STUDIES ON THE ENANTIOSELECTIVITY OF BACTERIAL LACTONASES

A thesis submitted in accordance with the requirements of the Faculty of Natural Sciences, University of Kent at Canterbury for the Degree of Doctor of Philosophy (Ph.D)

by

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or institute of learning

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DEDICATION

This thesis is dedicated to my parents Mr John Olaolu Onakunle (late) and Mrs Felicia Aderonke Onakunle. The love which you have bestowed upon me is what has got me this far.

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Abbreviations

ATP	adenosine-5'-triphosphate
CFE	cell free extract
ee	enantiomeric excess
FAD	flavin adenine dinucleotide
FPLC	Fast Protein Liquid Chromatography
g	force of gravity
GC	gas chromatography
HLADH	horse liver alcohol dehydrogenase
HLE	horse liver esterase
HSS	high speed supernatant
Km	Michaelis constant
M	molarity
min	minutes
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	nicotinamide adenine dinucleotide (phosphate) - reduced form
NCIMB	National Collection of Industrial and Marine Bacteria
NMR	nuclear magnetic resonance
pH	hydrogen ion, $-\log[H^+]$
PLE	pig liver esterase
rpm	revolutions per minute
v/v	volume/volume
Vmax	maximum velocity
w/v	weight/volume

ABSTRACT

Owing to their biological significance, increased efforts are being directed towards the production of lactones in optically pure form. One method of synthesis is via a microbial equivalent of the Baeyer-Villiger reaction. This reaction is common in microorganisms possessing a monooxygenase enzyme which is induced as part of the catabolic machinery responsible for the assimilation of cyclic alcohols or ketones. The stoichiometric requirement for NADPH as cofactor, however, makes this enzyme uneconomical for implementation on an industrial scale.

Microorganisms in which the monooxygenase enzyme have been induced also possess a lactone hydrolase (lactonase) enzyme which is responsible for the hydrolysis of formed lactones to hydroxy acids. It is the aim of this project to investigate the potential for the use of these lactonases for the optical resolution of racemic lactones in a bid to produce the latter in optically pure form.

Three inducible lactonases; δ-valerolactone hydrolase from Pseudomonas NCIMB 9872 grown on cyclopentanol, ε-caprolactone hydolase from Acinetobacter NCIMB 9871 grown on cyclohexanol and ε-caprolactone hydrolase from Rhodococcus coprophilus WT1 grown on cyclohexanol were selected for study. Growth studies, conducted in order to optimise lactonase production in each of the bacteria, revealed that in all three microorganisms, lactonase activity was induced throughout growth. High speed supernatants bearing the enzyme of interest were tested with a range of γ- (5-membered ring), δ- (6-membered ring) and ε- (7-membered ring) lactones. These lactonases only showed activity towards (δ- and ε-) lactones. Enantioselectivity studies using δ-decanolactone as substrate showed δ-valerolactone hydrolase from Pseudomonas NCIMB 9872 to have the greatest enantioselectivity with a %enantiomeric excess after 60% transformation for residual lactone of greater than 98%. The lactonases from Acinetobacter NCIMB 9871 and R. coprophilus WT1 only showed modest enantioselectivity towards this substrate with 6% and 15% enantiomeric excess after 60% transformation for residual substrate respectively.

δ-Valerolactone hydrolase was purified from high speed supernatant fractions of Pseudomonas NCIMB 9872 using a two step purification procedure involving anion exchange chromatography and hydrophobic interaction chromatography. This enzyme is a monomer with molecular weight of approximately 28kda and an isoelectric point of about 4.5. Kinetic studies indicate that this enzyme obeys classical Michaelis-Menten kinetics, has a pH optimum of about 7.5 and a temperature optimum between 28 and 30°C. In addition the first 15 amino acid residues starting at the NH_2 terminus are reported. Inhibitor studies suggest that this enzyme does not depend on sulfhydryl groups for its activity. However, there is a metal ion dependency since EDTA and citrate inhibited lactonase activity.

CHAPTER 1.

INTRODUCTION

1.1. Biotransformations

With a multiplicity of constitutive or inducible enzymes, microorganisms are capable of performing a vast number of chemical reactions which are essential for maintaining the life functions of the cell, including growth and reproduction. The exploitation of this capability of microorganisms forms the basis of a comparatively new technology developing at the interface of microbiology, biochemistry and synthetic organic chemistry; Biotransformations. A number of definitions have appeared in the literature describing biotransformations (Leuenberger, 1990; Yamada and Shimizu, 1988). However, these definitions are ambiguous and somewhat inaccurate, since there is the need to allow for future developments likely to occur in the emerging applications of plant biotechnology and animal cell-culture.

For the purpose of this thesis, a biotransformation is defined as a chemical reaction where the conversion of a specified substrate to a defined product is catalyzed by an enzyme system within or without its natural environment.

The technology of biotransformations has arisen due to improved knowledge of synthetic organic chemistry and enzymology. A vast number of organic compounds are synthesized by chemical means. However, limitations which arise due to extreme reaction conditions, lengthy reaction times, and lack of control over stereochemical configuration of products, has highlighted the need for an improved technology. As a route towards obtaining desired target compounds, investigations into the possibility of incorporatating enzyme catalysed steps into chemosynthetic pathways are being extensively carried out. The enzymes used in these biotransformations may be in the form of whole cells, cell extracts, or purified or partially purified enzymes. In whatever form the biocatalyst is used, these biotransformations differ

from more traditional fermentations, since the latter is the result of the actual life processes of a microorganism i.e the product is the result of the complex metabolism of the microorganism *and* involves growth on carbon and nitrogen sources. Thus, living or growing cells are required for fermentation, and the fermentation products are the natural products or derivatives of the fermentation. This is in contrast to biotransformation processes where it is insignificant whether the cell is alive or not. The life process of the organism is only necessary to produce the enzyme and hence the organisms serve as a sac for the enzymes or catalyst.

1.1.1. Practical Considerations

The use of enzymes and biocatalysts in preparative organic synthesis is a well defined area of research to which many reports and reviews have been devoted (Davies *et al.*, 1989; Jones, 1986; Santianello *et al.*, 1992). The rationale of incorporating an enzymatic step into a programme of multi-stage organic synthetis hinges upon a number of considerations:

- The desired reaction must be identified, and must be catalyzable by enzymes. Some reactions for which an enzyme catalysed equivalent have not yet been identified include the Diels-Alder reaction, the Copes rearrangement and the Claisen rearrangement described by Sogo *et al.* (1984).
- The stability of the substrate and/or product under the chemical reaction conditions employed must be assessed. If these are relatively unstable such that the reactions have to be carried out under mild reaction conditions, the use of a biocatalyst would be particularly favoured.
- The stereochemical requirement must be assessed. If there is an uncompromising requirement for optically active products, the use of biocatalysts would be highly favoured.

1.1.2. Methodology

Enzymes are categorized into six main groups, following the recommendations of the International Union of Biochemistry (see scheme 1)

Enzymes	Reaction Catalysed	Co-factor Requirement	Number of enzymes identified (1989)
Oxidoreductases	Oxidations/Reductions	yes	650
Transferases	Transfer of groups from one molecule to another	yes	720
Hydrolases	Hydrolysis, Condensations, Hydrations, Transesterifications	no	636
Lyases	Additions, Eliminations	no	255
Isomerases	Isomerizations	no	120
Ligases	Formations of bonds (C-X)	yes	80

Scheme 1

More than 2000 enzymes have been catalogued (Enzyme Nomenclature, 1979). Of these only about 15% are commercially available as partially purified protein. Various biotransformations have been successfully carried out with enzymes which are commercially available. However, the scope of commercially available biocatalysts is limited *and* in many

cases, it is necessary to search for a microorganism (sometimes by means of a laborious screening procedure) harbouring a tailor-made enyme capable of accomplishing the desired reaction. Pure cultures of microorganisms may be obtained from public culture collections or by isolation from natural sources, e.g. from soil samples. To increase the possibility of successful screening, microorganisms should be selected which are known (from personal experience or from the literature) to be capable of mediating the desired reaction type with structurally related compounds. Having obtained the microorganism, there is the need to generate sufficient viable biomass harbouring the enzyme of interest. This entails studies into the physiology of the microorganism, and requires optimising growth conditions, optimising harvesting conditions, and in cases where purified enzymes or cell extracts are desired, optimising cell breakage. In order to optimise the production of the desired enzyme, a pre-knowledge of the inducibility of the enzyme is required as well as knowledge of the induction mechanisms. A suitable enzyme assay must be developed. If levels of enzyme production are low, then efforts can be made at improving this by strain improvement techniques such as screening, mutation, and genetic engineering.

Biocatalysts can be employed either as whole cells or purified (or partially purified) enzyme. Purified or partially purified enzymes are the preferred choice when there is insufficient permeability of the substrate through the cell membrane leading to insufficient enzyme substrate interactions, or when there exists undesired side-reactions (due to the presence of other enzyme systems) which may serve to limit the yield of the desired product. Some modern techniques for enzyme purification have been summarized by Jakoby (1984). These techniques are usually tedious and expensive. Also the activity of many enzymes depends on the stoichiometric availability of specific cofactors such as NADH, $FADH_2$, NADPH or ATP, which are quite expensive. The feasibility of employing such enzymes on a preparative scale

is dependent on the development of a system to regenerate the required form of the cofactor. With the continual focus of research in this area, several systems have been developed for this purpose which involve chemical, enzymic or photochemical methods. Enzymic regeneration is generally the preferred method of choice (Chenault and Whitesides, 1987), although regeneration efficiency using this method may be low. Methods are now available that permit the economical research-scale use of NADPH or ATP in reactions where up to 1kg of substrate is converted to product (Findeis and Whitesides, 1984). To circumvent the need for co-factor regeneration during a biotransformation process whole cell systems can be used. Whole cells can be utilised as growing cultures, whereby the substrate is added to the growth medium at the time of inoculation or at a later stage of microbial growth, such that, growth and biotransformation occur simultaneously, or, as dormant cultures whereby the process of biomass (biocatalyst) propagation and biotransformation are separated.

When the objective of the biotransformation is to produce and recover a product, there must be a product isolation protocol, whereby the desired product is isolated in sufficient yield. The biocatalyst (whole cells or enzymes) is removed by filtration or centrifugation, then the product is concentrated or fractionated. Depending on the physical and chemical properties of the product, and on the chemical composition of the reaction broth, methods that are taken into consideration include: extraction, chromatography, adsorption to polymeric resins, precipitation and distillation.

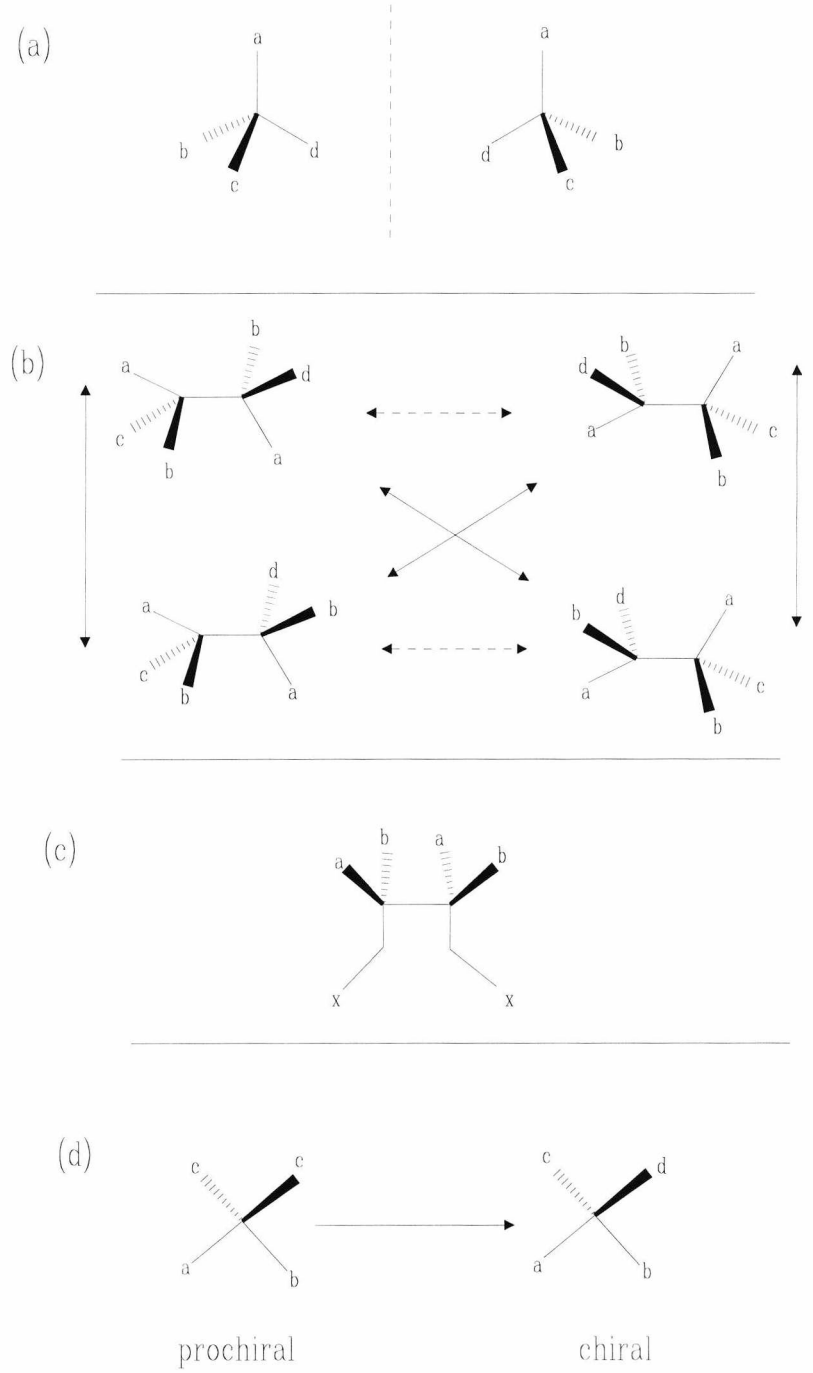
1.2. Chirality

Originating from the Greek word 'chiros' meaning hand, chirality describes the nature of an object which is not superimposable on its mirror image. The concept of chirality arose from

the pioneering research of Pasteur in 1848, with his physical resolution of dextro- and levo-rotatory tartaric acid through his observation of the hemihedral faces of the crystals of sodium ammonium tartarate. Inspired by the work of the French mineralogist Biot, who studied the rotation of polarized light by quartz plates, Pasteur was able to show a dextro- and levo-rotatory form of sodium ammonium tartarate which rotated polarized light in opposite directions, and also two other forms which did not rotate polarized light. This phenomenom of optical activity occurs in molecules in which a carbon atom has four different substituents attached to it. Such molecules exist in two forms called enantiomers (figure 1.1a) and have identical physical and chemical properties with exception to their interaction with plane polarized light. The dextro-rotatory (+)- enantiomer will rotate plane polarised light to the right while the levo-rotatory (-)- enantiomer will rotate plane polarised light to the left. An equimolar mixture of both enantiomers constitutes a racemate and does not rotate plane polarised light.

In most organic compounds the atom with the four different substituents attached is normally a carbon atom. This is known as the chiral center or stereo center. A compound may contain more than one chiral centre. When this is so, the number of possible stereoisomers is correspondingly larger. Compounds which are stereoisomers of one another, but are not enantiomers are called diastereomers (figure 1.1b) *and* are said to have a diastereomeric relationship. Some compounds may have more than one stereocentre and yet be achiral due to a plane of symmetry within the molecule. Such a compound is said to be a meso compound and is not optically active (fig 1.1c). A compound which does not contain any chiral centres, but which by a single replacement may be converted into a chiral compound containing an asymmetric centre is said to be prochiral (Alworth, 1971) (figure 1.1d).

Figure 1.1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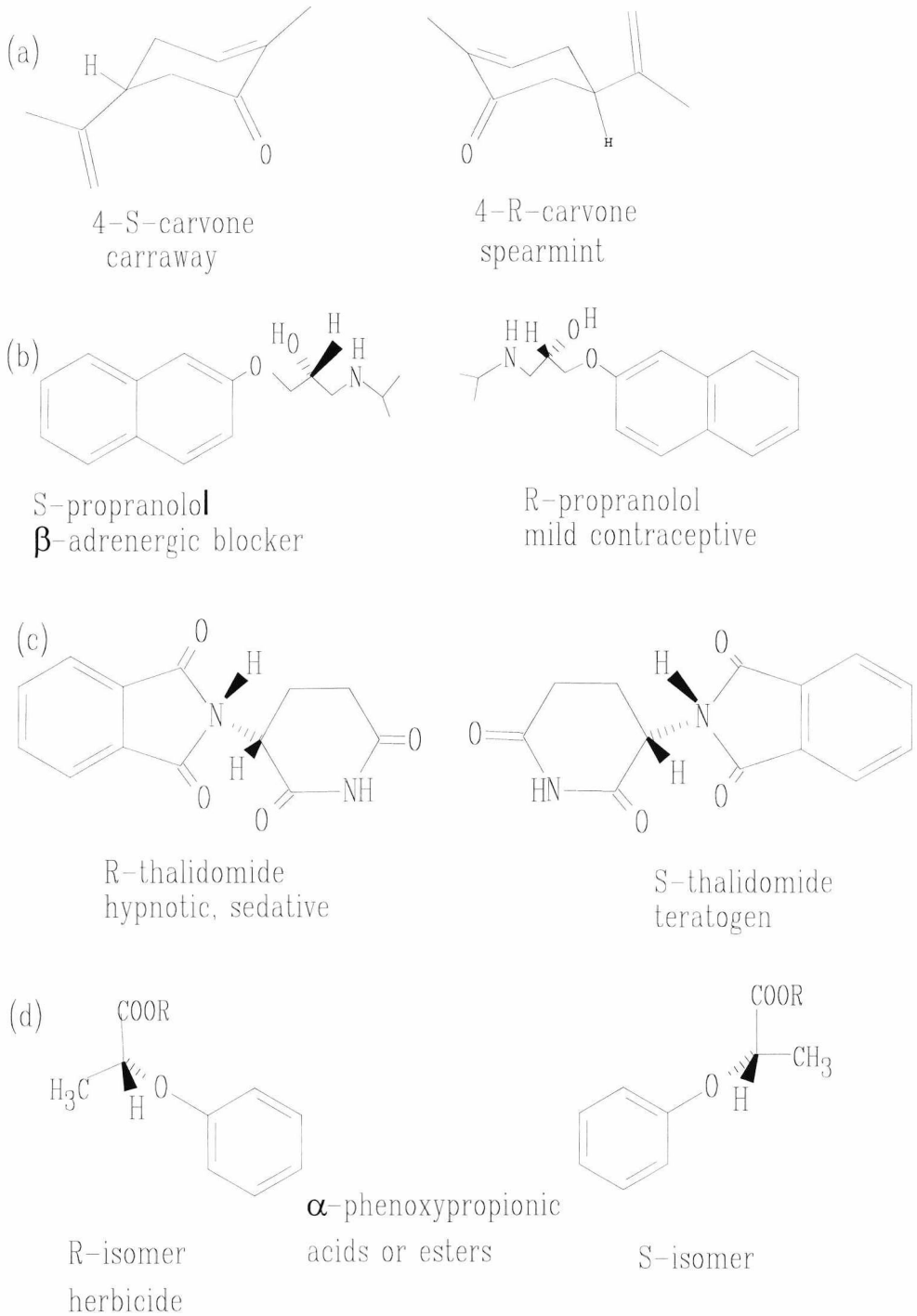
prochiral
chiral

1.2.1. The Importance of Chirality

Asymmetry and optical activity are widely distributed in biological systems as illustrated by sugars, proteins, enzymes and cell receptors. As a result, it would be expected that molecules and metabolites having stereogenic centres would interact stereospecifically with biologcal systems. According to Beckett (1991), the first recorded observations of differences in the biological actions of stereoisomers are attributed to A. Piutti in 1886, when he showed the capability of human taste receptors to distinguish between the sweet tasting (+) asparagine, and the bland tasting (-) asparagine. It is now recognised that stereochemistry plays an important role in the physiological activity of many natural products as well as the pharmacological response to many synthetic drugs. A few examples are illustrated in figure 1.2.

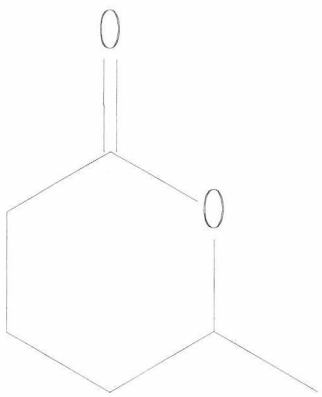
Figure 1.2: Biological effects of enantiomers



There is a great awareness of the significance of optical purity. The implications for the food, pharmaceutical and chemical industries cannot be emphasized too much. It is estimated that about 50% of all marketed drugs have chiral centres. Of these only 25% have been developed as the optically pure isomer. It is expected that this may change to the point where as many as 50% of all new products could be marketed as new isomers within the next 10 years (Macdonald, 1991). 1995 saw the value of the world pharmaceuticals market exceed $250bn. Of this, optically-pure compounds made up about $50bn (Cannarsa, 1996). Regulatory bodies world-wide have taken a similar stance as regards the production and marketing of stereoisomeric drugs and foods. In May 1992, the Food and Drug Administration (FDA) released its "Policy Statement for the Development of Stereoisomeric Drugs" which has significant implications not only to clinical, pharmacological or toxicological issues, but extend also to the chemist involved in the development and validation of analytical controls for chiral drug substances and products. Thus the individual enantiomers of an isomeric drug (for instance) have to undergo numerous clinical trials in order to establish their activity and possible deleterious effects.

1.3. Lactones

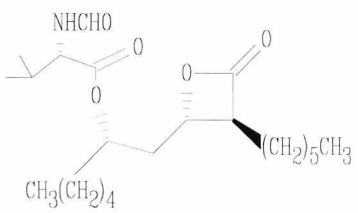
Lactones are cyclic esters:



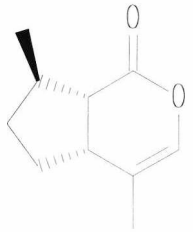
Lactone

The lactone functionality occurs widely in a large variety of natural products and biologically active compounds. Important lactone natural products include antibiotics, fungal toxins, steroids, and pheromones (figure 1.3). They play important roles in the food and fragrance industries, serving as flavour components. They can act as sex attractant pheromones in insects *and* they can also serve as building blocks for the synthesis of more complex macromolecules. Corey and Noyori (1970) have used derivatives of 2-oxabicyclo[3.3.0]oct-6-en-3-one in the total synthesis of postaglandin $F_{2\alpha}$. In many cases, the lactones are chiral, with the physiological activity being due to a single enantiomer. In some pheromones, the optical purity of the lactone can determine the biological activity (Tumlinson *et al.*, 1977). Due to their importance, much effort has been made towards the synthesis of various lactones in optically active form.

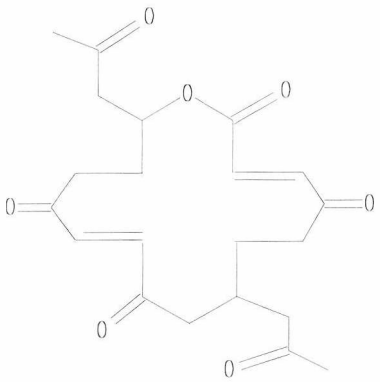
Figure 1.3: Some examples of naturally occuring lactones



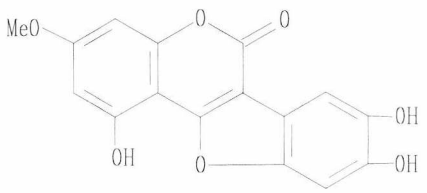
Valilactone



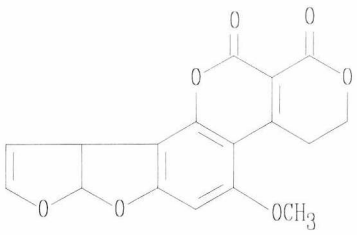
Nepatalactone



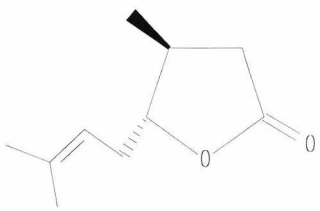
Vermiculine



Wedelolactone



Aflatoxin G1



Eldanolide

Valilactone - antibiotic produced by Streptomyces albologus (Chada *et al.*, 1991)

Nepatalactone - major attractant component of catnip (Sakurai, Ikeda and Mori, 1988)

Vermiculine - antibiotic produced by Penicillium vermiculatum (Boeckman, Fayos and Clardy, 1974)

Wedelolactone - Anti-fungal compound produced by several plant species.

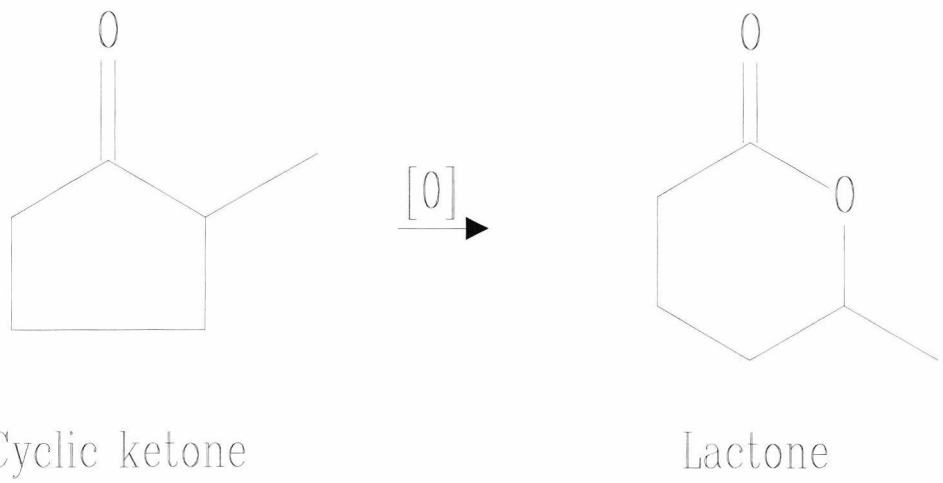
Aflatoxin G_1 - potent mycotoxin

Eldanolide - Pheromone produced by the male African sugar-cane borer Eldana saccharina (Uematsu, Umemura and Mori, 1983)

1.4. The Baeyer Villiger Reaction

Traditionally lactones have been synthesized by the Baeyer-Villiger reaction (Baeyer and Villiger, 1899). This reaction involves the insertion of an oxygen atom into one of the bonds adjacent to the carbonyl functional group of a ketone or lactone (figure 1.4), and can be effected by a peroxide or peroxyacid reagent (Lee and Uff, 1967; Rugero and Edwards, 1970; Mimoun, 1982).

