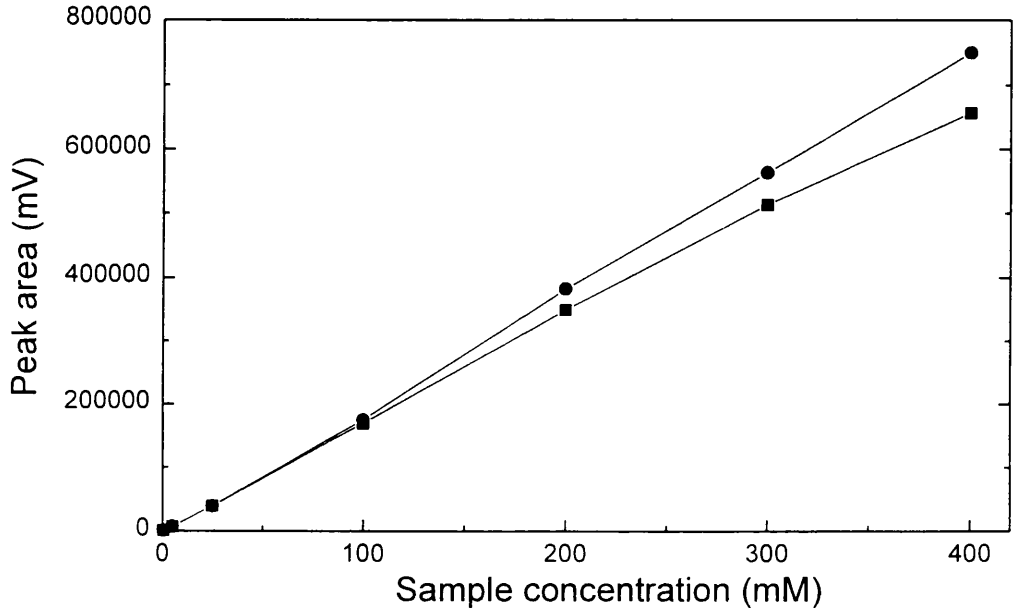

Figure A.3 Calibration curve and linear range for separation of the two enantiomers of 2-methylcyclohexanone by chiral GC where enantiomer 1 is represented by ■ and enantiomer two is represented by ●.

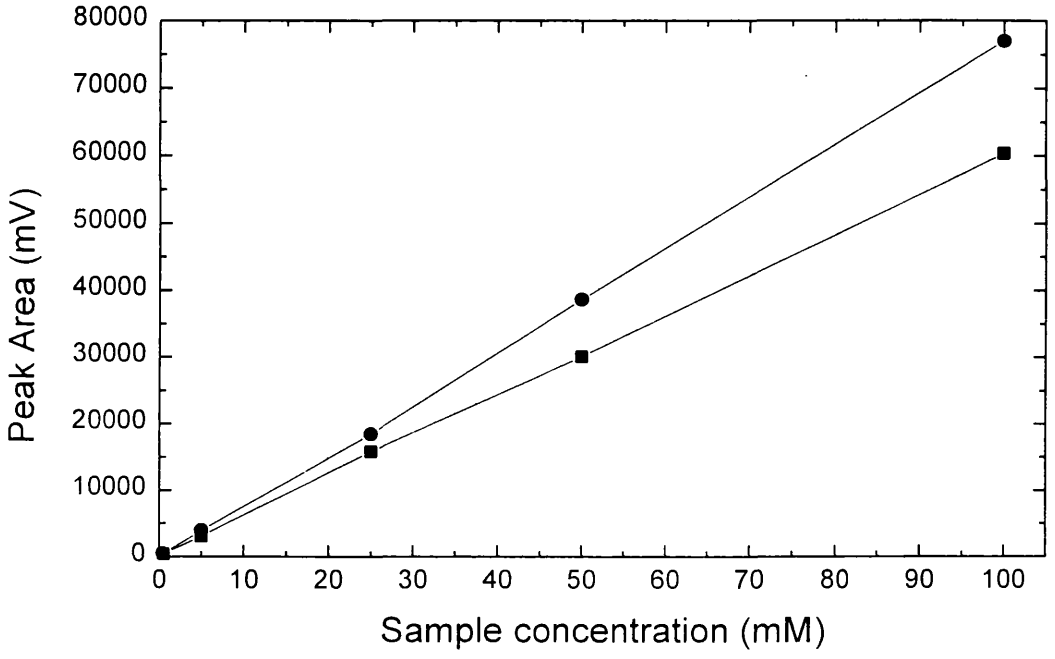

Figure A.4 Calibration curve and linear range for separation of the two enantiomers of 7-methyloxepan-2-one by chiral GC, where enantiomer 1 is represented by ■ and enantiomer two is represented by ●.