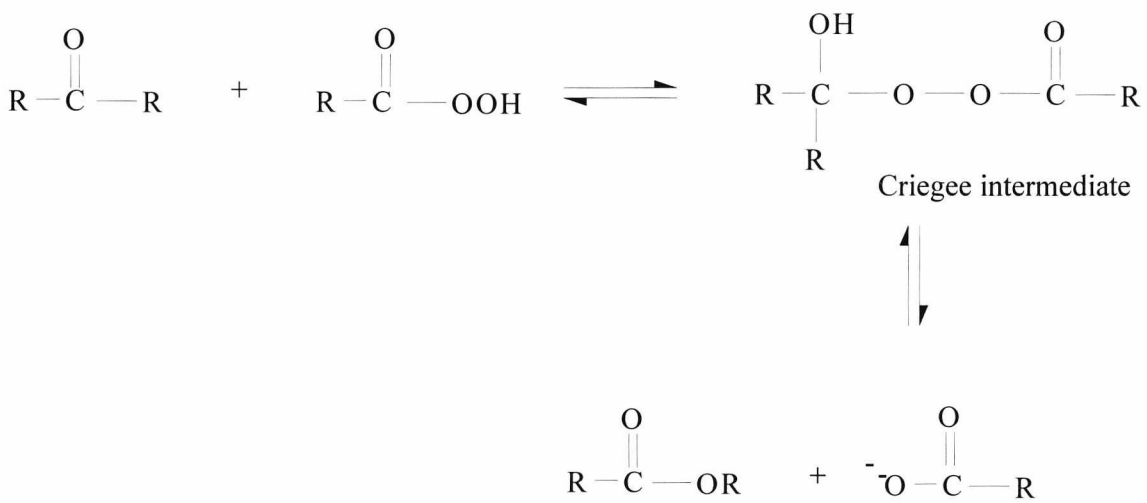
Figure 1.4: Baeyer-Villiger reaction



A two step reaction mechanism has been postulated in which the peroxide or peroxyacid reagent attacks the carbonyl group of the ketone to form a tetrahedral intermediate known as the "Criegee intermediate" (Criegee, 1948) (figure 1.5), the breakdown of which is the rate determining step (Hawthorne and Emmons, 1958; Mitsuashi, Miyadera and Simamura, 1970). In most cases, the prefered chemical effector for this reaction is a peroxyacid, because the reaction with simple peroxides proceeds in low yields or not at all, due to the

resistance of the tetrahedral peroxy adducts of ketones with peroxides to the alkyl migration step needed to generate the ester or lactone (Barton, Ollis and Stodart, 1979; Hiatt, 1971). Symmetrical ketones only give one product in the Baeyer Villiger oxidation.

Figure 1.5: Mechanism of Baeyer-Villiger reaction.



However, unsymmetrical ketones can give two oxidation products. Thus, when the two alkyl groups adjacent to the carbonyl carbon differ substantially a clear selectivity is observed, with oxygen insertion occuring towards the more substituted side of the carbonyl group. The regiochemistry of oxygen insertion can usually be predicted by assuming that the carbon atom best able to support a positive charge will migrate preferentially (Lee and Uff, 1967; Noyori *et al.*, 1980). Anomalous examples of regioselectivity in bicyclic ketones highlighted by Krow (1981), suggests that other factors may play a part in determining the regioselectivity of these reactions. Such factors include the electronic effects of electron

withdrawing groups (Grudzinski *et al.*, 1978; Takatsuto and Ikekawa, 1983) and the steric effects of bulky substituents (Dave *et al.*, 1984; Sauers and Beisler, 1964; Grieco *et al.*, 1975). Changing the peroxyacid/solvent combination can also have an effect on the regioselectivity as observed by Krow *et al.* (1982).

To date, only one report has appeared in the literature describing an enantioselective Baeyer-Villiger reaction (Bolm *et al.*, 1994) where up to 69% enantiomeric excess of lactone products occurred during the oxidation of racemic 2-phenylcyclohexanone using optically active nickel (II) complexes as catalysts.

The predictable regioselectivity coupled with a lack of enantioselectivity puts a limit to the amount of lactones that can be obtained using the Baeyer-Villiger reaction. This coupled with low yields, long reaction times (5-7 days), and the occasional need for harsh reaction conditions, makes this method unsatisfactory for the production of a wide variety of optically active lactones.

1.5. Baeyer-Villiger type reactions in Microbial Metabolism.

The capacity for xenobiotic degradation by microorganisms has been recognised (Dagley, 1978). Often central to this is the ability to incorporate molecular oxygen into recalcitrant organic compounds as a route to their assimilation into central cellular metabolic pathways. There have been numerous reports in the literature of organisms which are capable of Baeyer-Villiger type oxidations. This reaction is actually crucial to their capacity to degrade or metabolize certain organic compounds. Alicyclic hydrocarbons and their

derivatives such as ketones, alcohols and carboxylic acids are widely distributed in the biosphere and include components of the waxes of leaves, plant oils, insect secretions, the lipids of microorganisms, and a variety of plant secondary metabolites. The exploitation of fossil fuels, and the use of a wide variety of agrochemicals have added to the complexity of structures that tax the catabolic versatility of microorganisms in returning this organic carbon to the carbon cycle and thus maintaining environmental stability.

1.5.1. Biodegradation of Cyclic alkanes

Cycloalkanes are more resistant to microbial attack than most other groups of hydrocarbons, and as such, selective isolation of microorganisms capable of utilizing them as sole carbon sources can be quite difficult. Although large quantities of these compounds have been released into the biosphere, there is little evidence of their accumulation, suggesting that biodegradation of these compounds do occur in the natural environment (Perry and Scheld, 1968). The capacity of microorganisms to utilize linear alkanes does not have any bearing on their ability to degrade cycloalkanes. As such, attempts to isolate microorganisms capable of cycloalkane degradation by screening for linear alkane degraders have been largely unsuccessful (Komagata, Nakase and Katsuya, 1964; Pelz and Rehm, 1971; Beam and Perry, 1973, 1974; de Klerk and van der Linden, 1974).

It is possible that complete degradation of these compounds occur through co-metablism. Beam and Perry (1974) were able to show the complete oxidation of cyclohexane by a complex mixture of organisms present in marine mud. Stirling, Watkinson and Higgins (1977), were the first to successfully show the utilisation of a cycloalkane by a single

microorganism. They isolated a strain of Norcardia from estuarine mud that gave good growth with pure methylcyclohexane or cyclohexane as carbon source. Since then a number of reports have appeared in the literature describing the metabolism of cycloalkanes by pure strains of bacteria (Anderson, Hall and Griffin, 1980; Trower *et al.*, 1985).

In line with known pathways of linear alkane oxidation (McKenna and Coon, 1970; Jurtshuk and Cardini, 1971), methane metabolism (Tonge, Harrison and Higgins, 1977; Stirling and Dalton, 1979) and co-oxidation of alicyclic hydrocarbons (Ooyama and Foster, 1965), it was postulated that the initial step in cyclohexane metabolism by the Norcardia sp. was likely to be the introduction of an oxygen atom to form cyclohexanol. Although unable to directly demonstrate cyclohexane oxidation by the conventional procedures of substrate stimulated NADPH oxidation, Sterling (1977) was able to show the conversion of cyclohexane to cyclohexanol using radiolabelled-cyclohexane. Further elucidation of the metabolism of cyclohexanol was not as difficult. Organisms capable of the degradation of cycloalkanols and cycloalkanones are quite ubiquitous, and easily isolatable. In 1955, Posternak and Reymond, and, Posternak, Reymond and Friedli were able to demonstrate the ability of Acetobacter suboxydans to convert isomers of cyclopentane-1,2-diol, cyclohexane 1,2-diol, cycloheptane-1,2-diol, cyclohex-1-ene-2,3-diol and cyclohexane-1,2,3,4-tetrol into the corresponding monoketones and diketones. Further studies by Yugari (1961) of the metabolism of cyclohexane-1,2-diol assumed the initial involvement of NADH deydrogenases leading to the formation of cyclohexane-1,2-dione.

At first, it was postulated that further metabolism of these ketones involved hydrolytic fission of the cyclic ring, as observed during the cleavage of the aromatic counterpart,

catechol (Hayaishi and Hashimoto, 1950). However Bakule's and Long's (1963) observation of cyclohexane-1,2-dione's existence as a monohydrate in equilibrium with the enol form in solution contributed to the biochemical unusualness of this reation.

An alternative mechanism for ring cleavage was revealed by the work of Bradshaw *et al.* (1959) when they isolated a lactone as one of the metabolites from (+)-camphor metabolism by a strain of Pseudomonas. The pathway for (+)-camphor metabolism was further elucidated by Conrad, Dubus and Gunsalus (1961) and shown to involve two lactone forming ring insertion steps in a manner reminiscent of the Baeyer-Villiger reaction (figure 1.6). Ring cleavage in this pathway is thought to occur by spontaneous opening of these unstable lactones. An alternative route for (+)-camphor degradation by Mycobacterium rhodochrous proposed by Chapman *et al.* (1966) also involves a Baeyer-Villiger type oxygen insertion prior to ring cleavage. Studies of the metabolism of cyclohexanol by Nocardia globerula CL1 (Norris and Trudgill, 1971), and Acinetobacter NCIMB 9871 (Donoghue and Trudgill, 1975) (figure 1.7), and the metabolism of cyclopentanol by Pseudomonas NCIMB 9872 (Griffin and Trudgill, 1972) (figure 1.8) have confirmed the involvement of a monooxygenase in the key step for the degradation of these compounds.

Figure 1.6: Metabolism of camphor by Pseudomonas putida.

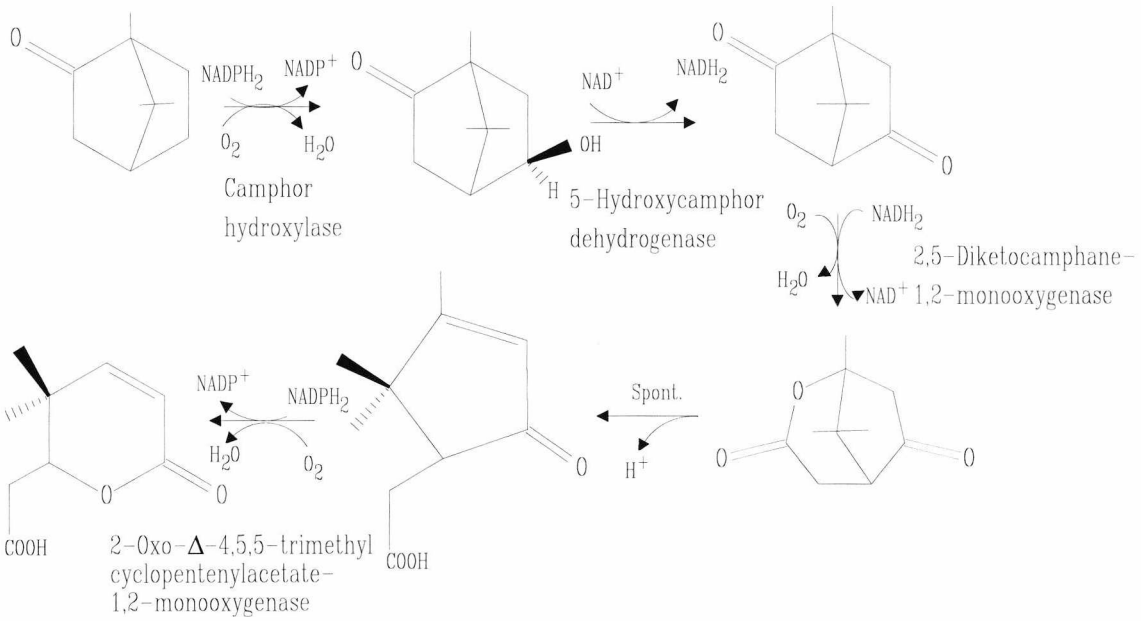


Figure 1.7: Reaction sequence of the oxidation of cyclohexanol to adipate by Acinetobacter NCIMB 9871 or Nocardia globerula CL1.

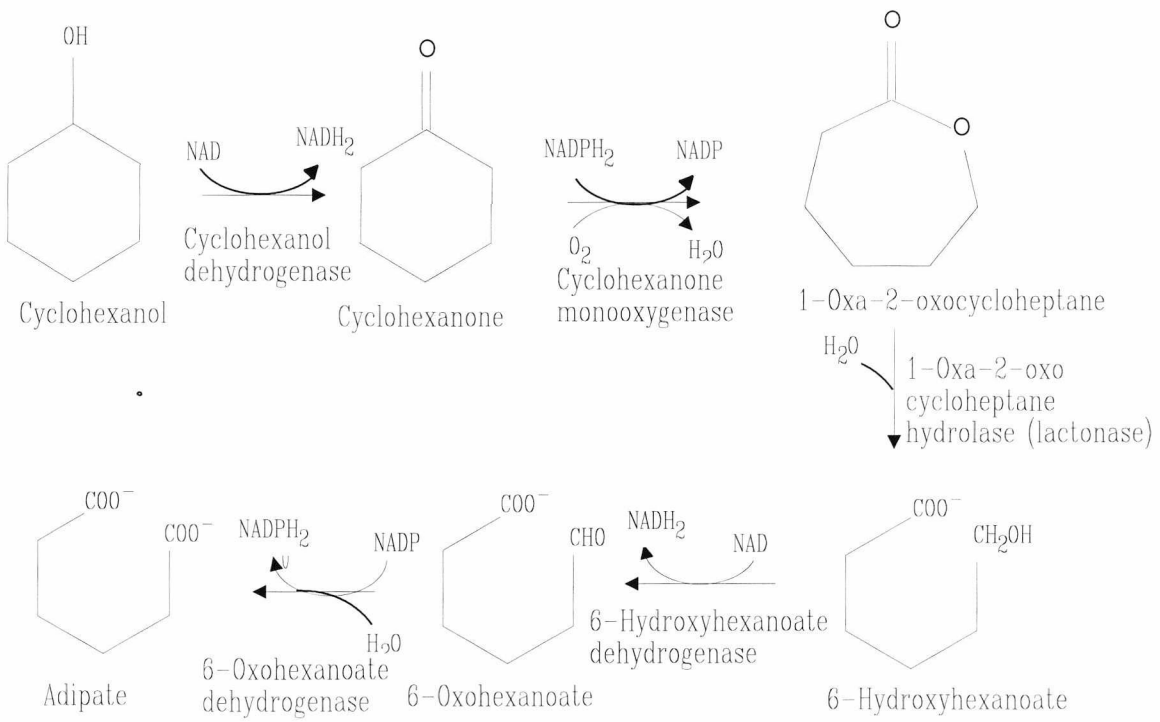
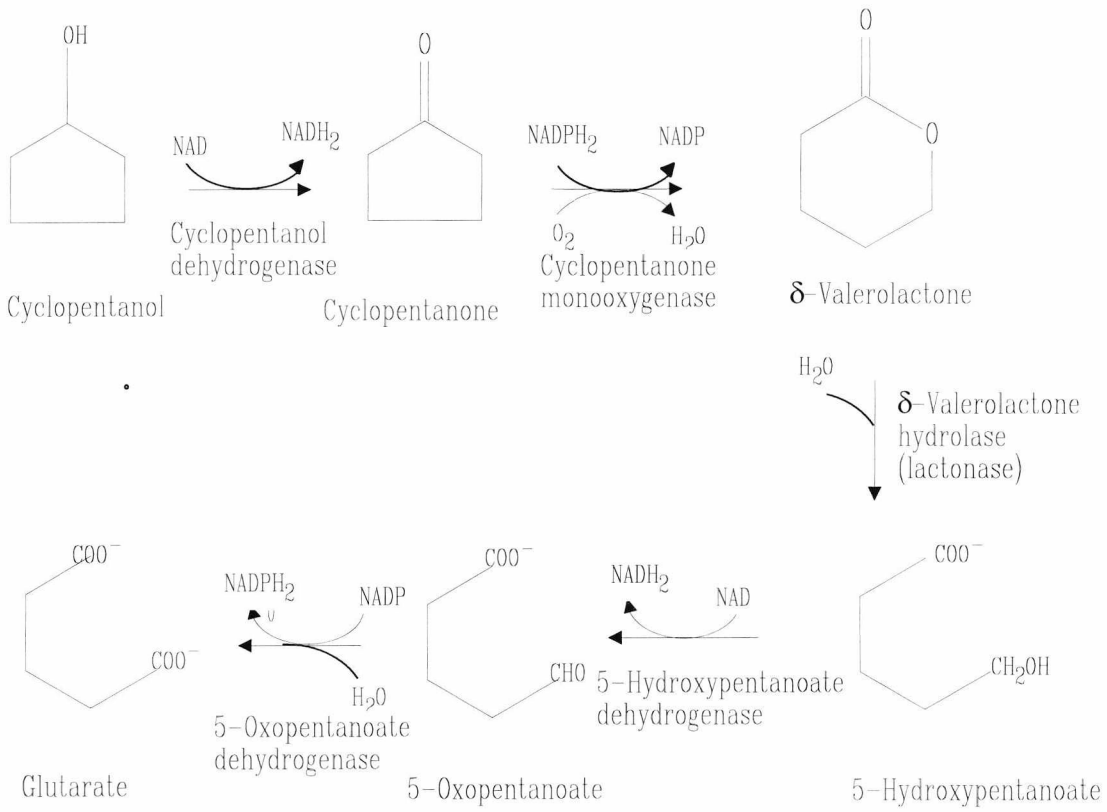


Figure 1.8: Reaction sequence for the oxidation of cyclopentanol to glutarate by Pseudomonas NCIMB 9872



1.6. Cyclohexanone Monooxygenase

This enzyme is responsible for the formation of 1-oxa-2-oxocycloheptane (ε-caprolactone) from cyclohexanone (figure 1.7). This is particularly interesting because the mode of formation is similar to the Baeyer-Villiger oxidation of ketones to lactones. Like the Baeyer-Villiger reaction, the enzyme-catalysed reaction exhibits retention of configuration at the migrating center, and preferential migration of the more substituted center (Schwab,

Li and Thomas, 1983). This enzyme has been characterised by Donoghue, Norris and Trudgill (1976). They achieved a 12-fold and 35-fold purification of the enzyme from Nocardia globerula CL1 and Acinetobacter NCIMB 9871 respectively. Thus, they constitute a high percentage of the total protein in the cell.

The purified cyclohexanone oxygenase exhibits the stoichiometry of typical monooxygenases, utilising NADPH and molecular oxygen in equal amounts. The enzyme is of the external monooxygenase category categorised by Hayaishi (1969) because of the requirement for an external electron donor. The systematic name for the enzyme is cyclohexanone NADPH:oxygen oxidoreductase (1,2-lactonizing) (EC1.14.13). The enzyme consists of a single polypeptide chain with a molecular weight of 50-60 kd. The enzyme carries one mole of FAD with a dissociation constant of 40nM for Acinetobacter. The flavin group for the Nocardia enzyme is easily dissociable. Both enzymes are absolutely specific for NADPH as the electron donor but have a broad specificity for ketone substrate. In both organisms the flavin prosthetic group of the enzyme is rapidly reduced in the absence of cyclohexanone on the addition of NADPH but not NADH. Both enzymes contain a functionally essential sulfhydryl group present at the catalytic active site. Inhibitor studies indicate the absence of functional metal ions. Protection against sulfhydryl active agents is afforded by NADPH but not cyclohexanone. The two enzymes differ slightly with respect to kinetic data. (Table 1.2)

Table 1.2: Comparison of kinetic properties of cyclohexanone monooxygenase from Nocardia globerula CL1 and Acinetobacter NCIMB 9871

	Nocardia globerula CL1 monooxygenase	Acinetobacter NCIMB 9871 monooxygenase
pH Optimum	8.4	9.0
K_m Cyclohexanone	1.56μM	6.9μM
K_m $NADPH_2$	31.3μM	17.8μM
Catalytic activity (ml substrate/min/mg	1018	1390

Similar properties have been reported for cyclopentanone 1,2 oxygenase of Pseudomonas NCIB 9872 (Griffin and Trudgill, 1976) which oxygenates cyclopentanone to 1-oxa-2-oxocyclohexanone as part of its metabolism of cyclopentanol. The cyclohexanone monooxygenase of Acinetobacter NCIB 9871 has been cloned into E. coli (Chen, Peoples and Walsh, 1988). The structural gene is 1626 nucleotides long and codes for a polypeptide of 542 amino acids, the complete sequence of which was derived by translation of the nucleotide sequence. Also reported are 399 nucleotides 5' and, 108 nucleotides 3' of the coding region. There is a potential flavin-binding site near the NH_2 terminus of the enzyme at residues 6-18, and there is a potential nicotinamide binding site extending from residue 176 to residue 208 of the protein. Other organisms with ketone-lactonizing enzyme systems have been isolated. Magor *et al.* (1986) have comparatively described the ability of 3 Xanthobacter species to metabolize cycloalkanes. These monooxygenases have been

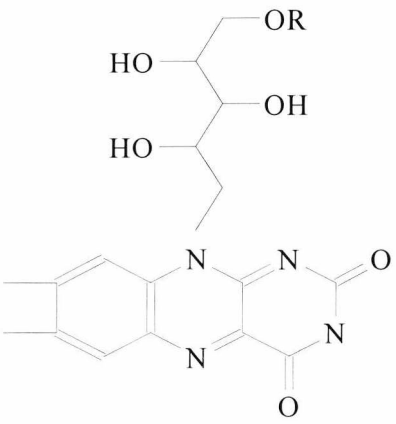
claimed to differ with respect of pH optima and co-factor requirements, and may be a useful feature in distinguishing between the different subgroups existing in this genus. Ougham, Taylor and Trudgill (1983) have reported the involvement of a unique monooxygenase enzyme in the metabolism of 2-oxo-4,5,5-trimethylcyclopentenylacetic acid (an intermediate of camphor metabolism) by Pseudomonas putida (see figure 1.6). This enzyme system has been reported to be specific for NADH and not NADPH as electron donor for reduction of the flavin prosthetic group.

1.6.1. Mechanism of reaction of cyclohexanone oxygenase

The key step in cyclic ketone carbon assimilation is the Baeyer-Villiger ring expansion to the seven membered ring lactone. Subsequent enzymic hydrolysis leads to the hydroxy acid which can be processed to standard C_2 and C_4 metabolites. Ryerson, Ballou and Walsh (1982), in an effort to elucidate the mechanism by which the oxygen insertion reaction on cyclohexanone is effected, presented information about the reaction mechanism of cyclohexanone oxygenase based on steady state kinetics, product analysis, pre-steady state kinetics and apo-enzyme reconstitution assays with flavin analogues of FAD.

The key component of the enzymatic Baeyer-Villiger transformation of cyclohexanone to ε-caprolactone is the flavin prosthetic group (figure 1.9).

Figure 1.9: Flavin prosthetic group.



R = H Riboflavin

R = PO_3-2FMN

R = ADP FAD

A mechanism has been proposed. See figures 1.10 and 1.11.

NADPH very rapidly binds to and reduces the enzyme bound FAD (E-FAD). The $FADH_2$ likewise reoxidises with oxygen much more rapidly than catalytic turnover to produce FAD-4a-OOH. The flavin hydroperoxide then forms the hydroperoxy anion and acts at the ketone carbonyl. The tetrahedral adduct so formed then decomposes in the forward direction by Baeyer-Villiger migration of one of the carbon-carbon bonds with fragmentation of the weak O-O bond to yield ester flavin 4a-OOH which returns to FAD by internal loss of H_2O.

Figure 1.10: Reaction of $FADH_2$ with O_2 to produce the reactive hydroperoxyflavin functional group (proposed by Walsh and Jack Chen, 1988). The flavin hydro-peroxide intermediate can either fragment to oxidized flavin and H_2O_2 or can oxygenate a co-substrate with concomitant generation of a 4a-hydroxyflavin which then breaks down to oxidized flavin and water.

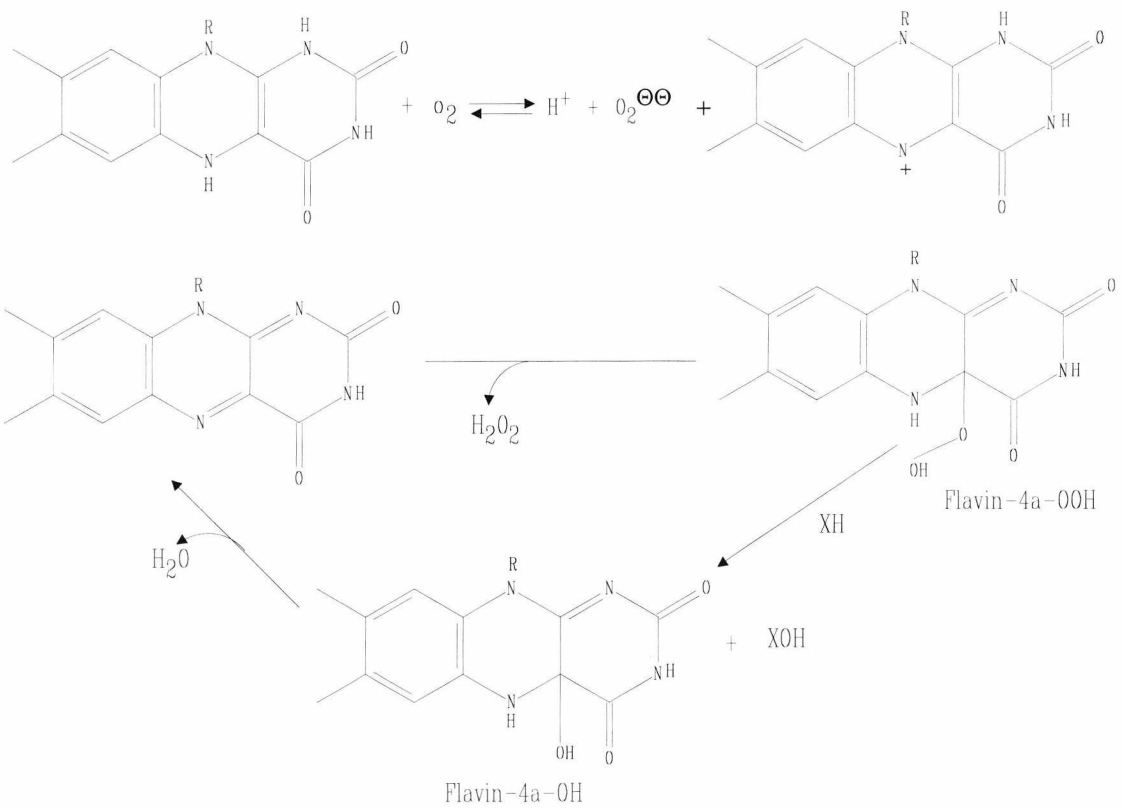
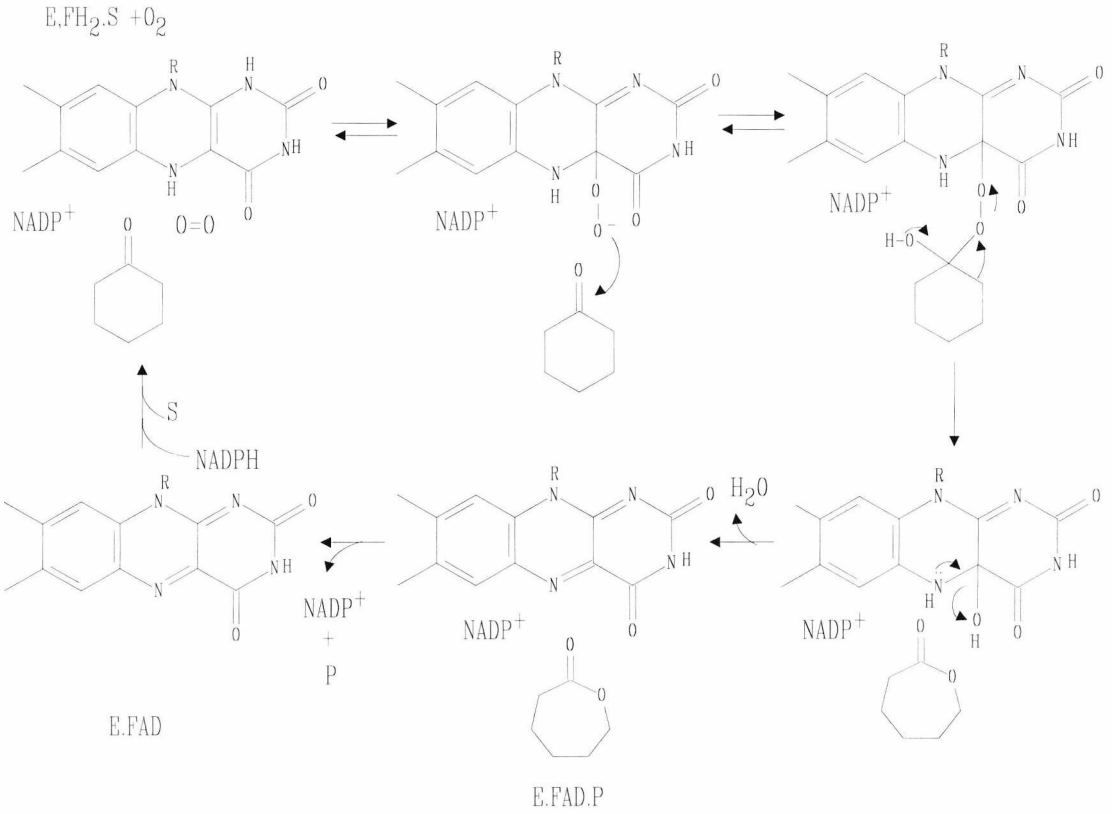


Figure 1.11: Proposed mechanism for the cyclohexanone monooxygenase catalysed oxygen insertion reaction with cyclohexanone (S) to yield ε-caprolactone (P).



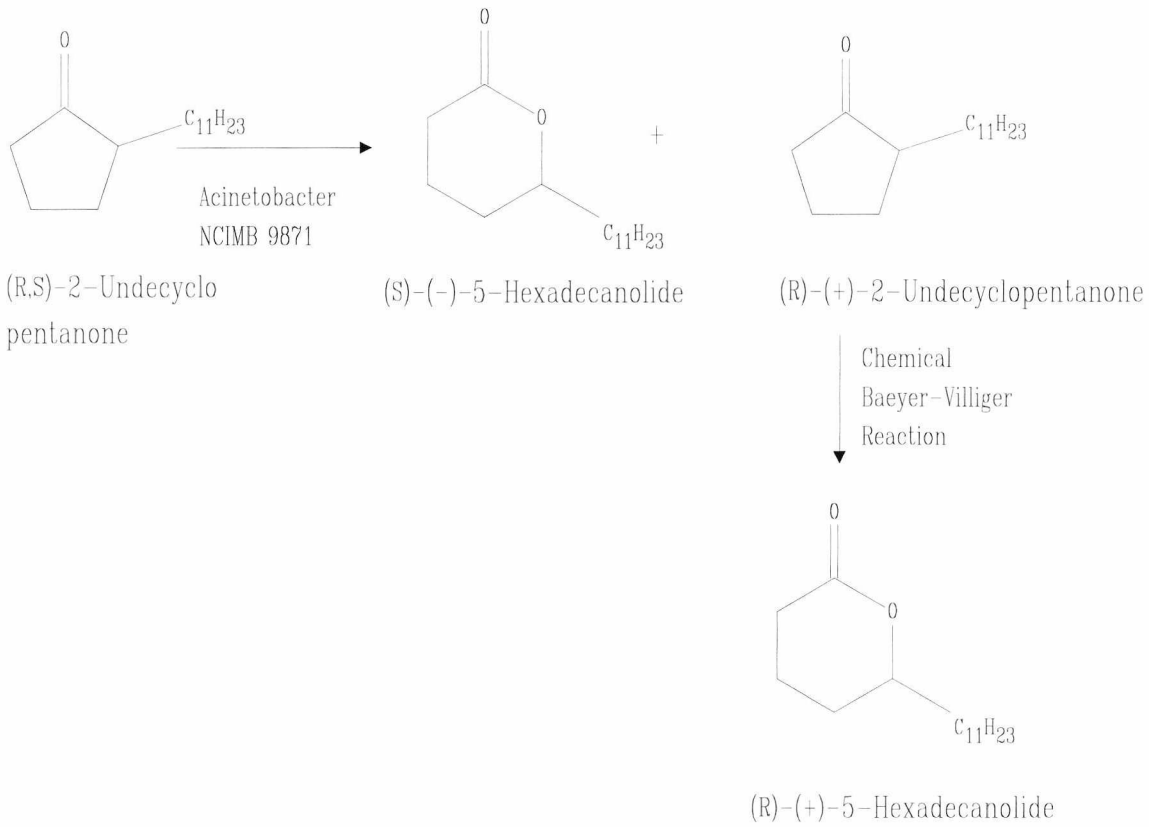
1.7. Baeyer-Villiger Monooxygenases in Chiral Lactone Synthesis

Ever since Ouazzani-Chahdi, Buisson and Azerad (1987) published a report describing the enantioselective Baeyer-Villiger type transformation of a simple cyclic dione, there has been considerable interest in the use of micro-organisms and microbial monooxygenases to produce optically active lactones from the corresponding cyclic ketones, particularly bicyclic ketones. Several examples of such biotransformations have appeared in the literature (Shipston, Lenn and Knowles, 1992; Levitt *et al.*, 1990; Carnell *et al.*, 1991; Carnell and Willetts 1992; Taschner and Pedadda 1992; Grogan, Roberts and Willetts 1993).

Taschner and Black (1988), described enantioselective synthesis of lactones from mesomeric cyclohexanones in their work directed toward the synthesis of 3-aryl tetrameric acid antibiotics. Their results however, gave no indication of the scale of the reaction, and, they were not able to demonstrate the absolute configurations of the products formed.

Alphand, Archelas and Furstoss (1990) using whole cells of Acinetobacter NCIMB 9871, described the use of these enzymes to perform a preparative enantioselective synthesis from racemic material, which allowed direct access to both (S-) and (R-) 5-hexadecanolide; a pheromone isolated from the oriental hornet, *Vespa orientalis* (figure 1.12). The 2S-enantiomer of the lactone product was produced with a %ee of 74% and isolated in 25% yield, while the 2R- enantiomer of the ketone substrate was left behind in 95% ee and 30% yield.

Figure 1.12: Bioconversion of 2-Undecyclopentanone by Acinetobacter NCIMB 9871



This afforded direct access to (R)-(+)-5-hexadecanolide; the sole bioactive enantiomer (Mori, 1989), by a chemical Baeyer-Villiger reaction. As a sequel to their work on 5-hexadecanolide, Alphand, Archelas and Furstoss (1990) investigated the effect of chain length (lipophilicity) on the feasibility, regioselectivity and enantiospecificity of the reaction, since this has been observed to have a profound influence in other biotransformation systems (Zhou *et al.*, 1983; Ladner and Whitesides, 1984). It was established that an increased lipophilicity of the ketone substrate led to increased yields of the lactone product, but lower e.e's.

In the one-step synthesis of 2-oxa-bicyclo[3.3.0]oct-6-en-3-one (a pivotal prostaglandin chiral synton) from bicyclo[3.2.0]hept-2-en-6-one, the same workers (Alphand, Archelas and Furstoss, 1989) observed that each one of the substrate enantiomers gave rise to a different lactone obtained through a highly regio- and enantio- specific microbial Baeyer-Villiger type process (figure 1.13). It appears that each one of the enantiomers reacts with a different regioselectivity for the O_2 atom insertion leading to the two different lactones, one through the normal Baeyer-Villiger reaction and the other through an unusual reverse pathway). A similar result was achieved using a wide range of microorganisms including Acinetobacter NCIMB 9871, Acinetobacter junii, Pseudomonas putida, Rhodococcus coprophilus and Rhodococcus fascians (Shipston, Lenn and Knowles, 1992).

An unexpected and unprecedented result was obtained from the microbial oxidation of bicylo[2.2.1]hepten-2-one (Konigsberger *et al.*, 1991). It was observed that bioconversion of this bicyclic ketone with Acinetobacter NCIMB 9871 did not give expected results but rather the strain performed reduction and oxidation concurrently, depending on the substrate enantiomer. However using purified cyclohexanone monooxygenase from Acinetobacter NCIMB 9871, Shipston, Lenn and Knowles (1992) were able to demonstrate unequivocally that this unusual regioselectivity was the result of a single enzyme (figure 1.13).

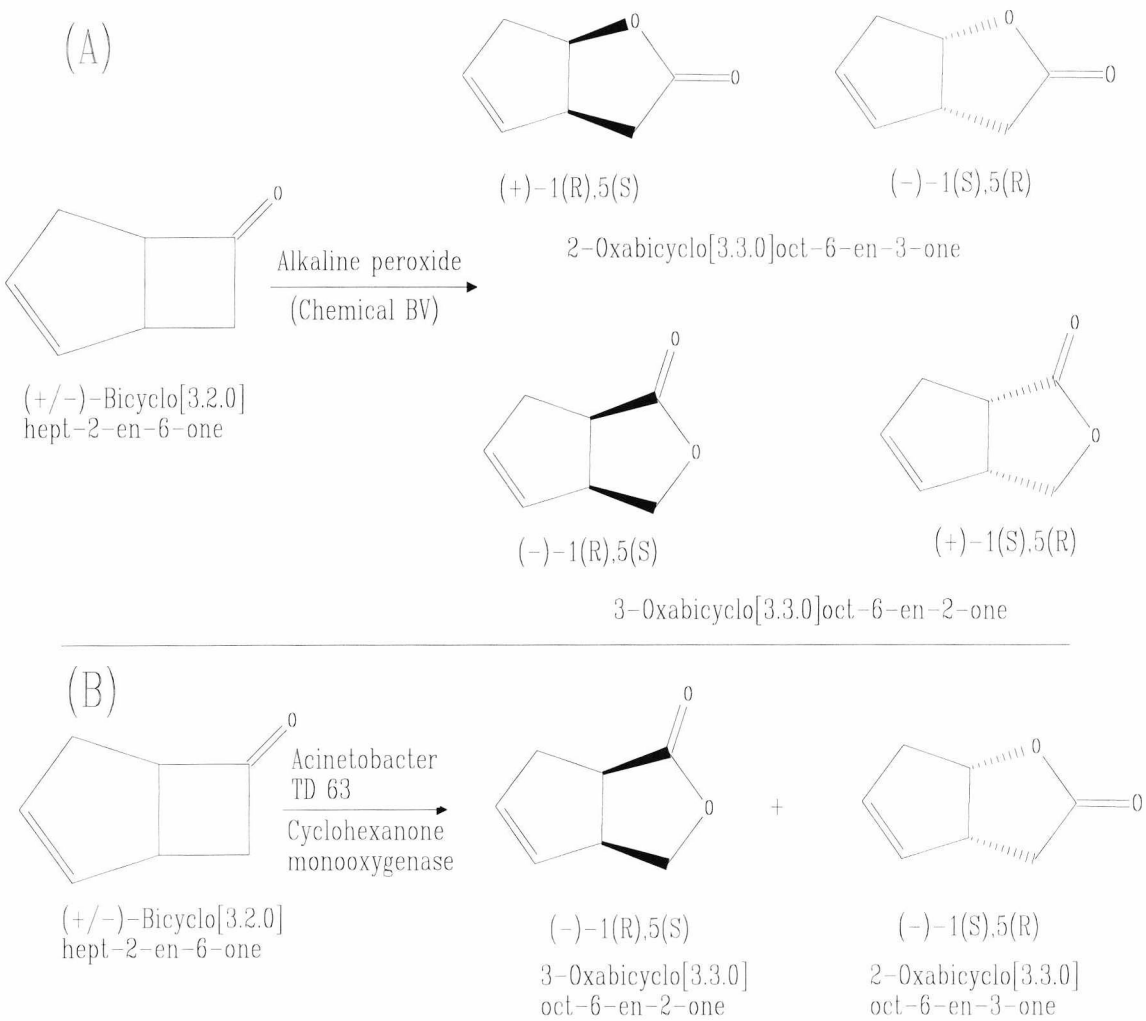
Following the observation that thiolactones can inactivate cyclohexanone monooxygenase (Latham and Walsh, 1987), enantiomerically enriched bicyclic thiolactones were used for inhibitor studies by Wright *et al.* (1994), presenting evidence in support of the presence of a single active site for the enzyme. Working on the assumption that the migrating C-C bond

of the peroxidic intermediate should be antiperiplanar to the peroxidic bond (Deslongchamps, 1983), and that the stereo- and regio- chemistry of the reactions is dictated by steric interference with the active site, models have been proposed to explain this regioselectivity (Carnell *et al.*, 1991; Alphand and Furstoss, 1992; Kelly *et al.*, 1995a,b).

1.7.1. Limitations to the use of Baeyer-Villigerases from Microorganisms.

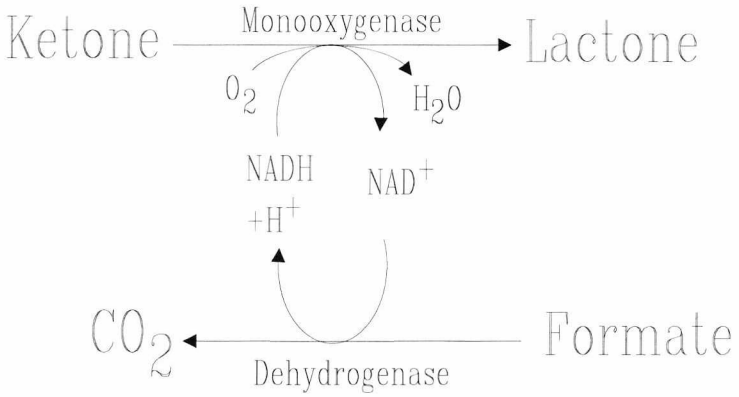
Limitations exist to the application of Baeyer-Villiger biocatalysts. Acinetobacter species are class 2 respiratory pathogens and as such are unsuitable for large-scale cultivation. In whole cell systems, the occurrence of additional reactions may serve to limit the accumulation of the desired lactone product. Use of purified enzymes require the stoichiometric addition of NADPH as cofactor, making this an expensive procedure which is difficult to scale up, even with cofactor recycling.

Figure 1.13: (A) Possible lactone products obtainable by Baeyer-Villiger oxidation of bicyclo[3.2.0]hept-2-en-6-one. (B) Lactone products obtained from biotransformation of bicyclo[3.2.0]hept-2-en-6-one by whole cells of Acinetobacter TD 63 or cyclohexanone monooxygenase.



Various workers have investigated the potential for the use of less pathogenic microorganisms bearing Baeyer-Villiger monooxygenases. Konigsberger *et al.* (1990), reported the oxidation of several bicyclic ketones using the fungus Cylindrocarpon destructans ATCC 11011. Unfortunately, this biocatalyst exhibited little enantioselectivity in its activity. Better enantioselectivity was shown by the fungus Curvularia lunata NRRL 2380 (Carnell and Willetts, 1990) which was shown to produce synthetically important chiral lactones in high yield. Grogan, Roberts and Willets (1992) investigated the claim by Taylor and Trudgill (1986) about the potential of Pseudomonas putida NCIMB 10007 as a Baeyer-Villiger catalyst. Biotransformations using this microorganism grown on (+)-camphor undertakes a different enantioselectivity towards racemic bicyclo[3.2.0]heptenone to that recorded with the monooxygenase from Acinetobacter NCIMB 9871, thus extending the range of synthons available for chemoenzymatic synthesis. Opposite enantiomers of the two regioisomers formed were observed for (+)- and (-)- camphor grown Pseudomonas putida (Wright *et al.*, 1994). An added advantage of the Baeyer-Villiger monooxygenase from P.putida was the requirement for NADH rather than NADPH as co-factor. Grogan, Roberts and Willetts (1992) were able to implement a system for co-factor recycling using the NAD-dependent formate dehydrogenase from Candida boidinii (figure 1.14)

Figure 1.14: Co-factor recycling using the NAD-dependent formate dehydrogenase from Candida boidinii.



1.8. Chiral Lactone Synthesis by Chemical Methods

Owing to their biological importance, chiral lactones have been the focus of much synthetic effort. Isolated reports have appeared in the literature whereby chemists have innduced chirality in the final lactone product by the use of chiral chemical reagents (Nagao *et al.*, 1992; Bravo, Resnati and Viani, 1985; Solladie and Matloubi-Moghadam, 1981; Sakamoto, Yamamoto and Oda, 1987). This asymmetric induction is based on the differentiation between enantiotopic groups in prochiral starting molecules, and requires critical control of the reaction conditions. This mode of synthesis has often given low yields of products, with relatively low optical purtiy.

Other chemists have reported lactone production in optically active form by transformations starting from chiral starting materials (Mori, 1974; Meyers and Mihelich, 1975; Roder *et al.*, 1980; Uematsu, Umemura and Mori, 1983; Sakuraim, Ikeda and Mori, 1988; Liu and Cohen, 1995). In these transformations, which are often lengthy and very complicated, the choice of the starting material is critical to the optical purity of the final product, and there must be little or no susceptibility to racemization during the course of the reaction. Ravid, Silverstein and Smith (1978) reported the synthesis of the pure enantiomers of several 4-alkyl (or alkenyl)-γ-lactones of known absolute configuration from glutamic acid enantiomers. Henrot, Larcheveque and Petit (1986) have also used chiral aspartic acid as a starting point for the synthesis of chiral butyrolactones in the course of their studies directed towards the synthesis of chiral pheromones. These reports are particularly interesting because these aminoacids are cheap and both the R- and S- configurations are commericially available.

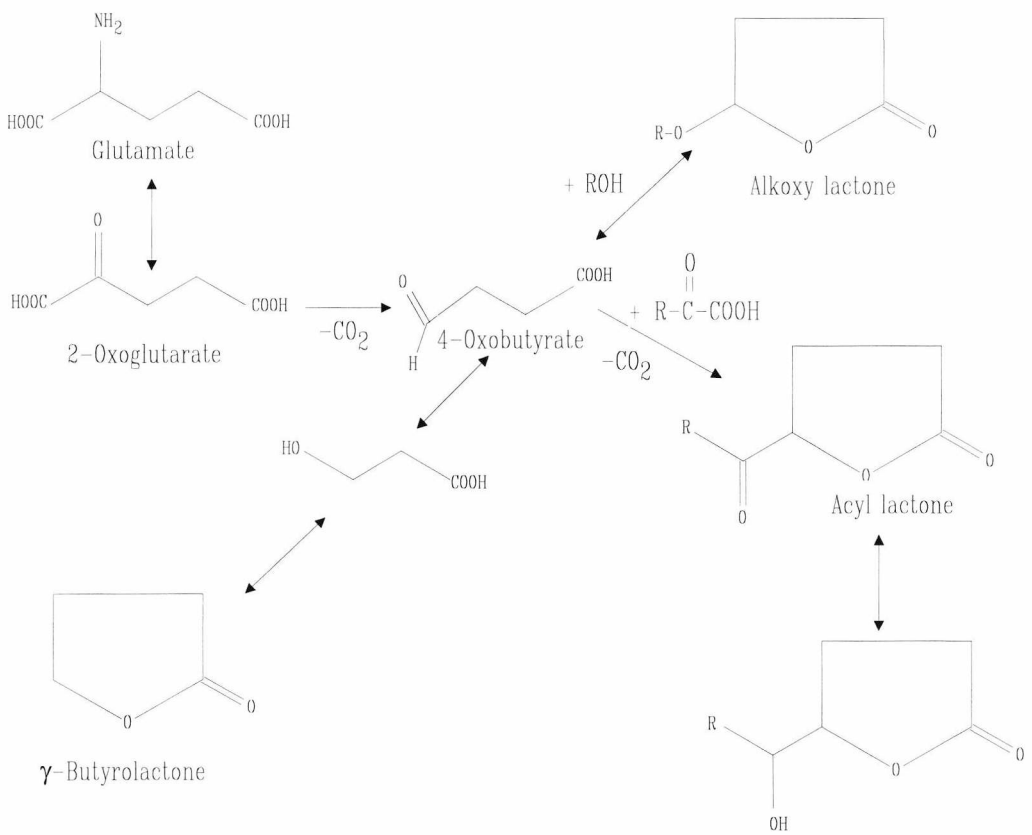
1.9. Formation of Chiral Lactones during Yeast Fermentations.

The formation of γ-butyrolactone, and a number of alkoxy and acyl lactones during yeast fermentations have been reported by Muller *et al.* (1972). These lactones which have been identified in wines, beers and sherry, are formed from simple precursors such as glutamate which are transformed via 2-oxoglutarate into 4-oxobutyrate. An alternate sequence of reactions which involves either a reduction, reaction with alcohols, or, aldolcondensation with 2-oxoacids, can lead to the formation of a variety of lactones (figure 1.15). The excretion during growth of a series of aliphatic γ- and δ- lactones by the yeast Sporobolomyces odorus has been reported (Tahara, Fujiwara and Mizutani, 1973; Tahara and Mizutani, 1975). Recently, the enantiospecific biosynthesis of R-δ-decanolactone from S- and R,S-13-hydroxy-(Z,E)-9,11-octadecadienoic acid was described by Albrecht *et al.* (1992). Chirospecific and mass spectrometric analysis of products generated from deuterium-labeled precursors have revealed an oxidation-reduction mechanism to be reponsible for the inversion of the configuration.

1.10. Formation of Chiral Lactones by Enzymatic Oxidation of Diols

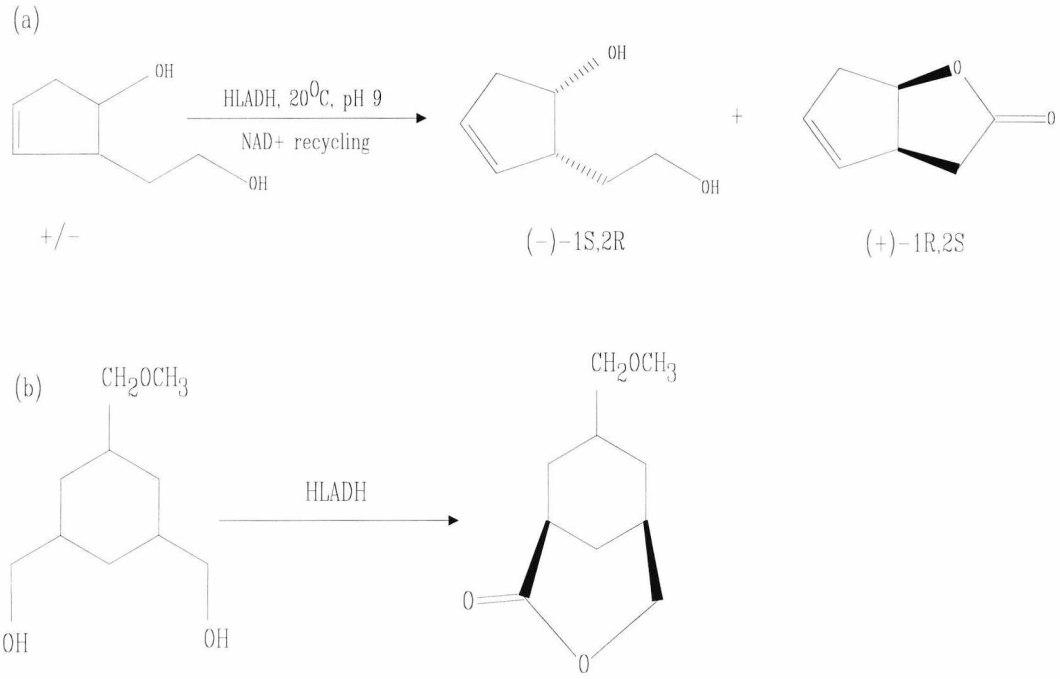
The synthetic applicability of alcohol dehydrogenases which catalyse oxidation reactions has been recognised (Jones and Beck, 1976). The most extensively studied of these enzymes is the NAD^+ dependent alcohol dehydrogenase from horse liver (HLADH).

Figure 1.15: Formation of γ-butyrolactone, alkoxy- and acyl- lactones during yeast fermentations.



This enzyme has been shown to retain its enantioselectivity while effecting regiospecific oxidation of only one of two unhindered hydroxyl groups within the same molecule (Irwin and Jones, 1977) (figure 1.16a), and has been used to effect stereoselective transformations of prochiral and mesomeric diols to form optically active monocyclic and bicyclic lactones in high enantiomeric purity (figure 1.16b) (Jakovac *et al.*, 1982; Bridges *et al.*, 1984; Lok, Jakovac and Jones, 1985).

Figure 1.16: Enantioselective and regiospecific oxidation of diols by HLADH

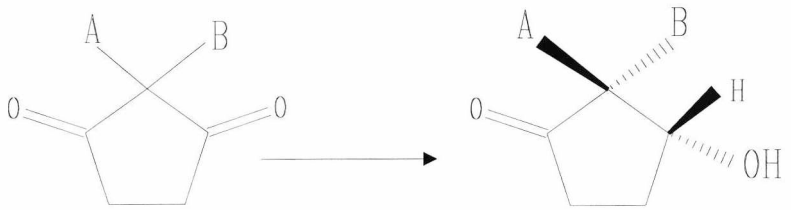


Recognising the disadvantage posed by the need for NAD^+ recycling, and the inconvinience of using whole mammalian cells for these transformations, Ohta, Tetsukawa and Noto (1982) investigated the ability of a range of Gluconobacter and Acetobacter species to catalyse diol and triol oxidations. Of the 16 strains of Gluconobacter and 9 strains of Acetobacter tested, Gluconobacter roseus IAM 1841 gave the best results with the diols

giving monocyclic lactones in up to 57% enantiomeric excess, while Gluconobacter scleroideus IAM 1842 showed better selectivity with the triols giving monocyclic lactones in up to 79% enantiomeric excess. It is believed that substituents on the prochiral center influence the rate and enantioselectivity of the oxidation in the same manner, i.e. the bulkier the substituent, the lower the rate and optical yield.

1.11. Chiral Lactone Formation by Microbial Reductions

Asymmetric microbial reductions of carbonyl-containing substrates with bakers yeast lead to optically active secondary hydroxy compounds (Akita *et al.*, 1982; Sih and Chen, 1984; Nakamura *et al.*, 1984). A number of prochiral diketones were reduced by the yeast Saccharomyces cerevisiae (Brooks, Mazdiyasni and Grothaus, 1987) yielding mono reduced ketols possessing two chiral centres.