Correlation between Optical density measurements and dry cell weight.

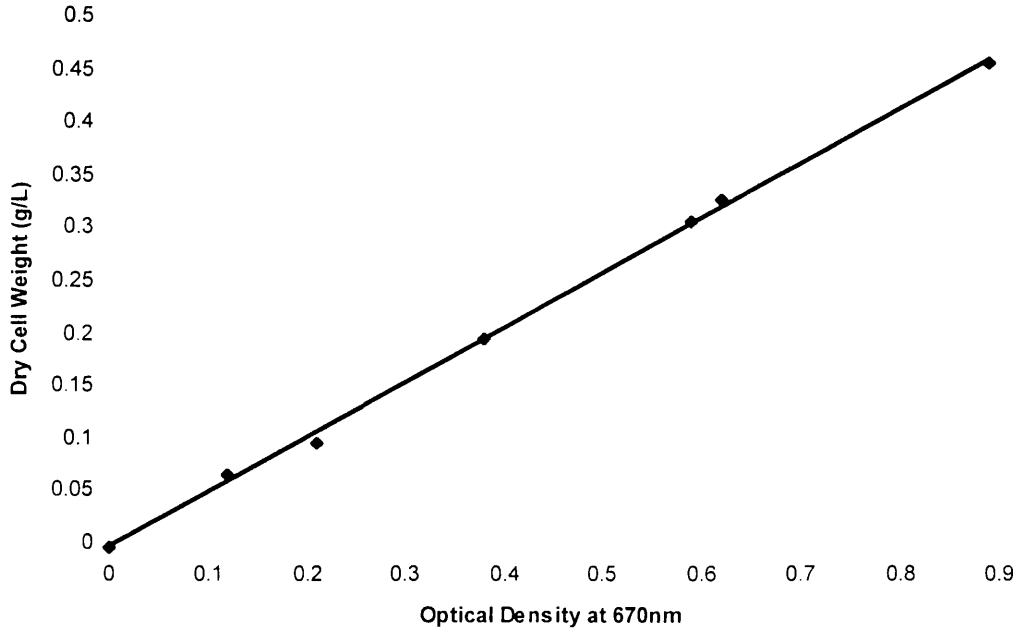

Figure A.5. Correlation between optical density and dry cell weight of *E. coli* fermentation broth.

APPENDIX C. SOLID CHEMICAL CATALYSTS INVESTIGATED.

Catalyst	Amount used (g/L)	Racemisation
Starlight C (0.14-0.25)mm	50	✗
Starlight C (0.5-1.0)mm	50	✗
Starlight C 250	50	✗
Starlight C 600	50	✗
Starlight K	50	✗
Starlight K	50	✗
Starlight K	50	✗
Starlight K prills	50	✗
Zeolite Na-Y	2.5	✗
Zeolite H-Y	2.5	✗
Aluminium oxide, activated base	50	✗
Amberlite IRA - 35	50	✗
Amberlite IRA - 68	50	✗
Amberlite IRA - 93	50	✗
Amberlite IRA - 400	50	✓
Amberlite IRA - 743	50	✗
Ambersorb 572	50	✗
Diaion SA 10A	50	✗
Diaion SA11A	50	✗
Diaion WK 100	50	✗
Dowex 1	50	✗
Dowex G-55	50	✗
Dowex MR-3	50	✗
Dowex SBR-C	50	✗
Duolite A-7	50	✗
Duolite A-368	50	✗
Ludox AM-30	50	✗
Ludox LS	50	✗
KF on silica gel (20% w/w)	250	✗
KF on calcium fluoride (20% w/w)	250	✗
KF on alumina (40% w/w)	125	✗
NaOH (pH 13.5)	N/A	✓