Prochiral diketone Chiral ketol

These chiral ketols can serve as valuable starting points for the production of optically active lactones by the Baeyer-Villiger reaction. Utaka, Watabu and Takeda (1985) reported the production of optically pure R(+)-5-hexadecanolide (pheromone of the oriential hornet) via the asymmetric reduction of 5-oxohexadecanoic acid by Saccharomyces cerevisiae, and later described the asymmetric reduction of the prochiral carbonyl group of a range of

aliphatic and δ-keto acids using the same microorganism (Utaka, Watabu and Takeda, 1987). Although affecting the yield, the optical purity of the products obtained was always greater than 98% ee regardless of the fermentation conditions employed, the length of the carbon chain, or, the position of the prochiral carbonyl group. Recently, Aquino *et al.* (1991) reported the synthesis of γ- and δ- lactones via bakers yeast reduction of the corresponding keto acids. The enantioselectivity of the reduction was strongly dependent on the nature of the keto acid, with the δ-lactones always obtained in %ee's higher than the γ- lactones, and ranged from 70% to 100%.

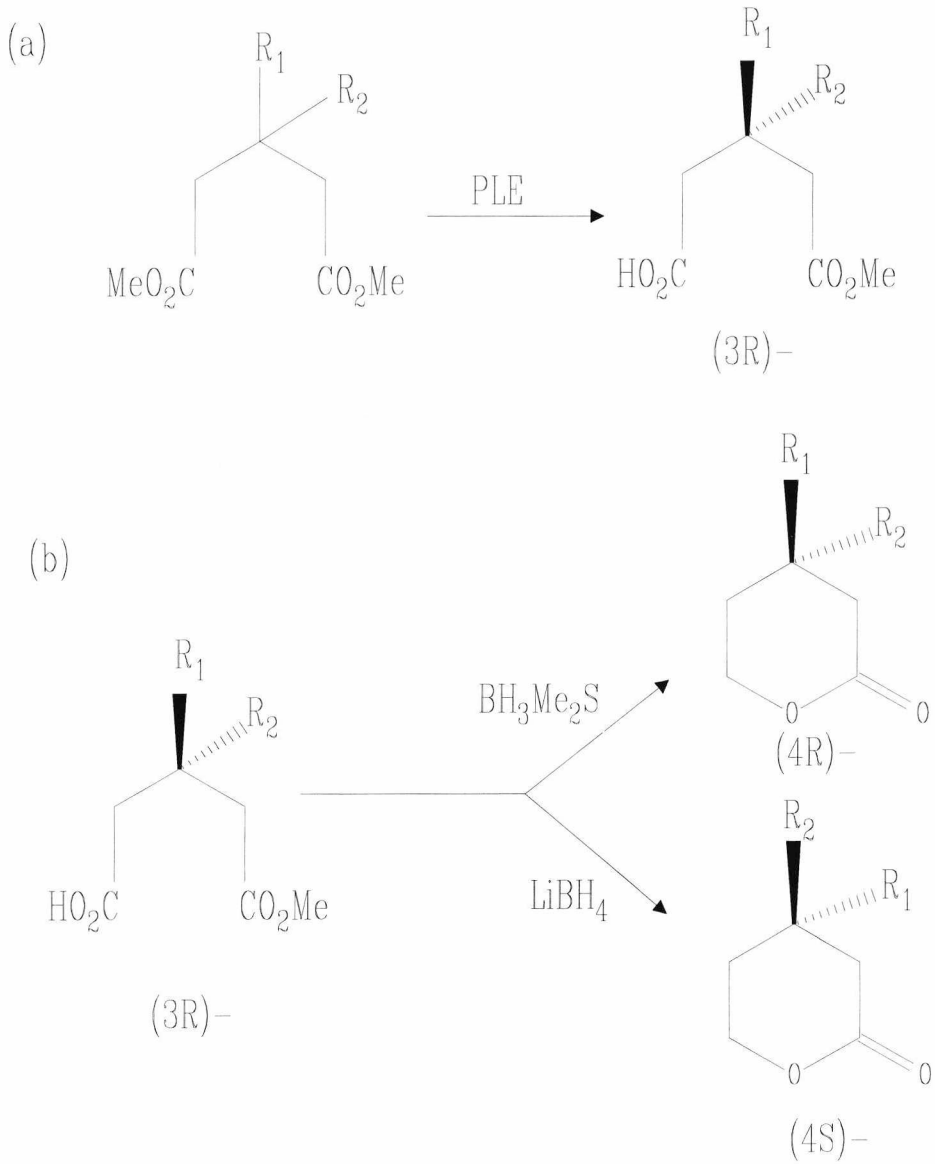
1.12. Use of Lipases and Esterases for the production of Chiral Lactones.

Commercially available hydrolytic enzymes are known to catalyze reactions with high stereoselectivity (Jones *et al.*, 1976). The possibility for the employment of these enzymes to the asymmetric synthesis of chiral lactones has been investigated by several workers, and are described here in relation to the mode of synthesis.

1.12.1. Asymmetric Hydrolysis of Diesters

Examples of enzymes that have been used for these reactions include pig liver esterase (PLE) and subtilisin Carlsberg (Francis and Jones, 1984; Bjorkling *et al.*, 1985; Sabbioni and Jones, 1987). These enzymes can catalyse reactions of the type shown in figure 1.17a. The enantiomerically enriched acid esters can then afford either of the two enantiomeric lactones by employment of different reduction conditions (figure 1.17b)

Figure 1.17: Pig liver esterase catalysed hydrolysis of prochiral diesters as a route to obtaining chiral lactones



1.12.2. Ring-Closure of Hydroxyacids

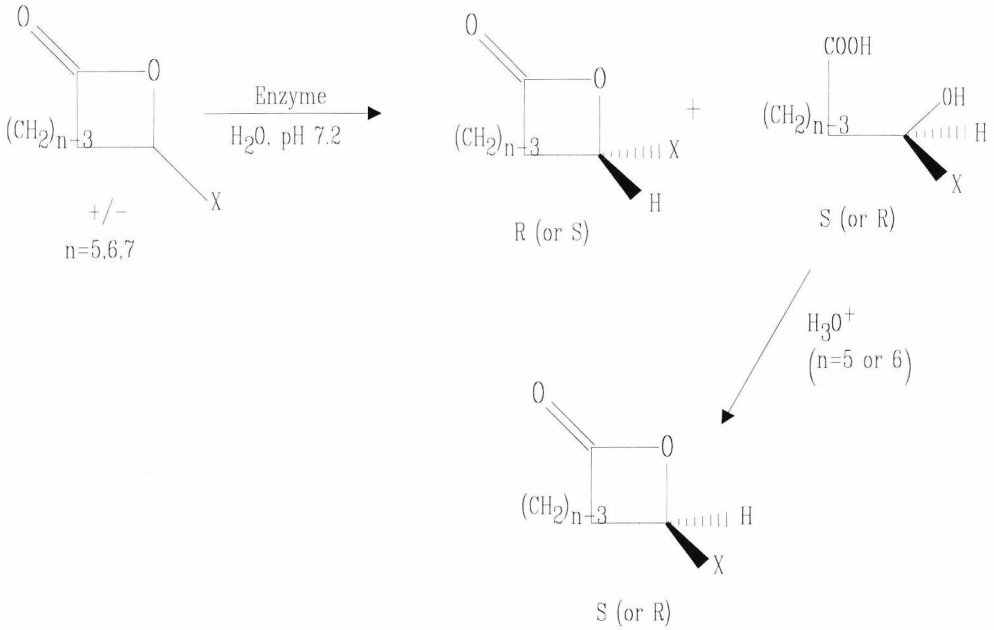
Many hydrolytic enzymes can function in mono- or bi- phasic organic media. Several factors that influence the efficiency and integrity of these enzymes and the reactions they catalyse in these media have been investigated *and* many reviews on this topic have appeared in the literature (Khmelnitsky *et al.*, 1988; Deetz and Rozzell, 1988; Dordick, 1989; Chen and Sih, 1989; Klibanov, 1989). Lipases in anhydrous organic solvents have been shown to catalyze the ring-closure esterification of methyl esters of hydroxyacids, forming lactones (Makita, Nihira and Yamada, 1987; Gutman, Zuobi, and Bravdo, 1990; Gutman, Zuobi and Boltansky, 1987; Sugai *et al.*, 1990; Robinson *et al.*, 1994). Under these conditions, the lipases exhibit both enantioselectivity and prochiral seclectivity, and this approach has been used to develop a convinient, high yielding and stereoselectivie route to several optically active γ-substituted γ-butyrolactones. Other workers have reported the enzymatic synthesis of macrocyclic lactones via intermolecular esterification in organic solvents starting with hydroxy acids or diols and diacids (Zhi-Wei *et al.*, 1988; Zhi-Wei and Sih, 1988).

1.12.3. Enzyme Catalysed Resolution of Racemic Lactones

The use of the inexpensive and commercially available hydrolases; pig pancreatic lipase (PPL) and horse and pig liver esterase (HLE and PLE), for the enantioselective hydrolysis of a range of racemic γ-, δ- and ε- lactones has been described by Blanco, Guibe-Jampel and Rousseau (1988), the main features of which are outlined in figure 1.18. For γ-

butyrolactones, the best resolutions were observed when PPL served as catalyst, with the S-configuration lactones serving as better substrates than their R- enantiomers.

Figure 1.18: Resolution of racemic lactones using esterases or lipases



For δ-valerolactones, HLE gave the best resolutions, although these lactones served as substrates for all three enzymes. In this case, enantioselectivity was inverted with the R-enantiomer serving as the better substrate. For ε-caprolactones, HLE and PLE gave satisfactory resolutions but showed opposite enantioselectivity towards the substrates. The same workers have extended the scope of their resolutions to racemic bicyclic lactones using HLE, and have obtained bicyclic lactones in up to 98% enantiomeric purity (Guibe-Jampel, Rousseau and Blanco, 1989). A similar approach has been adopted by Kataoka *et al.* (1995) for the production of optically active pantolactone using whole cells of Fusarium oxysporum. The racemic pantolactone was enantioselectively resolved into R-pantoic acid (90%ee) and S-

pantolactone. After removal of the latter, the remaining R-pantoic acid could be easily converted to R-pantolactone by heating under acidic conditions. (R)-(-)-Pantolactone is an important synthetic intermediate and chiral auxiliary reagent for asymmetric Diels-Alder reactions. Other workers have obtained this lactone in high enantiomeric excess by enantioselective hydrolysis of O-acetylpantoyl lactone using lyophilized cells of Saccharomyces cerevisiae and a lipase from Aspergillus species (Glanzer, Faber and Griengl, 1988)

1.13. Aims of the Study

The significance of lactone production in optically active form has been emphasized in the introduction of this thesis. As a result of the commercial potential of chiral lactones, numerous synthetic efforts have been employed (as highlighted previously) in the quest to achieve optically pure lactone production in high yields and with minimum cost.

Microorganisms capable of effecting Baeyer-Villiger type oxidation of ketones to lactones, which occurs during the metabolism of cyclic alcohols and ketones, have been shown to possess a lactone hydrolase, which functions to hydrolyse the formed lactone to the corresponding hydroxyacid. This facilitates complete assimilation of these metabolites via the enzymes involved in central metabolism.

In this study, the potential for the use of lactonase enzymes as enantioselective biocatalysts capable of lactone hydrolysis has been investigated. Three microorganisms possessing monooxygenase/lactonase systems have been proposed for study. These are Acinetobacter

NCIMB 9871, Rhodococcus coprophilus WT1, and Pseudomonas NCIMB 9872. Growth of these microorganisms has been conducted in batch culture *and* enzyme induction experiments carried out to establish the basal levels of enzyme activity with a view to optimising lactonase production and yield. The substrate specificity and enantioselectivity of the lactonases so obtained has been examined through a series of biotransformations of γ, δ- and ε- lactones. Lactonases exhibiting significant enantioselectivity have been studied to purify and characterise them.

Chapter 2

Materials and Methods

2.1 Microorganisms

2.1.1. Source

Acinetobacter NCIMB 9871 and Pseudomonas NCIMB 9872 were obtained from the National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen, Scotland.

Rhodococcus coprophilus WT1 was isolated by Lenn (1992) on cyclohexanol as the sole source of carbon and energy.

2.1.2 Maintenance

Acinetobacter NCIMB 9871 and R. coprophilus WT1 were maintained on 2% (w/v) oxoid purified agar containing M9 minimal salts (Miller, 1972), and $1mlL^{-1}$ of trace metals solution (Bauchop and Elsden, 1960). Cyclohexanol (10mM) was used as the source of carbon. This was filter-sterilized through a 0.25μm microfilter before being added to the autoclaved medium. For Pseudomonas NCIMB 9872, cyclopentanol replaced cyclohexanol as the source of carbon. In each case, the cultures were stored at $4^{o}C$ and subcultured at 2-monthly intervals.

2.1.3. Growth in Liquid Culture.

Microorganisms were inoculated from agar plates into 250ml Erlenmeyer flasks, which contained 50ml of a solution of M9 salts, $1mlL^{-1}$ tracemetals solution, and 10mM of either

cyclohexanol for Acinetobacter NCIMB 9871 or R. coprophilus WT1, or cyclopentanol for Pseudomonas NCIMB 9872. The cultures were incubated at 30°C in an orbital shaker at 200 revmin^{-1}. When the bacteria had grown, they were used to inoculate 2litre flasks containing 400ml of a solution of M9 salts, trace metals and either cyclohexanol or cyclopentanol, as a 5% (v/v) inoculum.

2.1.4. Estimation of Growth.

2.1.4.1. Absorbance

Growth was estimated by measuring the absorbance of the bacteria at 600nm. For all microorganisms used, exponential phase cultures obeyed the Beer-Lambert Law up to an absorbance of about 0.9 at 600nm. More concentrated samples were diluted to give absorbance readings that were less than 1.0.

2.1.4.2. Substrate utilisation

Substrate utilisation by growing bacteria was monitored by gas chromatography. Aliquots (1.5ml) were removed from growing cultures at timed intervals and the bacteria removed by centrifugation at full speed (13,000rpm) for 3minutes in a microcentrifuge. A sample (1ml) of the supernatant was transferred to a fresh Eppendorf tube and 3-cyanopyridine was added to a final concentration of 10mM. This served as an internal standard. The residual concentration of cyclohexanol, cyclohexanone, ε-caprolactone or δ-valerolactone in the

supernatant was determined by GC (see section 2.5.6), with reference to a standard response curve constructed using 2 - 10mM of each of the substrates.

2.1.5. Harvesting of bacteria

Bacteria were harvested by centrifuging at 10,000g for 10minutes at 4°C (Beckman, J2-21 centrifuge, JA14 rotor). The harvested bacteria were washed, resuspended in 50mM phosphate buffer pH7.1, and were either used immediately, or re-centrifuged, and the pellet stored at -20°C.

2.1.6. Disruption of cells

Washed bacteria, resuspended in 50mM phosphate buffer pH7.1 to give an absorbance at 600nm of approximately 20, were disrupted by sonication (Labsonic U, Braun) at 200 watts using a discontinuous cycle of 0.3second second^{-1} for a total time of 6minutes. The temperature was maintained at about 4°C by placing the sonication vessel in an ice bucket filled with ice. Cell debris was removed by centrifugation at 48,000g for 20minutes at 4°C. To obtain the high speed supernatant fraction, cell free extracts were centrifuged at 150,000g for 3 hours (Sorvall OTD, Combi) and the pellet removed. The supernatant fraction was either used immediately, or stored at -20°C.

2.1.7. Estimation of Protein

The protein contents of cell free extracts and high speed supernatants were determined using the method of Bradford (1976). Bovine serum albumin was employed as a standard.

2.2. Enzyme Assays

Unless otherwise stated, all enzyme assays were performed in triplicate and enzyme activity is expressed as μmol/min/mg protein

2.2.1 Cyclohexanone monooxygenase (Acinetobacter NCIMB 9871 and Rhodococcus coprophilus WT1)

Cyclohexanone monooxygenase was routinely assayed by measuring the decrease in absorbance at 340nm of 1ml of a mixture containing 0.1 - 0.3mg protein, 90μmol glycine-NaOH buffer pH 9.0, 0.08μmol of NADPH and 0.3μmol of cyclohexanone.

2.2.2. Cyclopentanone monooxygenase (Pseudomonas NCIMB 9872)

Cyclopentanone monooxygenase was routinely assayed by measuring the decrease in absorbance at 340nm of 1ml of a mixture containing 0.1 - 0.3mg protein, 60μmol of phosphate buffer pH7.1, 0.05μmol of NADPH and 0.2μmol of cyclopentanone.

2.2.3. Cyclohexanol dehydrogenase

Cyclohexanol dehydrogenase (Acinetobacter NCIMB 9871 and R. coprophilus WT1) was assayed by measuring the increase in absorbance at 340nm of 1ml of a mixture containing 0.1-0.3mg protein, 90μmol glycine-NaOH buffer pH10, 0.4μmol of NAD^+ and 1μmol of cyclohexanol. For cyclopentanol dehydrogenase (Pseudomonas NCIMB 9872), 1μmol cyclopentanol was used instead of cyclohexanol.

All the assays mentioned above were carried out at 30°C, and, the reaction mixtures were thoroughly shaken prior to analysis to ensure proper mixing and aeration of the reactants.

2.2.4. Lactonase

2.2.4.1. Assay by pH-dependent autotitration

ε-Caprolactone hydrolase activity was assayed by monitoring the rate of addition of 5mM NaOH from an autotitrator (Mettler), to a stirred reaction vessel (maintained at 30°C with the aid of a water bath) that contained an initial volume of 15ml distilled water adjusted to pH7, with 0.3-0.75mg of protein and 20μmol of ε-caprolactone. For δ-valerolactone hydrolase, 20μmol δ-valerolactone was used.

2.2.4.2. Assay by Gas Chromatography.

A 5ml reaction mixture was set up which contained, 250mmol tris HCl buffer pH7.1, 50μmol 3-cyanopyridine and 20μmol lactone incubated at 30°C. The reaction was started by the addition of 0.3-0.75mg of protein. Aliquots (1ml) of the reaction mixture were removed at 3 minute intervals and the reaction stopped by immersion in liquid nitrogen, and stored at -20°C. Residual lactone in the reaction mixture was determined by GC with reference to a standard response curve constructed using 0-4mM lactone prepared in the same way. 3-Cyanopyridine (10mM) was used as the internal standard.

2.2.4.3. Rapid procedure for lactonase activity determination in fractions collected during lactonase purification.

This method, carried out at room temperature, is based on the pH change that results upon lactone hydrolysis in a weakly buffered solution. A typical reaction mixture contained in 1ml, 10μmol phosphate buffer pH7.1, 0.05-0.1ml fraction from the purification process and 20μmol of lactone. The change in pH was detected using bromocresol purple as an indicator (pH range 5.2 - 6.8).

2.2.5. Confirmation of hydroxyacid production from lactone hydrolysis

High speed supernatant fraction (HSS) derived from Acinetobacter NCIMB 9871 that had been grown on cyclohexanol, was incubated with 0.5mmol ε-caprolactone in an autotitrator at pH 7 and room temperature. Progress of the reaction was followed by gas chromatography until no more ε-caprolactone could be detected. Comparison of NMR, IR and elemental

analysis of the extracted biotransformation product with that of chemically synthesized 6-hydroxyhexanoic acid (see below) confirmed that this was the product of ε-caprolactone hydrolysis.

2.2.6. Preparation of 6-hydroxyhexanoic acid

A solution of 50mmol ε-caprolactone in 100ml of 2M NaOH was incubated in a boiling water bath for 30min. After cooling to room temperature, the pH was adjusted to 4 with 2M HCl. The resultant mixture was immediately extracted into diethyl ether which was then dried over anhydrous magnesium sulphate. Excess solvent was driven off by rotary evaporation. The residual oily substance was subjected to NMR, IR and elemental analysis and confirmed to be 6-hydroxyhexanoic acid. The compound was repeatedly weighed in order to estimate the density. It was manually titrated against NaOH in order to determine the stoichiometry of neutralization.

2.3. Purification of δ-valerolactone hydrolase from *Pseudomonas* NCIMB 9872

2.3.1. Generation of Biomass

To generate sufficient biomass for enzyme purification, Pseudomonas NCIMB 9872 was grown in a 10L fermenter. A 50ml starter culture of Pseudomonas NCIMB 9872 was used to inoculate 400ml of M9 medium supplemented with cyclopentanol (10mM) as previously described. When the bacteria had reached late-exponential phase, the culture was used to inoculate 10L of M9 medium to which the following alterations had been made:

$(NH_4)_2SO_4$ at $30gl^{-1}$ instead of $3gl^{-1}$.

Double the normal trace element concentration.

Double the normal $MgSO_4/CaCl_2$ concentration.

Cyclopentanol was added to a final concentration of 10mM. The fermentation temperature was maintained at 30°C by the use of a heating pad placed at the base of the fermenter vessel. The pH was monitored but not controlled. Filter sterilised air was supplied at $3.5Lmin^{-1}$. Dissolved oxygen was monitored with the aid of an oxygen electrode attached to the control unit, and the stirrer speed was varied between 450 and 650 rpm depending on the dissolved oxygen levels. About 10ml of polypropylene glycol solution was added as an antifoam to curb excess frothing.

Growth was monitored by the absorbance at 600nm as previously described. Concentrated samples were diluted to an absorbance of less than 0.9. Substrate utilisation was monitored by packed column GC. When the concentration of cyclopentanol in the medium had dropped to below 2mM, more cyclopentanol was added to a concentration of 10mM. This was repeated several times until the absorbance of the culture was approximately 10. A final addition of about 5mM cyclopentanol was made just before harvesting.

Bacteria were harvested by centrifuging at 10,000g for 10min at 4°C. Pellets were resuspended and washed twice in 50mM phosphate buffer pH 7.1, before being stored at -20°C in a freezer.

To obtain cell free extracts (CFE), bacteria were prepared as a thick suspension in 20mM phosphate buffer pH 7.6. Bacteria were disrupted by sonication using a Braun Labsonic. This was operated using a repeat cycle of 0.3sec sec^{-1} at 200 watts. The sonication vessel was maintained at 4°C by placing in an ice bucket filled with ice. Cell breakage was monitored by

removing 1ml samples, which were centrifuged at full speed (13,000rpm) for 2min in a microfuge. The protein content and specific activity of δ-valerolactone hydrolase in the resulting supernatant was determined using protocols described previously. When cell breakage had been optimised, i.e the specific activity did not increase significantly with further sonication, the cell debris was removed by centrifugation at 48,000g for 20min at 4°C. To obtain the high speed supernatant fraction, the cell free extracts were centrifuged at 150,000g for 3 hours. The supernatant fraction was divided into equal aliquots, with each aliquot not exceeding 300mg total protein, and were either used immediately or stored at -20°C.

2.3.2. Anion Exchange Chromatography

Samples were applied at a flow rate of $1 ml min^{-1}$ to a pre-packed Hiload Q- Sepharose HP 16"/10 column (Pharmacia, Sweden) that had been equilibrated in 20mM phosphate buffer pH 7.5. The binding pH for δ-valerolactone hydrolase was crudely estimated using the protocol described below:

A 0.5ml wet volume of Q- sepharose matrix was placed in each of 10 test tubes. The matrix was equilibrated by washing 10 times in high ionic strength buffer (0.5M) with each of the tubes set up at a different pH ranging from pH 6.5 - 8.5. The matrix was then washed 10 times in a low ionic strength buffer (0.02M) at the pre-selected pH. About 0.5mg protein was added to each tube and mixed continuously for 10 min. Upon settling, the supernatant in each tube was assayed for lactonase activity by using the pH dependent autotitrator assay described previously.

2.3.3. Elution of δ-valerolactone hydrolase from Q- sepharose columns.

The column loaded with protein was washed with 40ml of the binding buffer, after which it was eluted with a linear gradient of 0 to 0.3M KCl, total volume 60ml; 2 ml fractions were collected. δ-Valerolactone hydrolase activity was assayed using the rapid pH-dependent colorimetric assay described earlier, and occurred in fractions corresponding to a salt concentration of 0.1-0.15M KCl. Fractions containing δ-valerolactone hydrolase activity were pooled.

2.3.4. Hydrophobic Interaction Chromatography (Butyl sepharose 4B)

2.3.4.1 Column

Butyl sepharose 4B matrix was purchased from Pharmacia and packed in a 16"/10 Pharmacia column. The bed volume was $10cm^3$, and was equilibrated in 50mM phosphate buffer pH 7.0 containing 1M $(NH_4)_2SO_4$.

2.3.4.2 Sample preparation and application

The ionic strength of pooled fractions from the Q- sepahrose anion exchange step was increased by the addition of $(NH_4)_2SO_4$ solution to give a final concentration of 1M. This was applied (at a flow rate of $1mlmin^{-1}$) to the equilibrated butyl sepharose column. The column with bound protein was washed with 50ml of phosphate buffer containing 0.4M $(NH_4)_2SO_4$ before being eluted with a linear gradient of 0.4 to 0.2 M $(NH_4)_2SO_4$ over 20min. Fractions (1ml) were collected and screened for lactonase activity. Fractions exhibiting the

desired activity were pooled, desalted and eventually concentrated using centriplus centrifugal concentrators obtained from Amicon.

2.4. Enzyme characterisation

2.4.1. Molecular weight determination.

2.4.1.1. Gel filtration chromatography.

A Superose 12 HR 10/30 gel filtration column (Pharmacia) was equilibrated at room temperature in 50mM phosphate buffer pH7. The column was operated at a flow rate of $0.75 ml min^{-1}$. The void volume (V_o) and total bed volume (V_t) were determined using blue dextran (mwt 2,000,000) and DNP-lysine mwt (349) respectively. The column was calibrated using the following marker proteins obtained from Sigma; cytochrome C, carbonic anhydrase, bovine serum albumin, alcohol dehydrogenase, and β-amylase. Pure δ-valerolactone hydrolase (200µl, $1 mg ml^{-1}$) was applied to the column, which was run under the same conditions. The molecular weight of the purified protein was determined by reference to a calibration chart constructed from the retention volumes of the molecular weight standards.

2.4.1.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This was done on a Midget Gel Electrophoresis system (LKB, Sweden) using a modified version of the discontinuous gel and buffer procedure of Laemeli (1970).

2.4.1.2.1. Gel Preparation

Gels were cast in a Midget Multicast Gel Casting Stand and were either 0.75mm or 1.5mm thick. Each comprised a 5% acrylamide stacking gel, pH6.8 and a 10% acrylamide resolving gel pH 8.8, both with added 10% sodium dodecyl sulphate (see appendix). Gel polymerization was initiated by the addition of ammonium persulphate and TEMED.

2.4.1.2.2. Sample Preparation

Samples (1mg/ml), mixed with loading buffer containing SDS and bromophenol blue (see appendix) in 5:1 ratio, and molecular weight marker proteins (BDH), were prepared for electrophoresis by boiling for 5min in a water bath, before being loaded (50µl) on to the gel.

2.4.1.2.3. Running the Gel

Samples were run on the gel in tris-glycine electrode buffer pH 8.3, containing SDS (see appendix) at a constant current of 20 milliamps, and an initial voltage of 100volts. When the samples had reached the resolving gel, the voltage was increased to 120volts. The gels were kept cool by passage of cold tap water through the jacket of the electrophoresis tank. Gels were run till the dye front reached the end of the resolving gel. Proteins were visualized by staining the gels with 0.0025% w/v coomassie brilliant blue R-250 dissolved in methanol:water:acetic acid (45:45:10) solution (see appendix), for 2 hours, followed by destaining with the same solution but without coomassie brilliant blue.

2.4.1.3. Native (non-denaturing) gels

Native gels were run to check the possibility of the existence of different conformational forms of δ-valerolactone hydrolase. The protocol for gel and sample preparation were similar to that for denaturing gels except that SDS was not added to gels, the loading buffer, or the electrode buffer, and, samples were not boiled before loading onto the gel.

2.4.2. Isoelectric point (pI) determination

Isoelectric point determination was performed by Mark Rendull of the Tissue Culture department, Research School of the Biosciences, University of Kent at Canterbury. This was done on Ampholine PAGplate (Pharmacia), calibrated with pI Calibration Kit Electran (wide range) pI 4.7 - 10.6 (BDH) The sample requirement was 20μl of $1 mgml^{-1}$ protein in salt free phosphate buffer.

2.4.3. N-terminal sequencing

N-terminal sequencing was performed by the Edman degradation using an Applied Biosystems 492 protein sequencer.

2.4.3.1. Sample Preparation

To facilitate sequencing, δ-valerolactone hydrolase was transferred to a polyviyrolidienediflouride (PVDF) membrane (problott) obtained from Applied Biosystems using a LKB 2051 Midget Multiblot Electrophoretic Transfer Unit. To do this, δ-

valerolactone hydrolase and pre-stained molecular weight markers obtained from Sigma were subject to SDS-PAGE as previously described. However, rather than being stained, the gel was soaked in 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) buffer pH11 (electroblotting buffer) for 5min, before being assembled as part of a transblotting sandwich. The transblotting sandwich comprised, proceeding from cathode to anode, a support mesh, a foam spacer, a sheet of Whatman No. 1 filter paper, the pre-run gel, a problot PVDF membrane, a sheet of filter paper, a foam spacer, and a support mesh. The transblotting sandwich was placed in the electroblott tank which contained electroblotting buffer stirred on a magnetic stirrer, and, cooled by the passage of cold tap water through a cooling jacket. Electrophoretic transfer of protein from the gel to the PVDF membrane was achieved at a constant voltage of 50volts (170mA - 100mA), for 30 minutes. After the transfer was complete, the PVDF membrane was saturated with 100% methanol for 10sec before being stained with 0.1% Coomassie Blue R-250 in 40% methanol v/v, 1% acetic acid solution for 1min. The PVDF membrane was destained with 50% methanol and rinsed extensively with de-ionised water. The band of interest was excised from the PVDF membrane and was subject to N-terminal sequencing

2.4.4. Determination of Kinetic Parameters

2.4.4.1. pH profile

A pH profile was done to determine the pH optimum for δ-valerolactone hydrolase from Pseudomonas NCIMB 9872. The standard autotitrator pH-stat assay and the GC assay (both described earlier) were used at controlled pH values ranging from 5-10 with the temperature

maintained at 30°C. For the GC assay, a suitable range of 0.2M buffers with pKa's ranging from 5-10 were employed (see appendix).

2.4.4.2. Temperature profile

A temperature profile was done to determine the optimum temperature for δ-valerolactone hydrolase activity. Enzymes were assayed using the autotitrator at controlled temperature values ranging from 20°C - 55°C with the pH maintained at 7.

2.4.4.3. K_m and V_{max} determination.

The K_m and V_{max} of δ-valerolactone hydrolase for δ-valerolactone was determined using the standard autotitrator assay at pH 7.0 and 30°C. The lactone concentrations used varied from 0.005mM-0.1mM. Each assay contained 50µl of 0.1mgml^{-1} protein.

2.4.4.4. Effect of Inhibitors

For each inhibitor studied the standard autotitrator assay for δ-valerolactone hydrolase was used with a 15ml reaction volume and 0.05mg of enzyme. The reaction mixture containing all the components with the exception of the δ-valerolactone was preincubated for 5min at pH 7 and at 30°C. Reactions were started by the addition of 20µmol of δ-valerolactone, and the reaction rates were compared with controls run under identical conditions (without the inhibitor)

2.5. Biotransformations

All biotransformations were carried out with the aid of an autotitrator.

2.5.1. Acinetobacter NCIMB 9871 and Rhodococcus coprophilus WT1

Biotransformations were carried out using cell free extracts derived from either microorganism grown on cyclohexanol. For each microorganism, the bacteria were harvested during the late-exponential phase of growth. Harvested bacteria were disrupted by sonication, as previously described, and, the cell debris removed by centrifugation. The cell free extract obtained was either used immediately for biotransformations, or stored at -20°C in the freezer.

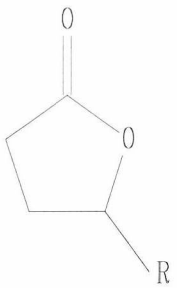
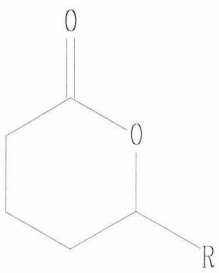
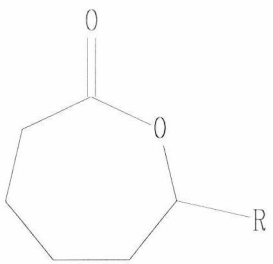
2.5.2. Pseudomonas NCIMB 9872

Biotransformations were carried out using cell free extracts or purified δ-valerolactone hydrolase derived from Pseudomonas NCIMB 9872 grown on cyclopentanol.

In all cases, biotransformations were carried out at 30°C, and, the pH maintained at 7.0 by the controlled addition of 5mM NaOH (unless otherwise specified) to an unbuffered reaction mixture, containing approximately 100μmol of the lactone substrate, and 3-5mg of cell free extract or 0.1mg of pure enzyme in 15ml of double distilled water previously adjusted to pH 7.0.

2.5.3. Investigation of substrate specificity

The following substrates were tested for their ability to stimulate lactonase activity in cell free extracts derived from Acinetobacter NCIMB 9871, R. coprophilus WT1 and Pseudomonas NCIMB 9872.

5-Membered ring lactones 	γ-butyrolactone (R=H) γ-valerolactone ($R=CH_3$) γ-caprolactone ($R=C_2H_5$) γ-octanoic lactone ($R=C_4H_9$) γ-decanolactone ($R=C_6H_{13}$)
6-membered ring lactones 	δ-valerolactone (R=H) δ-nonalactone ($R=C_4H_9$) δ-decanolactone ($R=C_5H_{11}$) δ-dodecanolactone ($R=C_7H_{15}$)
7-membered ring lactones 	ε-caprolactone (R=H) ε-decanolactone ($R=C_4H_9$)

2.5.4. Extraction of products from reaction mixtures

When the biotransformation had run for the desired time, the biotransformation mixture was saturated with sodium bicarbonate and the residual unreacted lactone was extracted into diethyl ether. The pH of the residual aqueous phase (now containing only the hydroxyacid product) was lowered to pH2 using 5M HCl and stirred for 15-30 minutes. This resulted in recyclisation of the hydroxyacid product back to the lactone and facilitated easy extraction into diethyl ether. Extracts were pooled and dried over anhydrous magnesium sulphate or sodium sulphate, before concentration by rotary evaporation.

2.5.5. Chiral Capillary Gas Chromatography

Chiral analysis of biotransformation products were carried out on a Chiraldex G-TA fused silica capillary column (0.25mm x 50, Technicol Ltd., USA). The operating conditions were; oven temperature 135°C, injector/FID detector temperature 250°C, Helium carrier gas 1.4 bar, split ratio 1:75.

2.5.6. Packed Column Gas Chromatography

Cyclohexanol, cyclohexanone, ε-caprolactone and δ-valerolactone concentrations were determined by GC, using a packed glass column (4mm X 1.52m) which contained Porapak PS (80 - 100mesh) (Jones Chromatography). The operating conditions were: oven temperature 225°C, injector/detector temperature 250°C, Helium carrier gas 60mlmin^{-1}.

2.5.7. Nuclear magnetic resonance

Compounds were characterised using 270mhz ^{1}H nuclear magnetic resonance spectroscopy (GX270, JEOL). Tetramethyl silane was used as a reference

Chapter 3

Growth of the organisms and induction of lactonase activity

3.1. Growth of the organisms and basal levels of lactonase activity

3.1.1. Acinetobacter NCIMB 9871

Acinetobacter NCIMB 9871 was maintained on cyclohexanol supplemented agar plates. Growth of this organism in a minimal salts liquid medium was rapid when cyclohexanol was supplied as the sole source of carbon and energy. There was a short lag phase of 1-2 hours, after which exponential growth occurred (figure 3.1). The stationary phase was attained after about 9 hours, with a maximal absorbance at 600nm of 1.5, when 10mM cyclohexanol was supplied to the medium. This corresponded to a dry weight of about $0.38gl^{-1}$. GC analysis of cyclohexanol in the medium showed that its concentration decreased during the exponential phase of growth, and it was completly depleted at the onset of the stationary phase. There was no buildup of cyclohexanone or any other intermediate of cyclohexanol metabolism during growth on this substrate, when monitored by GC.

When cyclohexanone was supplied to the medium as the sole source of carbon and energy, the growth curve obtained was similar to that for growth on cyclohexanol, with a short lag phase of about 1-2 hours followed by an exponential phase. Growth typically lasted about 10 hours when 10mM cyclohexanone was supplied as carbon source, and, the maximum absorbance at 600nm achieved was about 1.4 (figure 3.2). GC analysis of residual cyclohexanone in the medium showed that it was consumed during exponential growth. There was no buildup of cyclohexanol or any intermediates of cyclohexanone metabolism when analysed by GC.

Figure 3.1: Growth of Acinetobacter NCIMB 9871 on cyclohexanol as sole source of carbon.

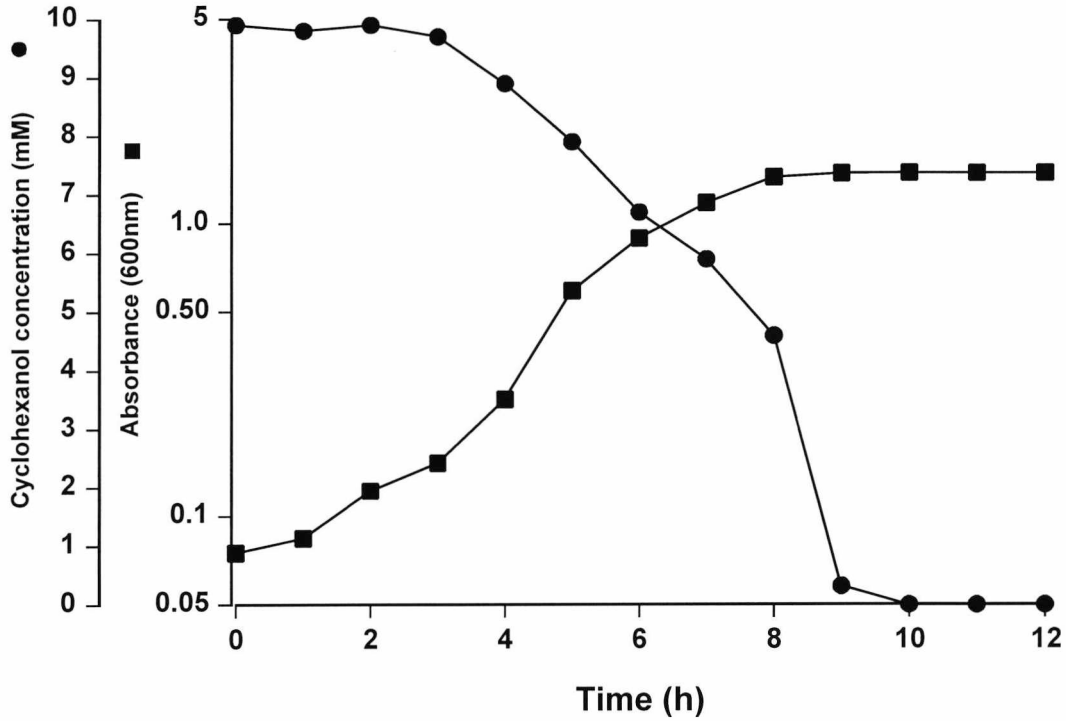
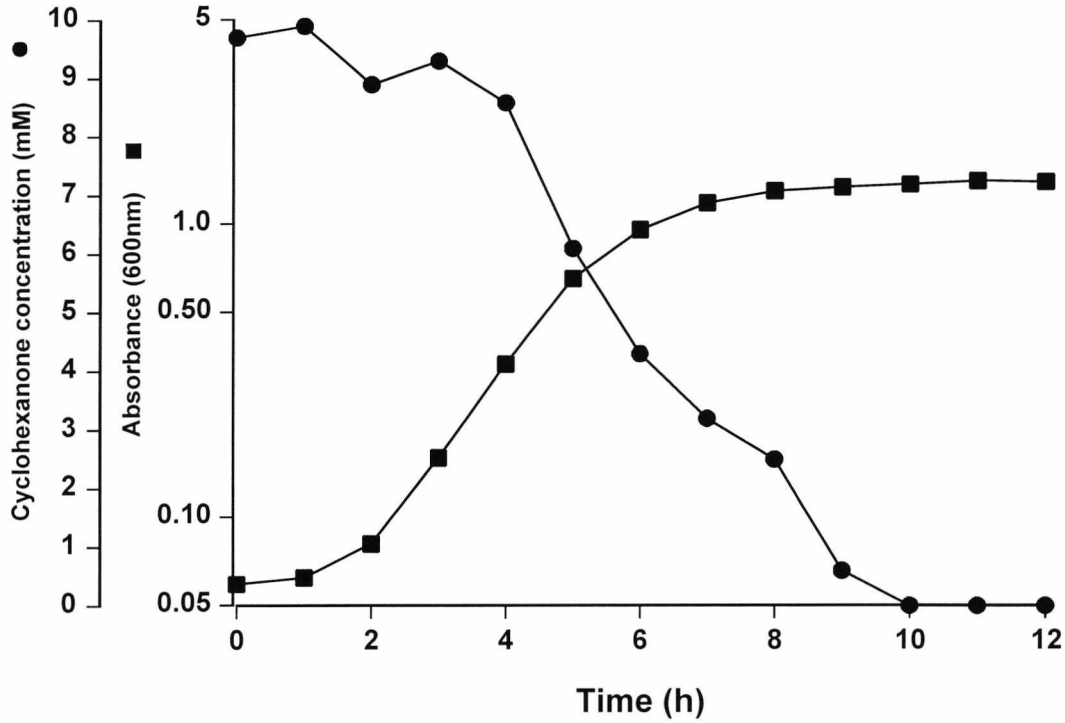


Figure 3.2: Growth of Acinetobacter NCIMB 9871 on cyclohexanone as sole source of carbon.



Acinetobacter NCIMB 9871 did not show appreciable growth on 10mM ε-caprolactone, taking about 24 hours to achieve an absorbance at 600nm of about 0.5. GC analysis of residual ε-caprolactone showed only a small decrease in ε-caprolactone concentration in the medium.

3.1.2. Pseudomonas NCIMB 9872

Pseudomonas NCIMB 9872 was maintained on cyclopentanol supplemented agar plates. Growth of this microorganism in a minimal salts liquid medium was rapid when cyclopentanol was supplied as the sole source of carbon. There was a short lag phase of about 1 hour after which exponential growth occurred. Growth lasted about 10-12 hours on 10mM cyclopentanol with a maximum absorbance at 600nm of about 1.6 (figure 3.3). This corresponded to a dry weight of approximately $0.4gl^{-1}$. GC analysis of residual cyclopentanol in the medium showed its consumption during growth. The decrease in cyclopentanol was most rapid towards the late exponential phase of growth. No formation of cyclopentanone or δ-valerolactone could be detected in the medium.

Cyclopentanone illicited a similar growth profile to cyclopentanol in Pseudomonas NCIMB 9872 when supplied as sole carbon source (figure 3.4). The lag phase lasted about 1 hour, after which exponential growth occurred. Growth lasted about 11 hours when 10mM cyclopentanone was supplied as the carbon source. Absorbance at 600nm for fully grown cultures was about 1.5. GC analysis of residual cyclopentanone in the medium showed

Figure 3.3: Growth of Pseudomonas NCIMB 9872 on cyclopentanol as sole source of carbon.

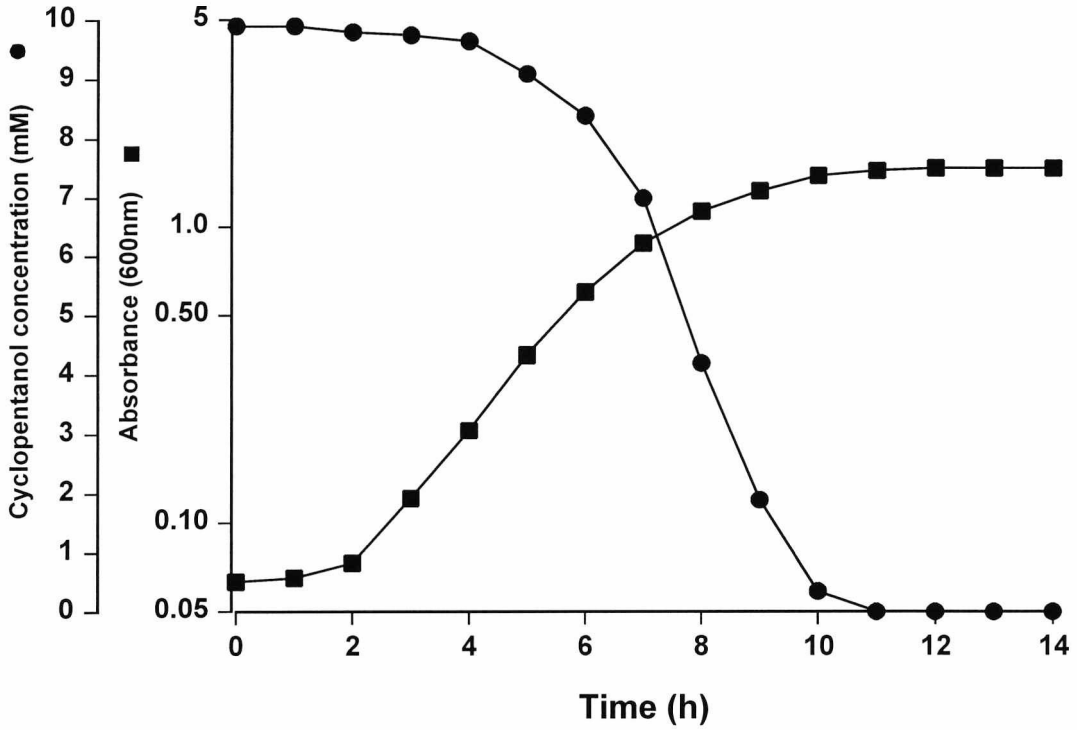
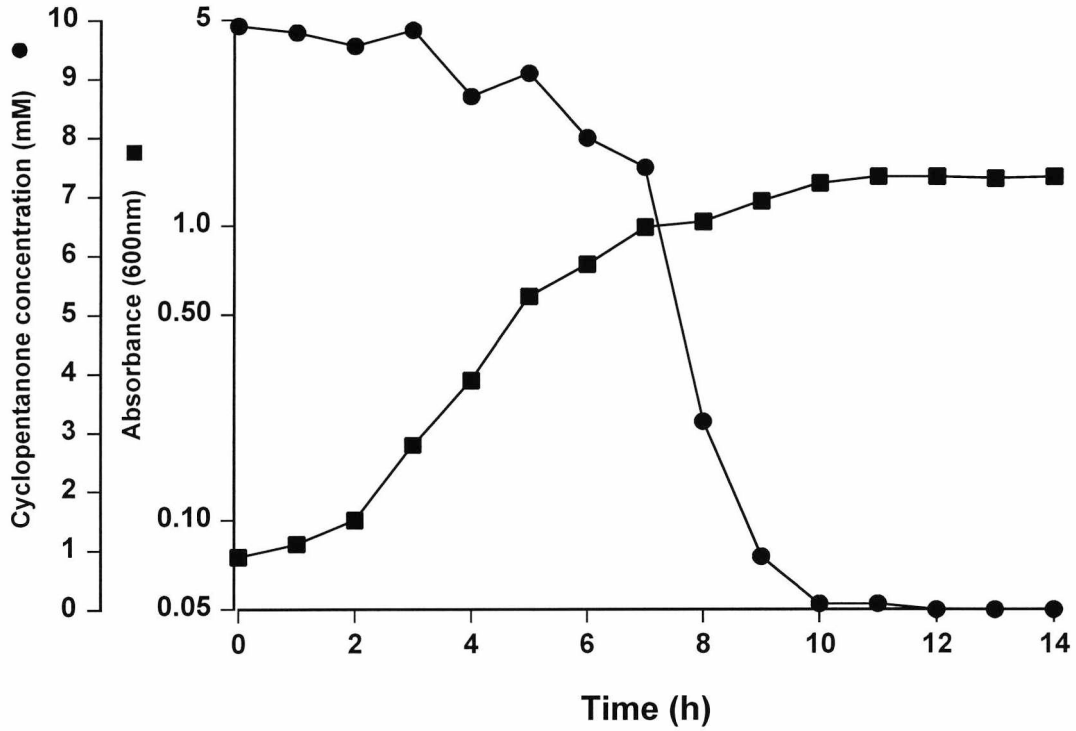


Figure 3.4: Growth of Pseudomonas NCIMB 9872 on cyclopentanone as sole source of carbon.



complete consumption of this substrate with growth. No intermediary metabolites were detected during growth on this substrate.

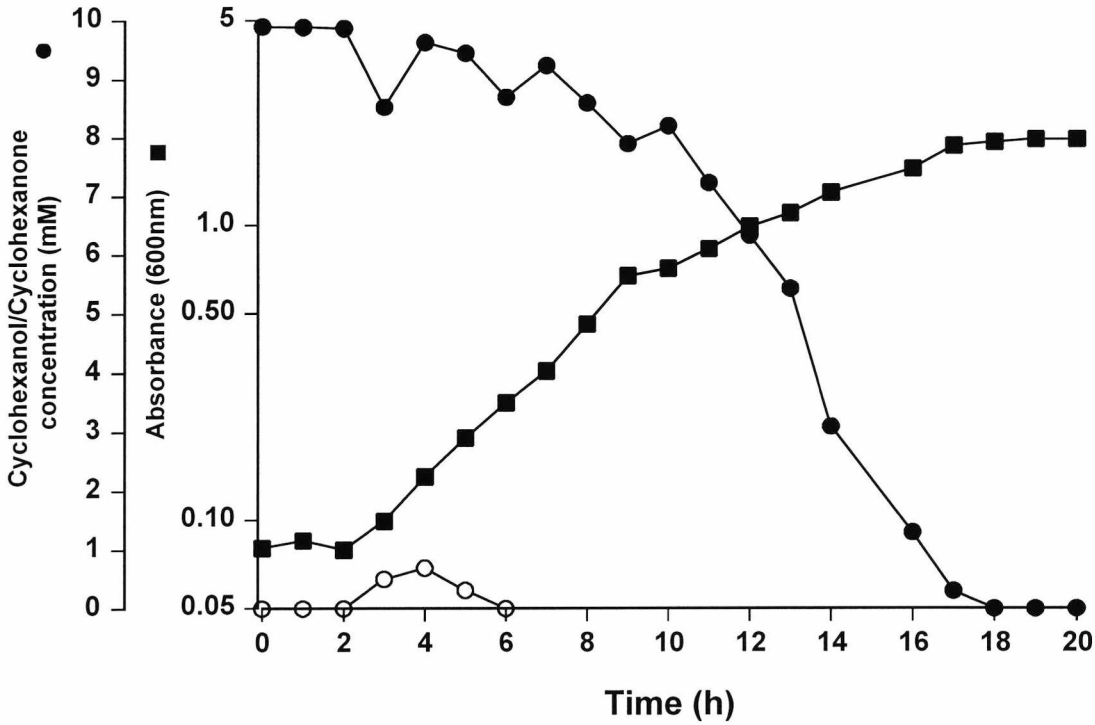
δ-Valerolactone did not support good growth of Pseudomonas NCIMB 9872. Growth on this substrate yielded an absorbance at 600nm of about 0.4 after 28 hours. GC analysis of residual δ-valerolactone in the medium showed that this substrate had hardly been consumed at this stage.

3.1.3. Rhodoccus coprophilus WT1

Rhodococcus coprophilus WT1 was isolated in our laboratory by Lenn (1992) and maintained on nutrient agar plates. Growth of this microorganism in a minimal salts liquid medium on cyclohexanol is shown in figure 3.5. This microorganism exhibited a short lag phase of about 3 hours after which exponential growth occurred. Growth lasted about 16-18 hours with a maximum absorbance at 600nm of 2.0 when 10mM cyclohexanol was supplied as source of carbon and energy. GC analysis of residual cyclohexanol in the medium showed complete utilisation of the growth substrate as the bacteria entered into the stationary phase. In some growth experiments trace amounts of cyclohexanone (up to 0.6mM) were detected in the medium early in the exponential phase of growth.

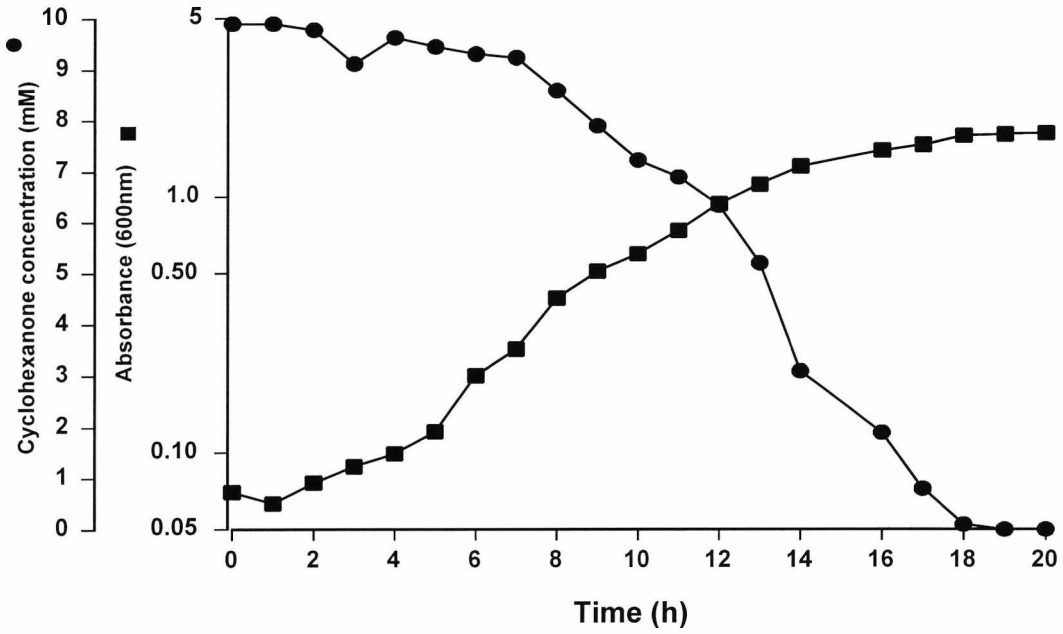
Growth on cyclohexanone (figure 3.6) was similar to growth on cyclohexanol, with a short lag phase of about 2 hours followed by exponential growth. Cyclohexanone

Figure 3.5: Growth of Rhodococcus coprophilus WT1 on cyclohexanol as sole source of carbon.



o - Cyclohexanone (mM)

Figure 3.6: Growth of Rhodococcus coprophilus WT1 on cyclohexanone as sole source of carbon.



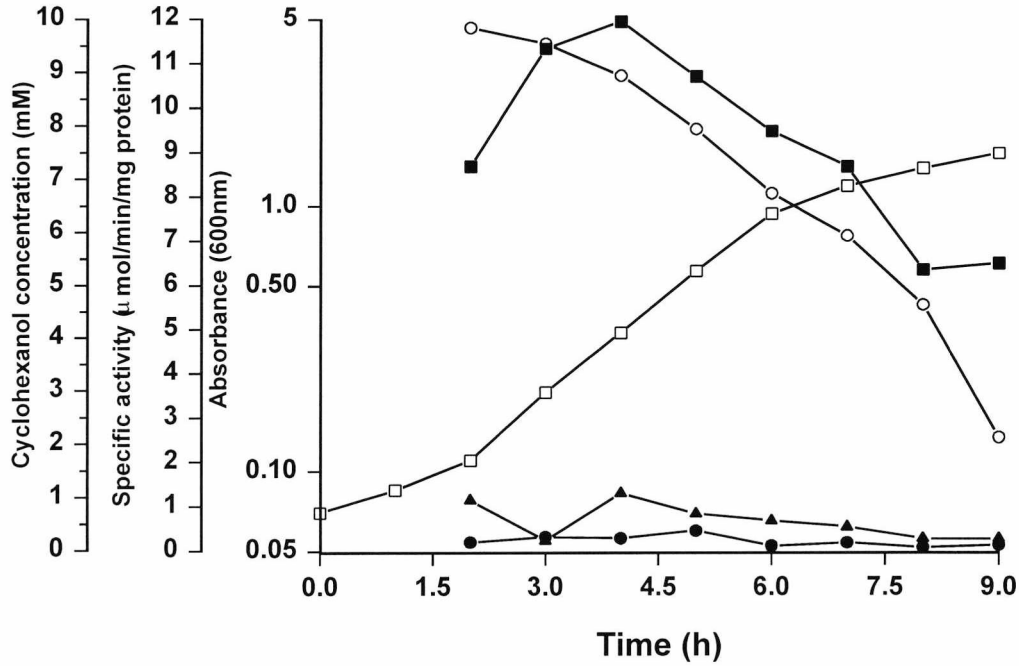
(10mM) supported growth for about 17 hours with a maximum absorbance at 600nm of about 1.8. GC analysis of residual cyclohexanone showed its complete consumption by the end of the exponential growth phase. No intermediary metabolites were detected during growth on this substrate.

3.2. Enzyme Induction Profile during Cyclohexanol and Cyclopentanol metabolism

Figures 3.7 and 3.8 show the activity of cyclohexanol dehydrogenase, cyclohexanone monooxygenase and ε-caprolactone hydrolase in cell free extracts derived from Acinetobacter NCIMB 9871 and R. coprophilus WT1 during growth on cyclohexanol respectively. In both microorganisms lactonase activity was induced throughout growth and was 20-30 fold greater than either the dehydrogenase or monooxygenase activities. In Acinetobacter NCIMB 9871, specific activities for cyclohexanol dehydrogenase and cyclohexanone monooxygenase were fairly constant throughout growth, whereas the lactonase specific activity increased rapidly during the early exponential phase of growth, and tailed off towards the end of the exponential phase. In R. coprophilus WT1, the specific activities of cyclohexanone monooxygenase and ε-caprolactone hydrolase increased during the exponential phase of growth, but tailed off towards the end, when cyclohexanol had been almost depleted from the growth medium. Cyclohexanol dehydrogenase activity could not be demonstrated during growth of R. coprophilus WT1 on cyclohexanol.

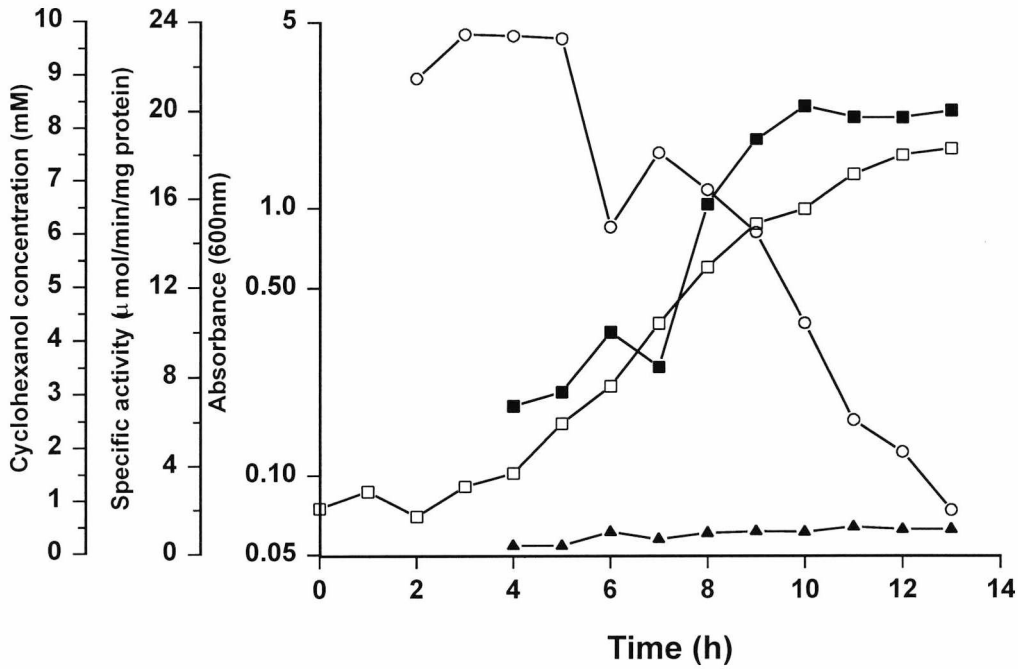
Pseudomonas NCIMB 9872 showed a similar growth profile when grown on cyclopentanol (figure 3.9). δ-Valerolactone hydrolase (lactonase) activity was about 15-20 fold greater

Figure 3.7: Enzyme induction profile of key enzymes involved in cyclohexanol metabolism by Acinetobacter NCIMB 9871 during growth on this compound



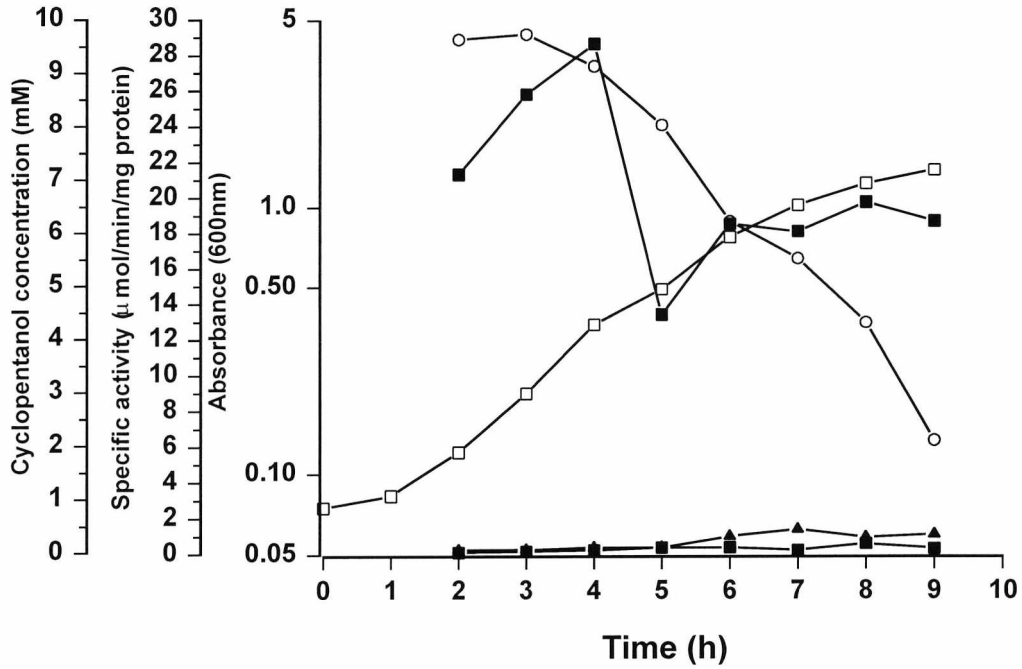
□- Absorbance at 600nm , o - cyclohexanol, ● - cyclohexanol dehydrogenase, ▲- cyclohexanone monooxygenase, ■ - ε-caprolactone hydrolase (lactonase)

Figure 3.8: Enzyme induction profile of key enzymes involved in cyclohexanol metabolism by Rhodococcus coprophilus WT1during growth on this compound.



□- Absorbance at 600nm, o - cyclohexanol, ▲- cyclohexanone monooxygenase, ■ - ε-caprolactone hydrolase. Cyclohexanol dehydrogenase activity was not detected.

Figure 3.9: Enzyme activity profile of key enzymes involved in cyclopentanol metabolism by Pseudomonas NCIMB 9872 during growth on this compound.



□- Absorbance at 600nm, o - cyclopentanol, ● - cyclopentanol dehydrogenase, ▲- cyclopentanone monooxygenase, ■ - δ-valerolactone hydrolase

than the cyclopentanol dehydrogenase and cyclopentanone monooxygenase activities throughout growth. Specific activities for all three enzymes increased during the exponential phase of growth tailing off towards the end of the exponential phase when cyclopentanol concentration in the medium became limiting.

3.3. Induction of enzymes responsible for cyclohexanol or cyclopentanol metabolism.

For measurements of basal (non-induced) enzyme activities, p-hydroxybenzoate was used as a growth substrate since none of the microorganisms showed appreciable growth when glucose or any of the following compounds were used as a source of carbon: citrate, glycerol, lactate or acetate. Cells were harvested in the late-exponential phase of growth and the specific activities of the relevant enzymes in the high speed supernatant (HSS) fraction were compared with the HSS derived from bacteria grown to the late exponential phase on cyclohexanol, cyclohexanone or ε-caprolactone (cyclopentanol, cyclopentanone or δ-valerolactone in the case of Pseudomonas NCIMB 9872). In all three microorganisms, the relevant enzyme activities were not detectable when p-hydroxybenzoate was used as the source of carbon and energy for growth.

In Acinetobacter NCIMB 9871, growth on cyclohexanol resulted in a greater than 100-fold increase in all three specific activities. Cyclohexanol dehydrogenase activity observed in cyclohexanone grown cultures, was about one-tenth the activity observed in cyclohexanol grown cultures. In both cultures, ε-caprolactone hydrolase activity was about 10 times the activity of cyclohexanone monooxygenase (Table 3.1). ε-Caprolactone did not stimulate

Table 3.1: Synthesis of key enzymes involved in cyclohexanol metabolism by Acinetobacter NCIMB 9871.

Carbon source	Cyclohexanol dehydrogenase μmol/min/mg protein	Cyclohexanone monooxygenase μmol/mim/mg protein	ε–caprolactone hydrolase μmol/min/mg protein
Cyclohexanol	0.37	0.84	9.61
Cyclohexanone	0.037	0.79	9.2
ε-caprolactone	0.030	0.007	3.2
p-hydroxybenzoate	ND	0.001	0.0013

The bacteria were grown on 10mM of the indicated substrate and were harvested in the mid-late exponential phase of growth. Key enzymes were assayed in the high speed supernatant fraction as detailed in the Methods section.
(ND = not detected)

significant activity of the appropriate dehydrogenase or monooxygenase, when used as growth substrate for this microorganism.

For Pseudomonas NCIMB 9872, growth on cyclopentanol also resulted in a significant increase in specific activity of cyclopentanol dehydrogenase, cyclopentanone monooxygenase and δ-valerolactone hydrolase activity when compared to p-hydroxybenzoate grown cultures. Growth on cyclopentanone also resulted in a significant increase in the specific activity of the three assayed enzymes, while δ-valerolactone grown cultures did not show significant dehydrogenase or monooxygenase activity (Table 3.2). δ-Valerolactone hydrolase activity was observed in these cultures albeit of a lower magnitude than that observed in cyclopentanol and cyclopentanone grown cultures.

No cyclohexanol dehydrogenase acitivity was detected in HSS from R. Coprophilus WT1 when grown on any of the carbon sources (Table 3.3). However cyclohexanone monooxygenase activity and ε-caprolactone hydrolase activity from cyclohexanol and cyclohexanone grown cultures were greater than 100 times that observed when p-hydroxybenzoate was used as the carbon source. ε-Caprolactone grown cultures exhibited only ε-caprolactone hydrolase activity.

Table 3.2: Synthesis of key enzymes involved in cyclopentanol metabolism by Pseudomonas NCIMB 9872.

Carbon source	Cyclopentanol dehydrogenase μmol/min/mg protein	Cyclopentanone monooxygenase μmol/mim/mg protein	δ-valerolactone hydrolase μmol/min/mg protein
Cyclopentanol	0.25	0.65	13.4
Cyclopentanone	0.14	0.43	8.1
δ-valerolactone	0.030	0.018	2.53
p-hydroxybenzoate	ND	ND	ND

The bacteria were grown on 10mM of the indicated substrate and were harvested in the mid-late exponential phase of growth. Key enzymes were assayed in the high speed supernatant fraction as detailed in the Methods section.
(ND = not detected)

Table 3.3: Synthesis of key enzymes involved in cyclohexanol metabolism by Rhodococcus coprophilus WT1.

Carbon source	Cyclohexanol dehydrogenase μmol/min/mg protein	Cyclohexanone monooxygenase μmol/mim/mg protein	ε–caprolactone hydrolase μmol/min/mg protein
Cyclohexanol	ND	0.43	15.4
Cyclohexanone	ND	0.54	12.3
ε-caprolactone	ND	ND	1.32
p-hydroxybenzoate	ND	ND	ND

The bacteria were grown on 10mM of the indicated substrate and were harvested in the mid-late exponential phase of growth. Key enzymes were assayed in the high speed supernatant fraction as detailed in the Methods section.
(ND = not detected)

Chapter 4

Purification and Characterisation of δ-Valerolactone hydrolase from Pseudomonas NCIMB 9872

4.1. Purification of δ-valeroactone hydrolase from *Pseudomonas* NCIMB 9872

4.1.1 Generation of biomass

In order to achieve quantitative purification of δ-valerolactone hydrolase from Pseudomonas NCIMB 9872, large quantities of the enzyme had to be obtained. Since this enzyme was induced throughout growth on cyclopentanol, Pseudomonas NCIMB 9872 was grown in a 10Litre fermenter as a fed batch culture. The concentration of cyclopentanol in the medium was monitored, and, when this fell below a set limit (about 2mM), more cyclopentanol was added to a final concentration of 10mM. Figure 4.1 shows fed batch growth of Pseudomonas NCIMB 9872 on cyclopentanol in a 10 litre fermenter. Growth lasted about 27 hours with a maximal absorbance at 600nm of about 10. Immediately prior to harvesting, a final addition of cyclopentanol was made, to give a concentration of 5mM in the medium. This was to ensure that δ-valerolactone hydrolase remained fully induced during harvesting. There was accumulation of cyclopentanone in the medium up to 3mM, particularly after replenishment of utilised cyclopentanol.

4.1.2. Generation of the High Speed Supernatant Fraction (HSS)

Harvested cells of Pseudomonas NCIMB 9872 were resuspended in twice its volume of 20mM phosphate buffer, pH 7.6, and, lysed by sonication. Optimal breakage occurred with about 25 minutes sonication. Further sonication did not affect protein release or the level of δ-valerolactone hydrolase activity (figure 4.2). Cell free extracts (CFE) were obtained by low speed centrifugation (48,000g for 20min) of the sonicant to remove cell debris. To

Figure 4.1: Growth of Pseudomonas NCIMB 9872 on cyclopentanol in 10 litre fed-batch culture. Initial cyclopentanol concentration was 10mM. Cyclopentanol concentration was made up to 10mM at 11, 16 and 25 hours

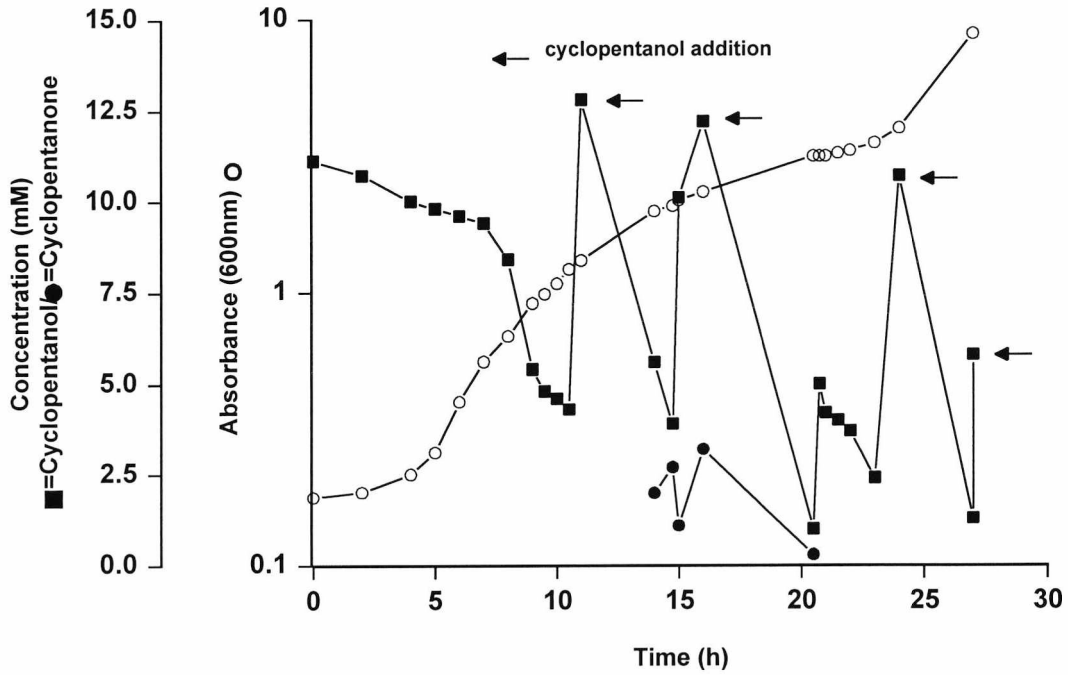
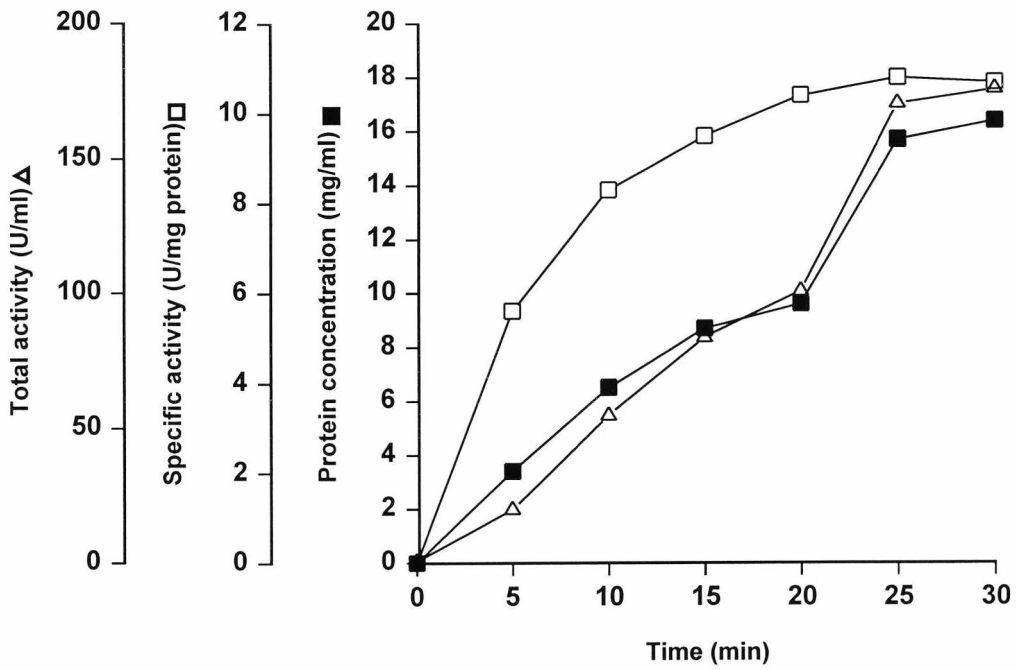


Figure 4.2: Optimization of cell breakage by sonication. The yield of protein, lactonase specific activity and total lactonase activity in cell free extract obtained by sonication of Pseudomonas NCIMB 9872 biomass.



1unit (U) - defined as the amount of protein that would catalyse the conversion of 1μmol of substrate to 1μmol of product.

obtain high speed supernatant, CFE were subjected to ultra-speed centrifugation (150,000g for 3hours).

4.1.3. Q- Sepharose anion exchange chromatography

δ-Valerolactone hydrolase bound to Q- sepharose ion-exchange matrix at pH values above pH 7.3. Subsequent anion exchange separations were therefore carried out at pH 7.6. Figure 4.3 shows a typical elution profile of δ-valerolactone hydrolase from Q- sepharose at pH 7.6.

4.1.4. Butyl sepharose 4B hydrophobic interaction chromatography

Figure 4.4 shows a typical elution profile of δ-valerolactone hydrolase from butyl sepharose 4B at pH 7. Fractions exhibiting high lactonase activity were pooled, desalted and concentrated before storing at -20°C.

δ-Valerolactone hydrolase was purified essentially to homogeneity (section 4.2.1.2) using the steps outlined above. A summary of a typical purification process is shown in table 4.1.

Figure 4.3: Q- Sepharose anion exchange chromatography (FPLC). 150mg protein (15mg/ml) in 20mM phosphate buffer pH 7.6 was applied to the column pre-equilibrated to pH 7.6 with 20mM phosphate buffer at 1ml/min. Bound protein was eluted with a linear gradient of 0-0.3M KCl and 2ml fractions were collected. Lactonase activity occurred between 40 and 55 mls of eluant corresponding to fractions 20 - 26.

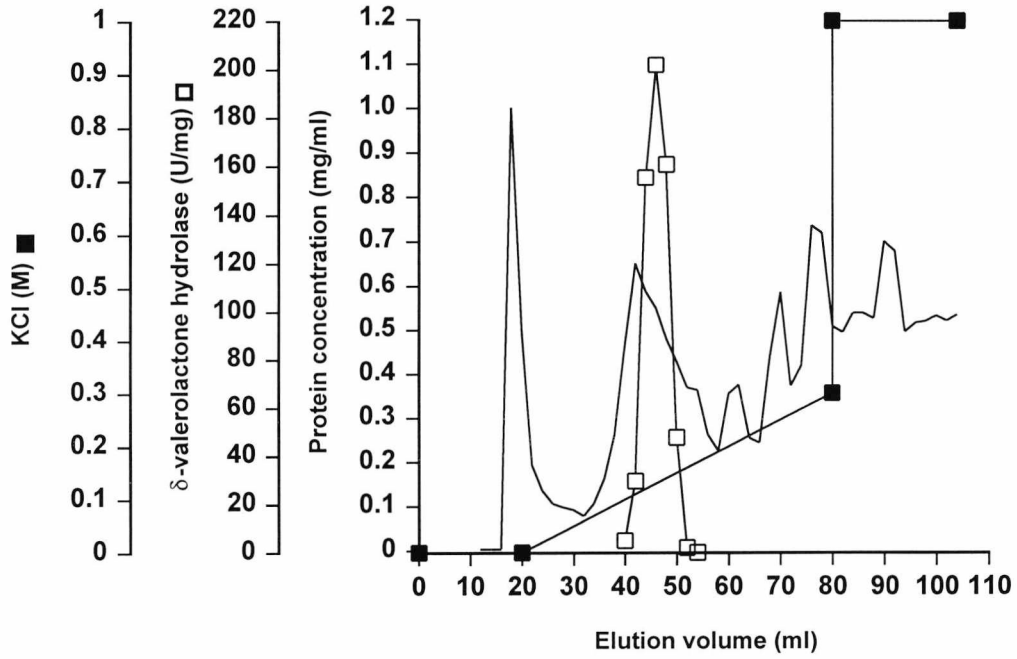


Figure 4.4: Butyl Sepharose-4B Hydrophobic Interaction Chromatography. Ionic strength of eluted protein from ion exchange step was increased by the addition of $(NH_4)_2SO_4$ to a final concentration of 1M. This was applied to the column equilibrated with 50mM phosphate buffer containing 1M $(NH_4)_2SO_4$. The column was washed with 40mls of phosphate buffer containing 0.4M $(NH_4)_2SO_4$ before desired enzyme was eluted with a linear gradient of 0.4 - 0.1M $(NH_4)_2SO_4$. Lactonase activity occurred between 63 and 70mls eluant volume.

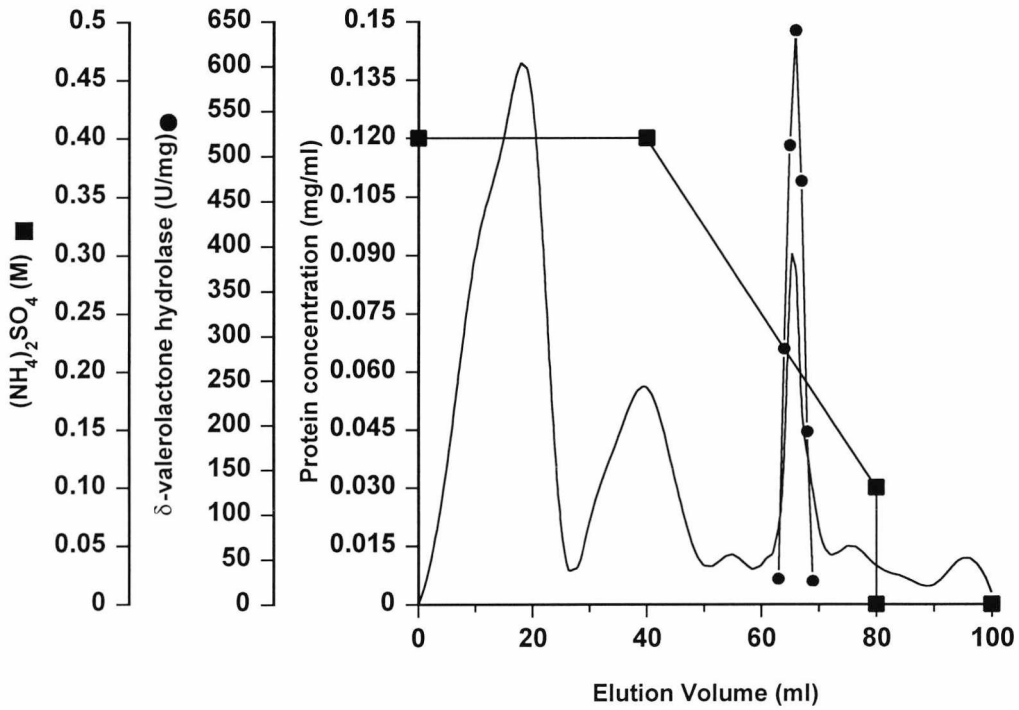


Table 4.1: Summary of purification of δ-valerolactone hydrolase from Pseudomonas NCIMB9872. Units of activity are expressed as μmol δ-valerolactone hydrolysed min^{-1}

Fraction	Volume (mls)	Protein conc. (mg/ml)	Total (mg)	Specific Activity (Umg^{-1})	Total U	Yield %	Fold Purif.
CFE	10	17.5	175	5.2	910	-	-
HSS	9	14.35	129	8.5	1096.5	1.21	1.5
IEX	8	0.50	4	150	600	66	17.6
HIC	6	0.07	0.42	580	244	27	112

CFE = Cell free extract; HSS = High Speed Supernatant; IEX = Q- Sepharose Ion Exchange Chromatography Fractions; HIC = Butyl Sepharose 4B Hydrophobic Interaction Chromatography Fractions.

4.2. Characterisation of δ-valerolactone hydrolase from *Pseudomonas* NCIMB 9872

4.2.1. Molecular weight determination

4.2.1.1. Gel filtration.

Purified δ-valerolactone hydrolase from Pseudomonas NCIMB 9872 was applied to a calibrated superose 12/30 gel filtration column. The active protein eluted as a single peak corresponding to a molecular weight of about 21kda (figure 4.5).

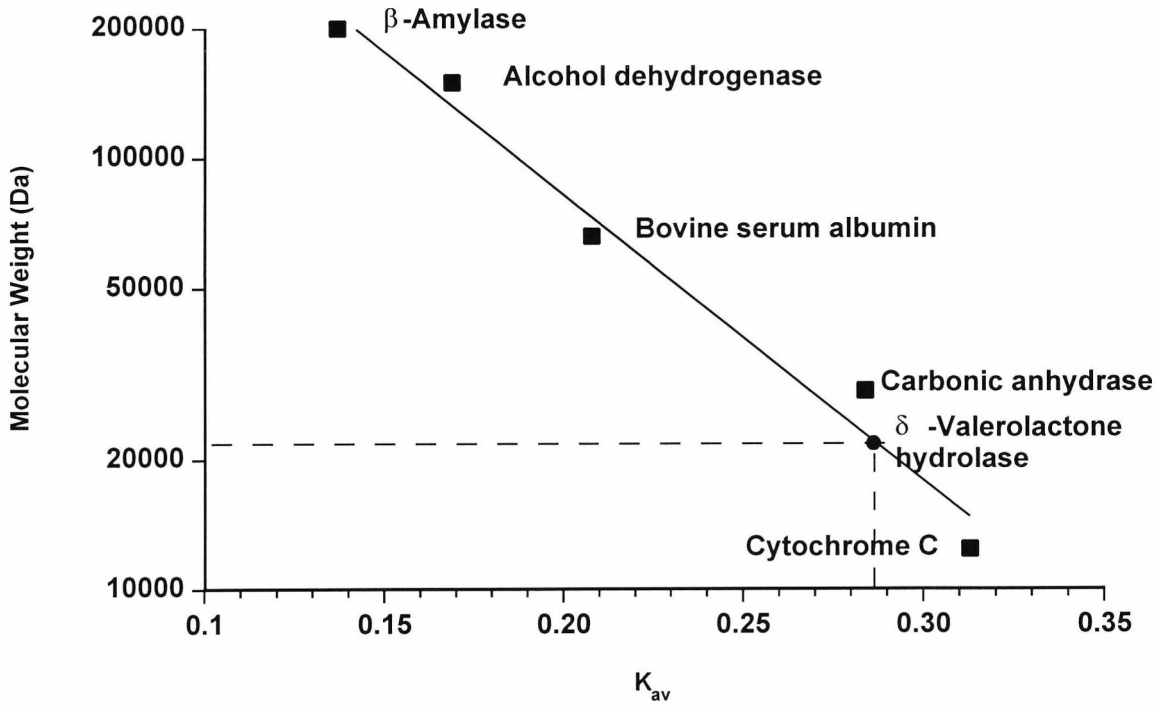
4.2.1.2. SDS-PAGE electrophoresis.

Figure 4.6 shows an SDS-PAGE analysis of the purification of δ-valerolactone hydrolase. Only minor traces of impurities were detected by careful visual examination of the gel. δ-Valerolactone hydrolase migrated as a single band with a subunit molecular weight that corresponded to approximately 28kda.

4.2.2. Absorption spectra

The absorption spectrum of this enzyme showed the absorbance due only to aromatic amino acids with no distortions or additional absorbance indicative of the presence of non-protein prosthetic groups.

Figure 4.5: Estimation of molecular weight of δ-valerolactone hydrolase by Gel filtration (Superose 12/30). Calibration curve showing K_{av} values for standard molecular weight markers.



4.2.3. Native gel electrophoresis

When subject to native gel electrophoresis, δ-valerolactone hydrolase migrated as a single band (figure 4.7). It is thus unlikely that this enzyme exists in different conformational forms.

4.2.4. Isoelectric focussing.

The pI of δ-valerolactone hydrolase was determined to be approximately 4.5 by isoelectric focussing (figure 4.8).

4.2.5. N-terminal amino acid sequence.

The amino acid sequence of the first 15 residues at the NH_2- terminus of δ-valerolactone hydrolase was determined by Edman degradation. The sequence is:

xxx-lys-xxx-arg-glu-val-his-thr-val-val-asp-gly-phe-thr-tyr

When submitted to the SwissProt protein sequence database for comparison to other known sequences, no matching sequences were found.

Figure 4.6. SDS-PAGE analysis of fractions obtained during the purification of δ-valerolactone hydrolase from Pseudomonas NCIMB 9872. Each lane contained approximately 50μg of protein sample.

Lane 1: High speed supernatant fraction

Lane 2: Q- sepharose anion exchange pooled fractions

Lane 3: Butyl Sepharose 4B hydrophobic interaction pooled fractions

Lane 4: Molecular weight markers (Sigma, Dalton mark VII). Myosin (rabbit muscle) 205,000; β-Galactosidase (E.coli) 116,000; Phosphorylase b (rabbit) 97,400; BSA, 66,000; Ovalbumin 45,000; Carbonic anhydrase, 29,000; Trypsin inhibitor, 20,100.

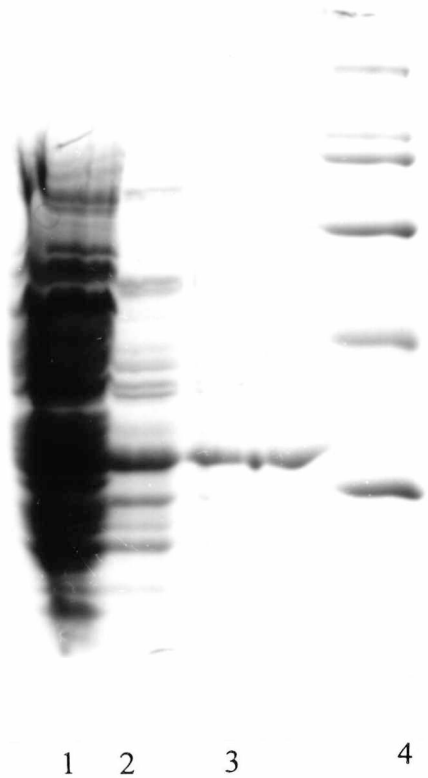


Figure 4.7: Non-denaturing gel electrophoresis of δ-valerolactone hydrolase from Pseudomonas NCIMB 9872. Electrophoresis was done at room temperature. The gel was stained with Coomassie blue R-250. Each lane contained approximately 50μg of δ-valerolactone hydrolase

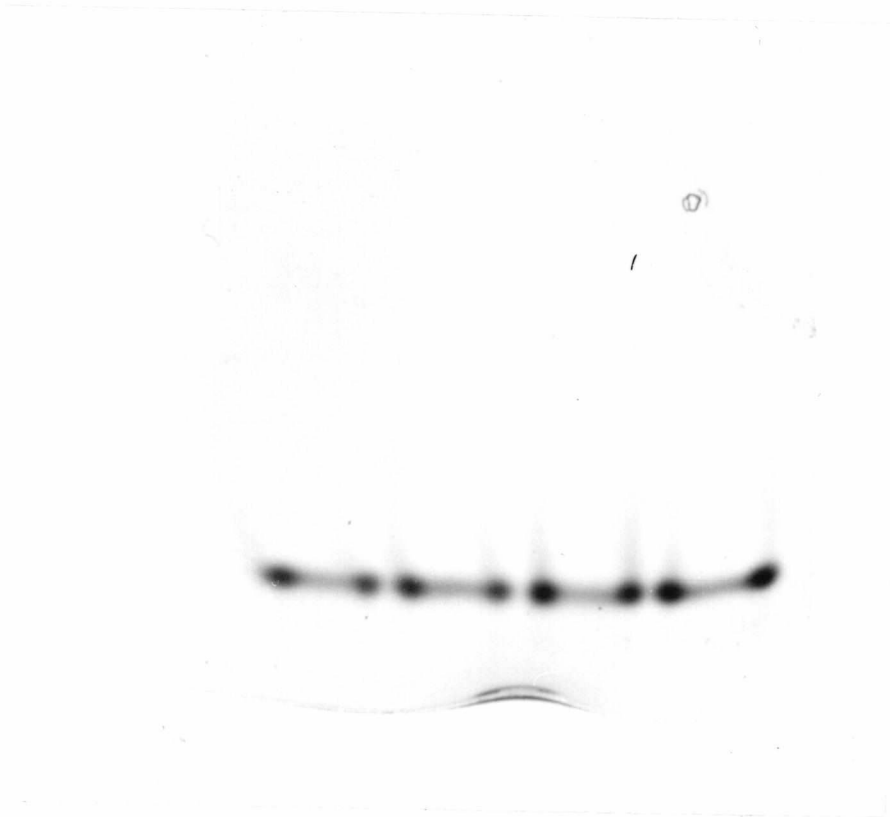
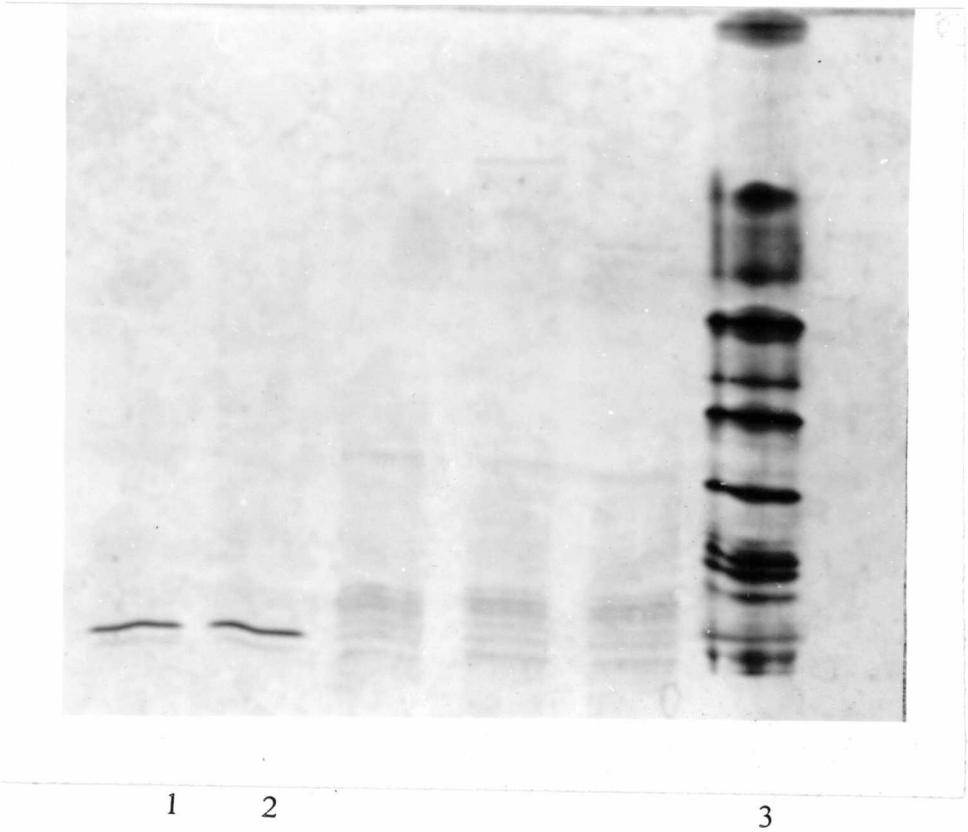


Figure 4.8: Determination of pI of δ-valerolactone hydrolase by Isoelectric Focussing. IEF was performed on Ampholine PAGplate (Pharmacia), calibrated with pI Calibration Kit Electran (wide range) pI 4.7 - 10.6 (BDH).

Lane 1 and Lane 2: δ-valerolactone hydrolase (~20μg)
Lane 3: pI markers (~20μg)

Details of the markers used are:

C-Phycocyanin (A.nidulans) pI 4.75, 4.85
Azurin (P. Aeruginosa) pI 5.65
Trifluoroactylated Myoglobin Met (porcine) pI 5.9
Myoglobin Met (porcine) pI 6.45
Myoglobin Met (equine) pI 7.3
Myoglobin Met (sperm whale) pI 8.3
Cytochrome C (horse heart) pI 10.6



4.2.6. pH-activity profile for δ-valerolactone hydrolase.

Figure 4.9 shows the effect of pH on the activity of d-valerolactone hydrolase. The pH dependent change in activity was small over the pH range 7-8.5 with maximum activity occuring at pH 7.5.

4.2.7. Effect of temperature.

δ-Valerolactone hydrolase showed maximal activity between 28° and 35°C (figure 4.10). There was significant loss of activity above 45°C

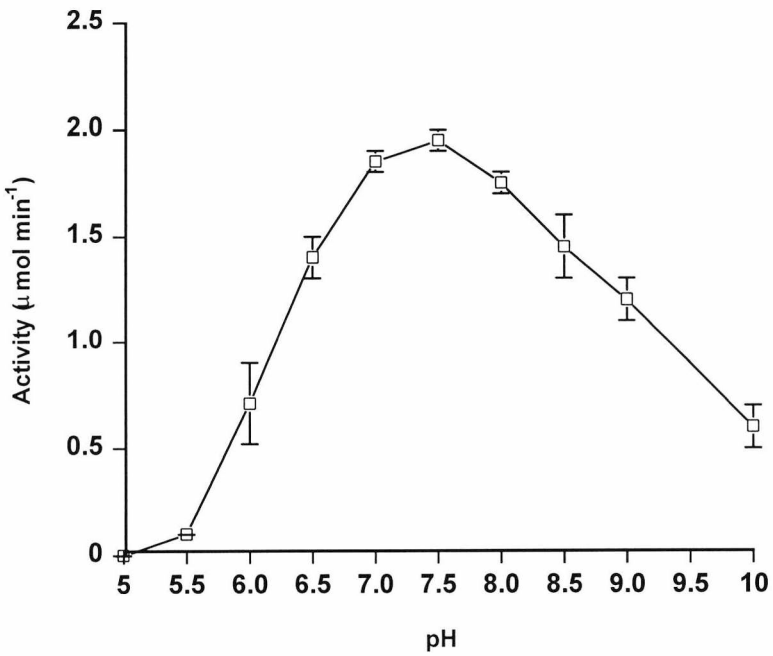
4.2.8. Stability

The enzyme was stable for several weeks at -20°C without significant loss of activity ,and did not require the addition of a protective agent.

4.2.9. Effect of inhibitors.

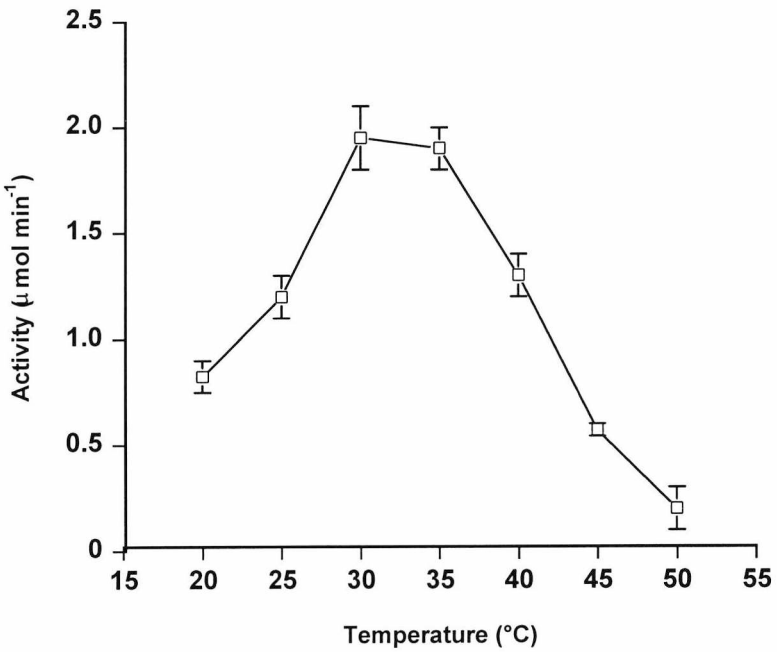
Table 4.2 shows the effect of a range of potential inhibitors on the activity of δ-valerolactone hydrolase. This enzyme is sensitive towards metal chelating reagents such as EDTA and citric acid. In addition p-chloromercuribenzoate (a sulphydryl reagent) strongly inhibited lactonase activity suggesting that a thiol group is essential for enzyme activity. Serine-protease inhibitors did not appear to inhibit the activity of this enzyme suggesting

Figure 4.9: Effect of pH on the activity of δ-valerolactone hydrolase



For each pH tested, 5μg of purified enzyme was used.

Figure 4.10: Effect of temperature on δ-valerolactone hydrolase activity. δ-valerolactone hydrolase was incubated for about 5minutes at the desired temperature before being assayed for activity.



For each temperature tested, 5μg of purified enzyme was used.

Table 4.2: Effect of various compounds on δ-valerolactone hydrolase activity.

Type of Reagent	Chemical Inhibitor	Final Concentration (mM)	Relative Activity (%)
None	None		100
Thiol Specific	4-Chloromercuribenzoate	0.1	20
	5,5'-Dithiobis(2-nitrobenzoate)	0.1	105
	Iodoacetate	0.1	82
		1	99
Serine-OH Specific	Phenyl methane sulfonyl Fluoride	1	103
Metal Chelators	EDTA	1	2.9
	Citrate	1	18
		10	8.8
Metal Ions	$CuCl_2$	0.1	2.6
	$NiCl_2$	0.1	18
	$ZnCl_2$	0.1	ND
	$CaCl_2$	0.1	27
	$MgCl_2$	0.1	107
	$CoCl_2$	0.1	120
	$MnCl_2$	0.1	200

The standard autotitrator pH stat assay was used with a 15ml reaction volume and about 1μg of purifed enzyme. The reaction mixture containing all the components with the exception of δ-valerolactone was preincubated for 3 minutes at pH 7 before starting the assay by the addition of 20μmol of δ-valerolactone.

that serine OH groups were not essential for activity. Metals such as Mn and Mg enhanced activity whilst Ni and Cu inhibited actitity.

4.2.10. Km and Vmax.

Michaelis-Menten treatment of data obtained from kinetic studies conducted at 30°C and pH7 yielded a Km value for δ-valerolactone of 0.91 ± 0.36 mM, and a Vmax of 777.7 ± 112.9 $\mu molmin^{-1}mg^{-1}$ (figure 4.11). Lineweaver-Burke analysis of the same data yielded a Km value of 1.4 mM and a Vmax of 877.2 $\mu molmin^{-1}mg^{-1}$ (figure 4.12)

Figure 4.11: Michaelis-Menten representation of the hydrolysis of δ-valerolactone by δ-valerolactone hydrolase purified from Pseudomonas NCIMB 9872.

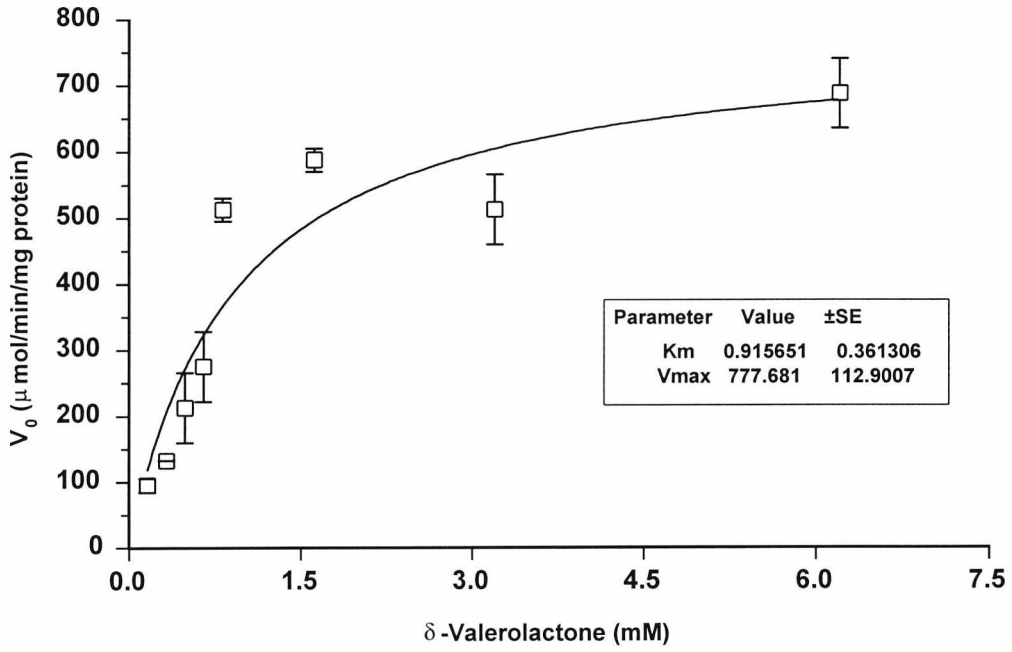
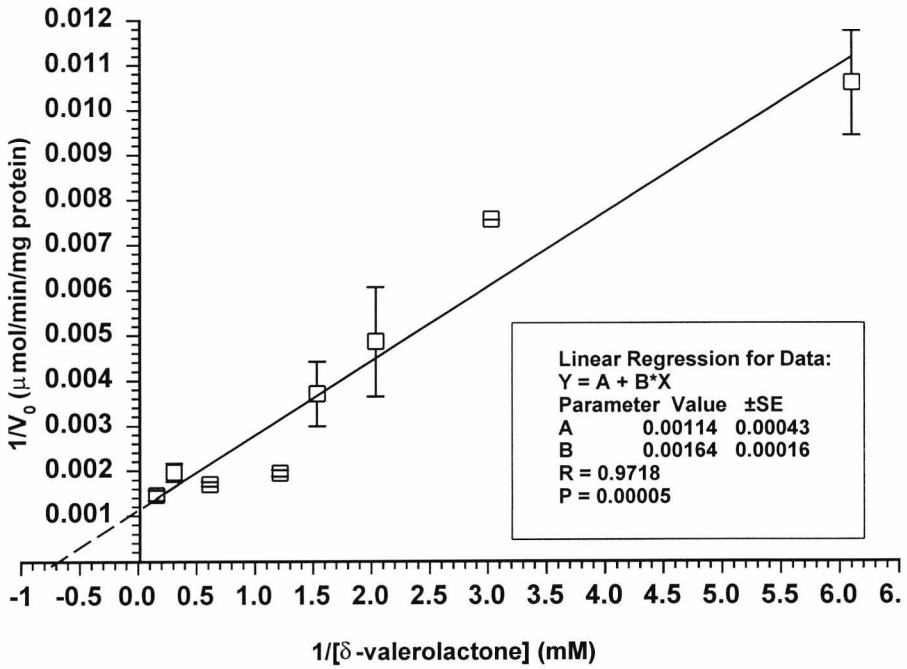


Figure 4.12: Lineweaver-Burke plot for the hydrolysis of δ-valerolactone by δ-valerolactone hydrolase purified from Pseudomonas NCIMB 9872.



Chapter 5

Biotransformation of lactones by δ-valerolactone hydrolase from Pseudomonas NCIMB 9872, and ε-caprolactone hydrolase from Acinetobacter NCIMB 9871 and Rhodococcus coprophilus WT1

5.1. Substrate Specificity

5.1.1. Pseudomonas NCIMB9872

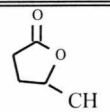
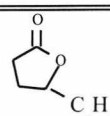
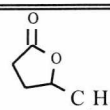
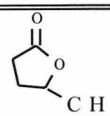
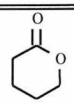
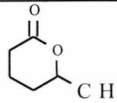
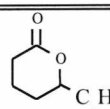
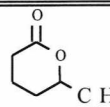
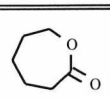
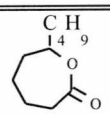
The activity of δ-valerolactone hydrolase from Pseudomonas NCIMB 9872 was tested using a range of monocyclic lactones. Biotransformations were carried out using either cell free extracts or purified enzyme derived from the microorganism grown on cyclopentanol. Both the cell free extract and the purified δ-valerolactone hydrolase showed identical substrate specificities. The extent of the biotransformation was determined by monitoring the amount of NaOH added to maintain the pH of the biotransformation mixture at the pH7. δ-Valerolactone hydrolase showed significant activity towards 6- and 7- membered ring monocyclic lactones, but little or no activity towards γ-butyrolactone or any of the other 5-membered ring lactones tested (Table 5.1). Maximal activity occurred with δ-valerolactone as substrate. Activity towards the substituted δ- lactones was lower than towards unsubstituted δ-valerolactone; activity decreased with increasing chain length. Activity towards ε-caprolactone (a 7-membered ring lactone) was approximately 10% of the activity observed when δ-valerolactone was the substrate. δ-Valerolactone hydrolase did not show appreciable activity towards ε-decanolactone.

5.1.2. Acinetobacter NCIMB 9871 and Rhodococcus coprophilus WT1

Biotransformations were carried out using cell free extracts derived from either organism grown on cyclohexanol. Lactonases from both microorganisms showed similar substrate specificities, with maximal activity occurring towards ε-caprolactone (Table 5.1). Activity

Table 5.1. Substrate specificity of lactonases present in HSS derived from Acinetobacter NCIMB 9871, Pseudomonas NCIMB 9872 and Rhodococcus coprophilus WT1.

100% activity corresponds to about 10μmol/min/mg protein for HSS derived from Acinetobacter NCIMB 9871 and R. coprophilus WT1, and, 11μmol/min/mg protein for HSS derived from Pseudomonas NCIMB 9872. 100% activity of pure δ-valerolactone hydrolase from Pseudomonas NCIMB 9872 corresponds to about 300μmol/min/mg protein. (ND= not detected)

Substrate	Relative Lactonase activity (Acinetobacter NCIMB 9871) %	Relative Lactonase Activity (Pseudomonas NCIMB 9872) %		Relative Lactonase Activity (Rhodococcus coprophilus WT1) %
		Pure enzyme	HSS	
 γ-butyrolactone	ND	Not tested	0.03	ND
 γ-valerolactone	ND	Not tested	ND	ND
 γ-caprolactone	ND	Not tested	ND	ND
 γ-octanoic lactone	ND	Not tested	ND	ND
 γ-decanolactone	ND	Not tested	ND	ND
 δ-valerolactone	10	100	100	20
 δ-nonalactone	7.5	12	10	15
 δ-decanolactone	7.5	12	9.3	12
 δ-dodecanolactone	1.1	ND	1.3	5
 ε-caprolactone	100	10	9.87	100
 ε-decanolactone	80.5	ND	ND	60

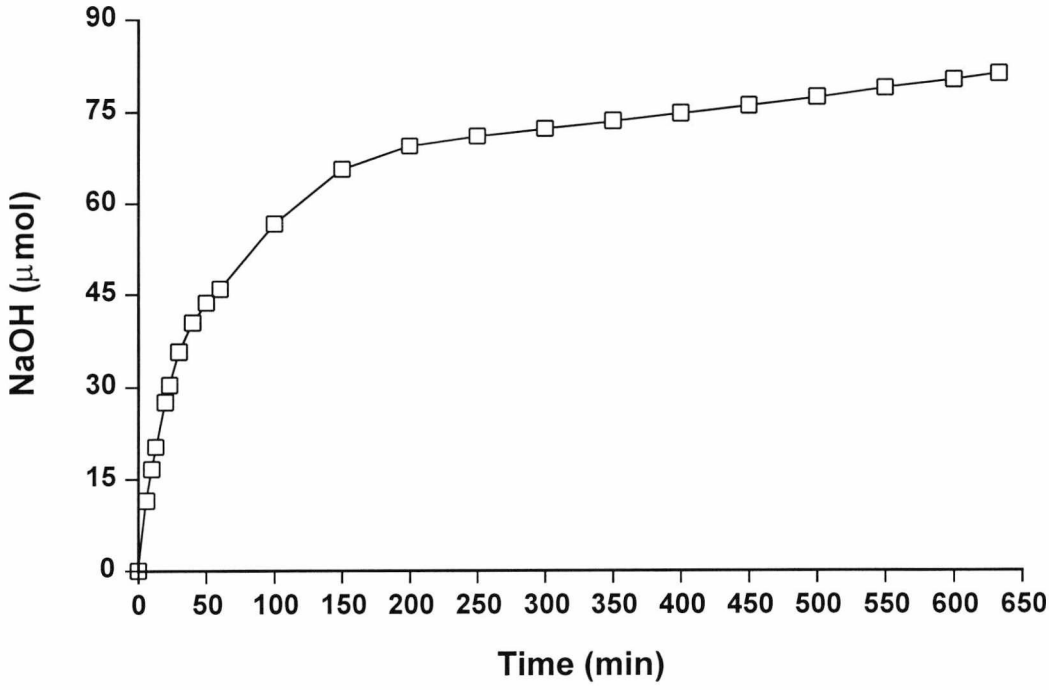
towards ε-decanolactone was about 80% of the activity observed towards ε-caprolactone whereas with δ-valerolactone the activity was only about 10% of the maximal activity. Activity towards the substituted δ-lactones decreased with increasing chain length, with δ-dodecanolactone stimulating the lowest activity of the substituted lactones tested. Neither lactonase showed appreciable activity towards γ-butyrolactone, or any of the substituted γ-lactones tested.

5.2. Enantioselectivity

5.2.1. δ-Valerolactone hydrolase from Pseudomonas NCIMB 9872

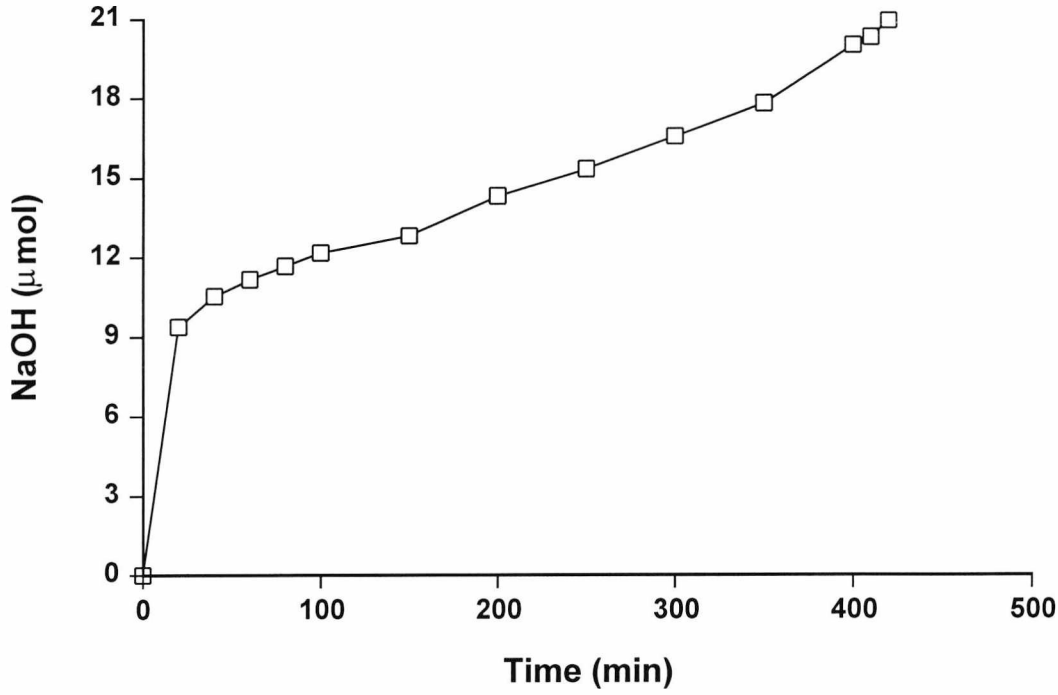
Two distinct rates of NaOH addition to the biotransformation mixture were observed during the hydrolysis of racemic δ-decanolactone, and racemic δ-nonalactone, by cell free extracts derived from Pseudomonas NCIMB 9872 (figure 5.1 and figure 5.2). The initial more rapid rate was re-attained upon re-addition of either substrate. In contrast, hydrolysis of δ-valerolactone (a non-chiral substrate), using the same cell free extracts, did not show this differential activity (figure 5.3), slowing down only towards the end of the overall biotransformation. All attempts to purify and analyse the hydroxyacid formed from δ-decanolactone or δ-nonalactone hydrolysis by chiral GC were unsuccessful. This could be because δ-hydroxyacids spontaneously undergo intramolecular condensation at acidic pH's making it difficult to extract them into solvent. Hence, products were analysed by separating the hydroxyacid formed from the residual untransformed lactone (see Methods section). After separation, the hydroxyacid spontaneously converted back to give the lactone on acidic treatment, hence affording analysis by chiral GC. Figure 5.4 shows a time

Figure 5.1: Biotransformation of δ-decanolactone (112μmoles) by cell free extracts derived from Pseudomonas NCIMB 9872 grown on cyclopentanol.



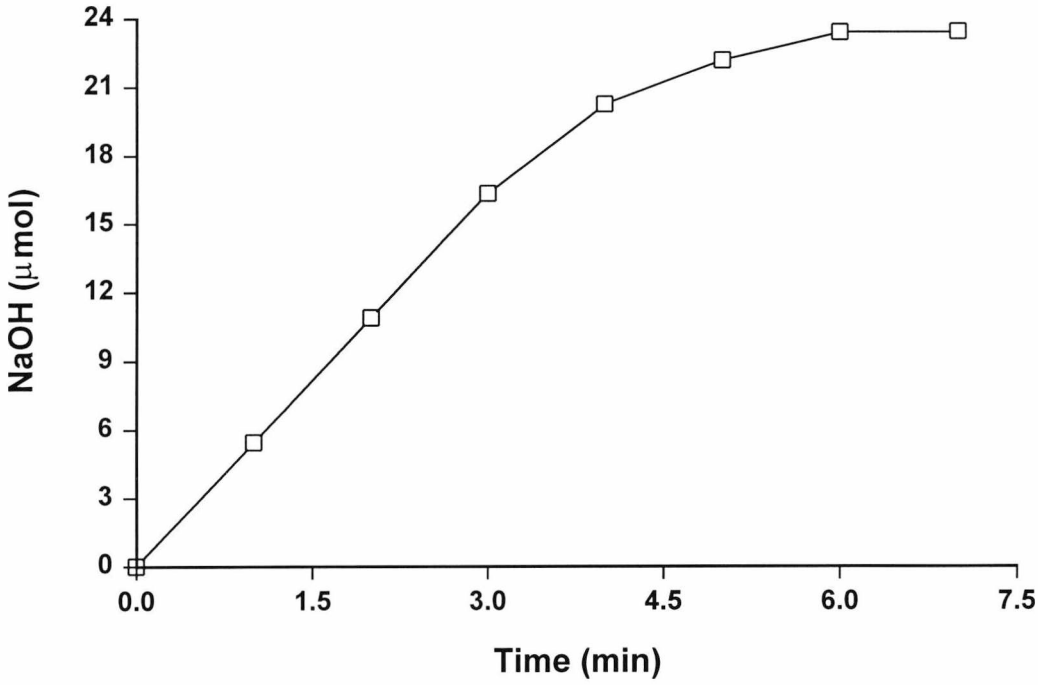
Reaction carried out in autotitrator pH stat 7.0 at 30°C

Figure 5.2: Biotransformation of δ-nonalactone (25umoles) by cell free extracts derived from Pseudomonas NCIMB 9872 grown on cyclopentanol



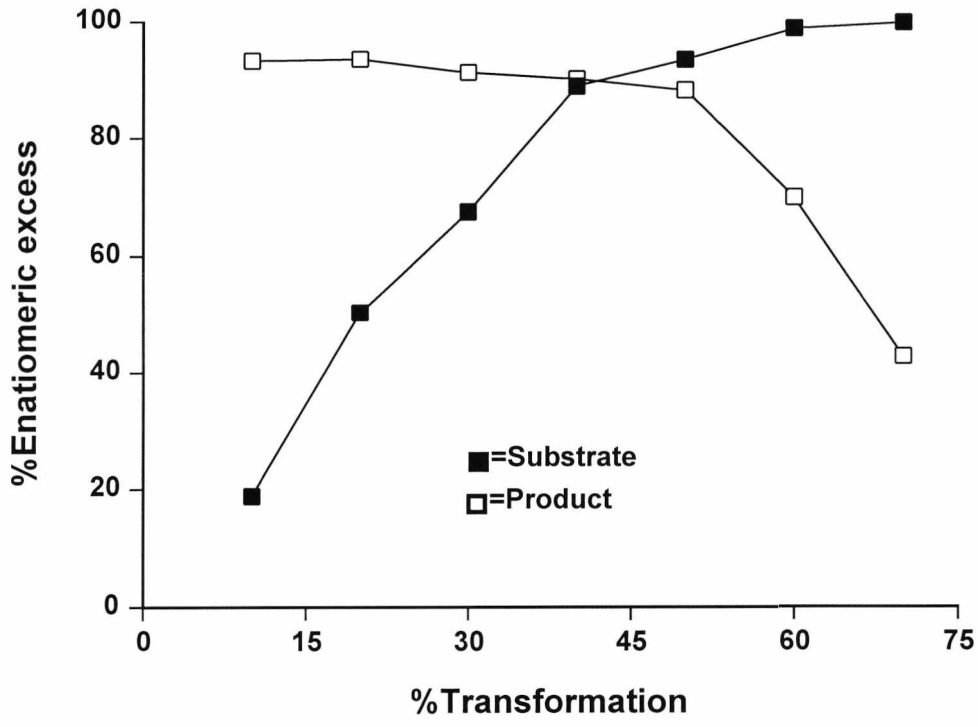
Reaction carried out in autotitrator pH stat 7.0 at 30°C

Figure 5.3: Biotransformation of δ-valerolactone (20umoles) by cell free extracts derived from Pseudomonas NCIMB 9872 grown on cyclopentanol.



Reaction carried out in autotitrator pH stat 7.0 at 30°C

Figure 5.4: Time course profile of the enantiomeric excess of residual lactone substrate and the hydroxyacid formed during the biotransformation of δ-decanolactone by cell free extract derived from Pseudomonas NCIMB 9872 grown on cyclopentanol



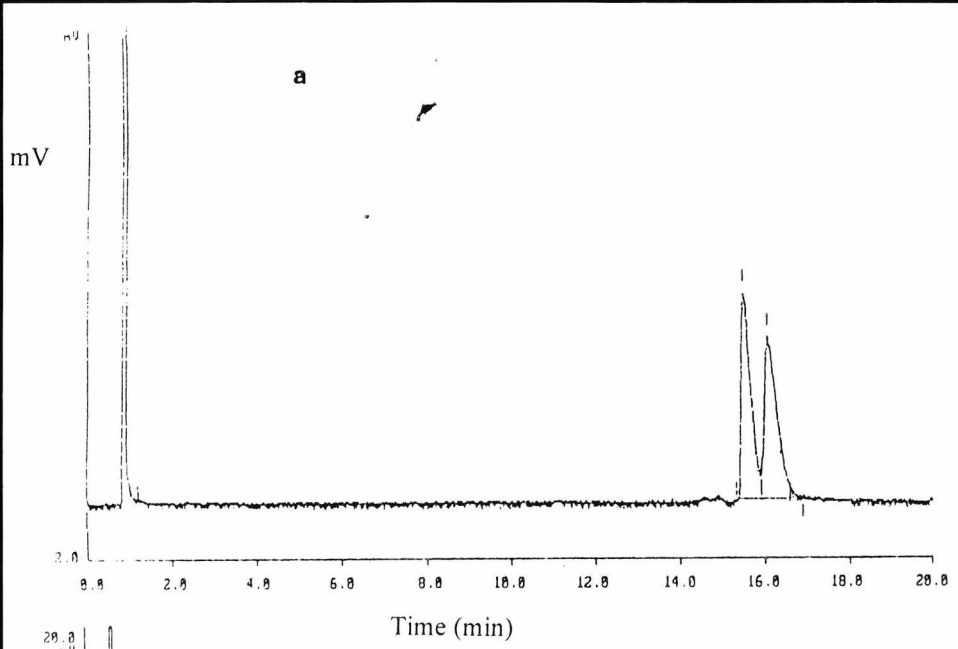
course profile for the biotransformation of δ-decanolactone by cell free extracts derived from Pseudomonas NCIMB 9872. In the initial phase of the biotransfomation, the lactone substrate was in low enantiomeric excess whereas the resultant hydrolysis product was in high enantiomeric excess. As the biotransformation proceeded towards the 40% transformation stage, the enantiomeric excess of the residual lactone substrate increased to greater than 80%, whereas the hydroxyacid product's enantiomeric excess did not significantly change. However by 70% transformation, the enantiomeric excess of the residual lactone was greater than 98% but the enantiomeric excess of the hydroxy acid decreased to about 55%. Figure 5.5 shows the GC traces of the enantiomers of the residual lactone at 0%, 20%, 40% and 65% transformation.

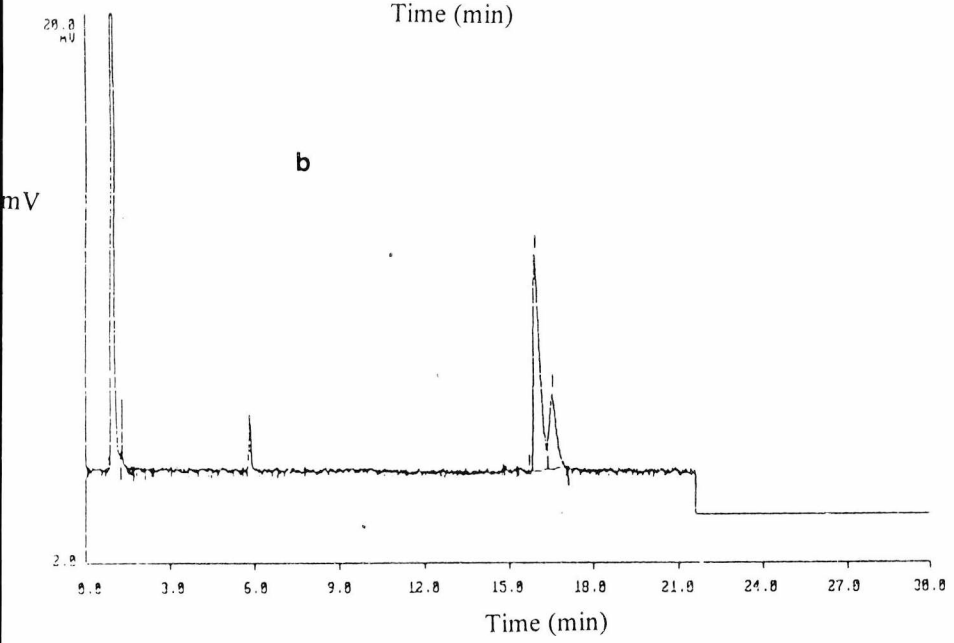
In order to assign absolute configurations to the products of the biotransformations, individual enantiomers of racemic δ-decanolactone and δ-nonalactone were isolated and purified, since authentic samples of the individual enantiomers of the products were not commercially available. About 0.5mmol of each substrate was transformed by about 65% using cell free extract from Pseudomonas NCIMB 9872. The residual lactone was separated from the biotransformation mixture, and chiral GC analysis showed this to be present in > 99% ee. Further analysis was carried out by nmr (figure 5.6) and polarimetry. Comparison to previously reported optical rotations (Utaka et. al, 1987) confirmed, in both cases, that the less preferred, residual lactone enantiomer was of the S- configuration (Table 5.2). In order to obtain the R- enantiomer of the lactone in high enantiomeric excess, the biotransformation was terminated after approximately 40% transformation, and the hydroxyacid that had been produced was reconverted to the lactone by chemical means. In this manner %ee values of up to 90% for the R- enantiomer were obtained (figure 5.7).

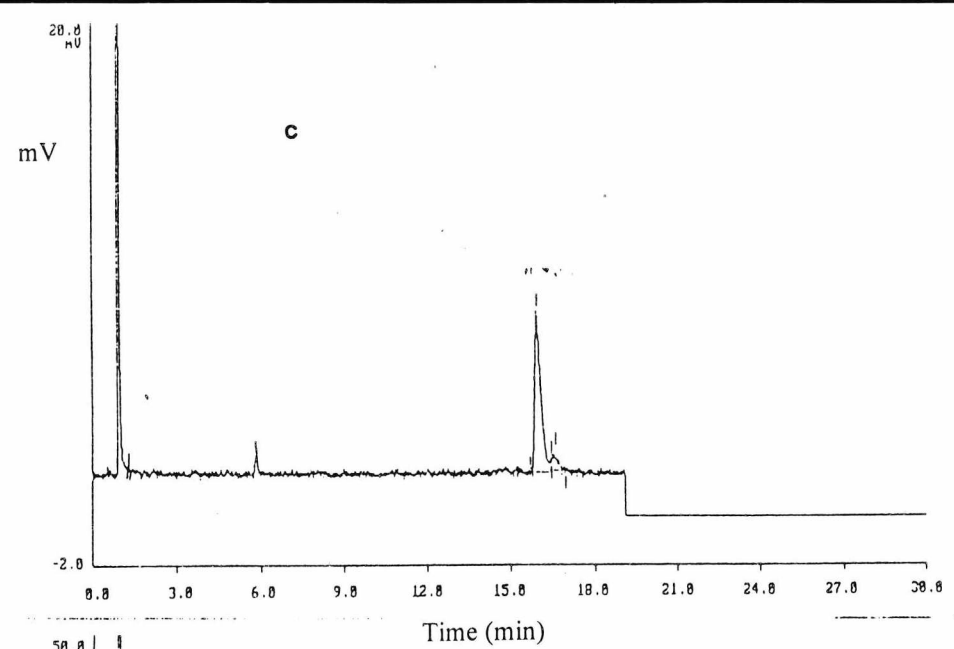
Figure 5.5: Chiraldex G-TA chiral capillary gas-chromatographic analysis of residual lactone obtained during the biotransfomation of δ-decanolactone by cell free extracts obtained from Pseudomonas NCIMB 9872.

Residual lactone was analysed at 0%, 20%, 40% and 65% transformation. %Ee's obtained at the various biotransformation stages are;

(a) 0% transformation; 0.2%ee
(b) 20% transformation; 43.6%ee
(c) 40% transformation; 82.08%ee
(d) 65% transformation; >>98%ee


a
mV
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
Time (min)


b
mV
2.0
0.0
3.0
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0
30.0
Time (min)


c
mV
20.0
-2.0
0.0
3.0
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0
30.0
Time (min)

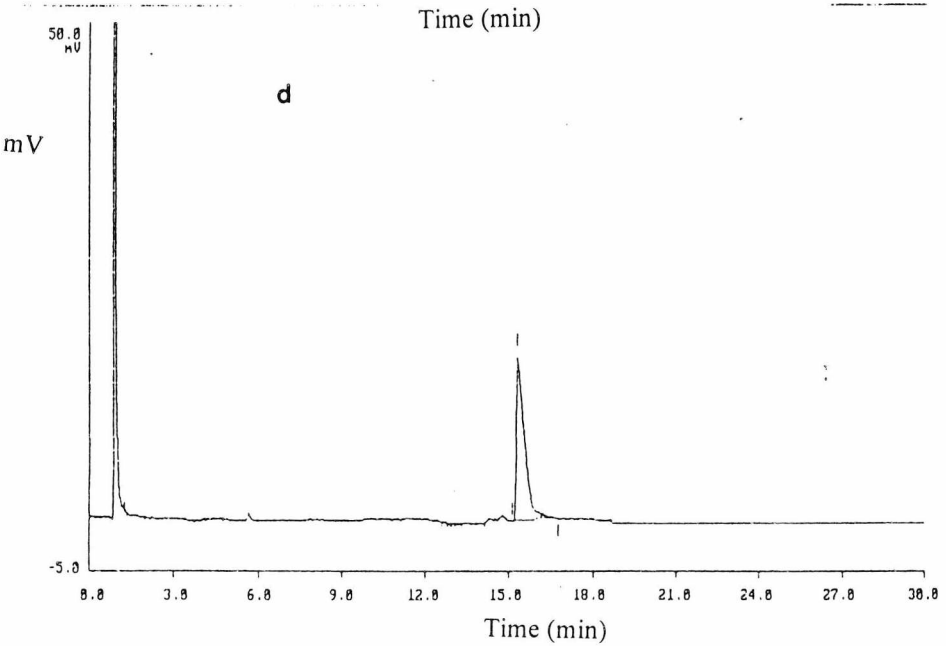
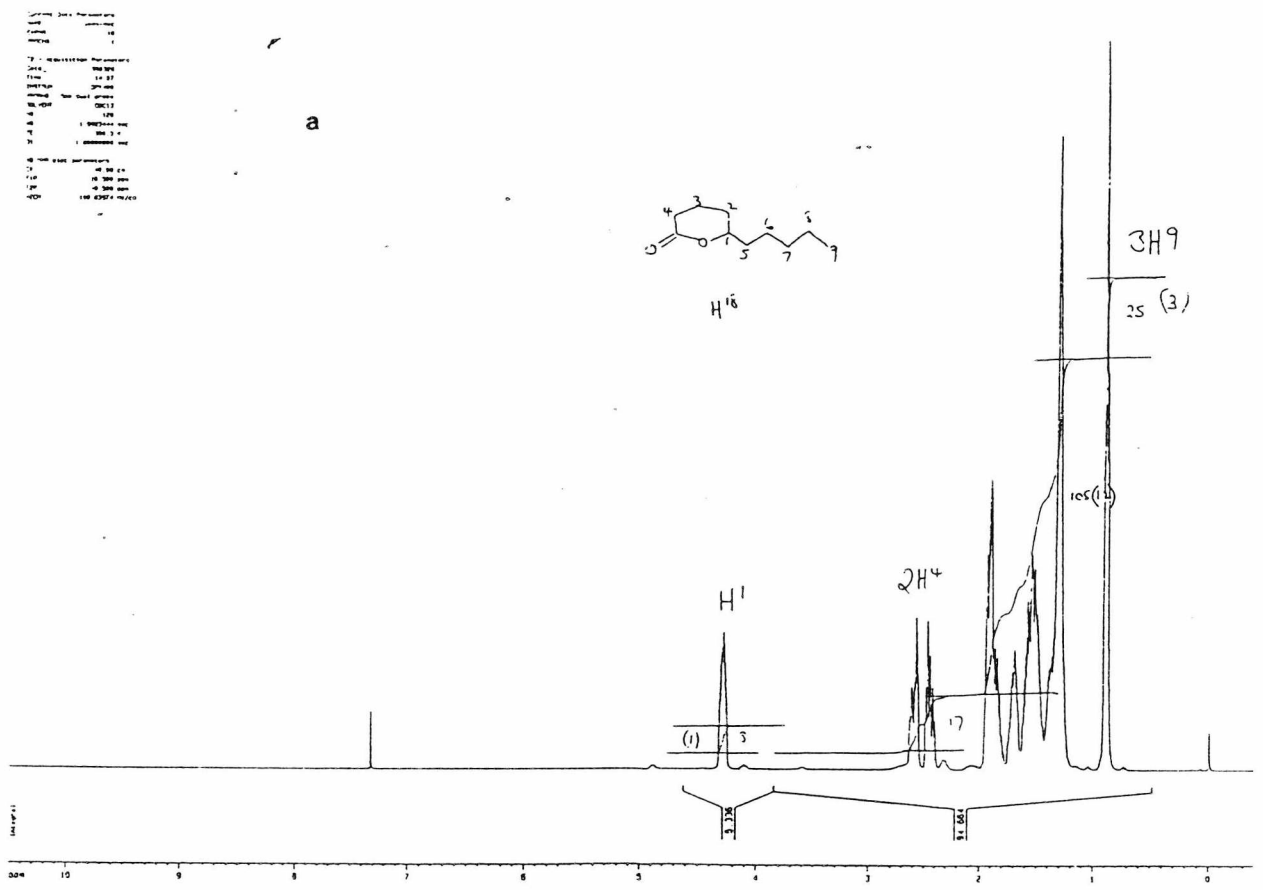

d
mV
50.0
-5.0
0.0
3.0
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0
30.0
Time (min)

Figure 5.6: NMR spectrum for (a) δ-decanolactone, and (b) δ-nonalactone. Comparison of optical activity to that of the literature confirms both compounds to be of the S-configuration (see table 5.2).


a
3H9
2H4
H1
ppm

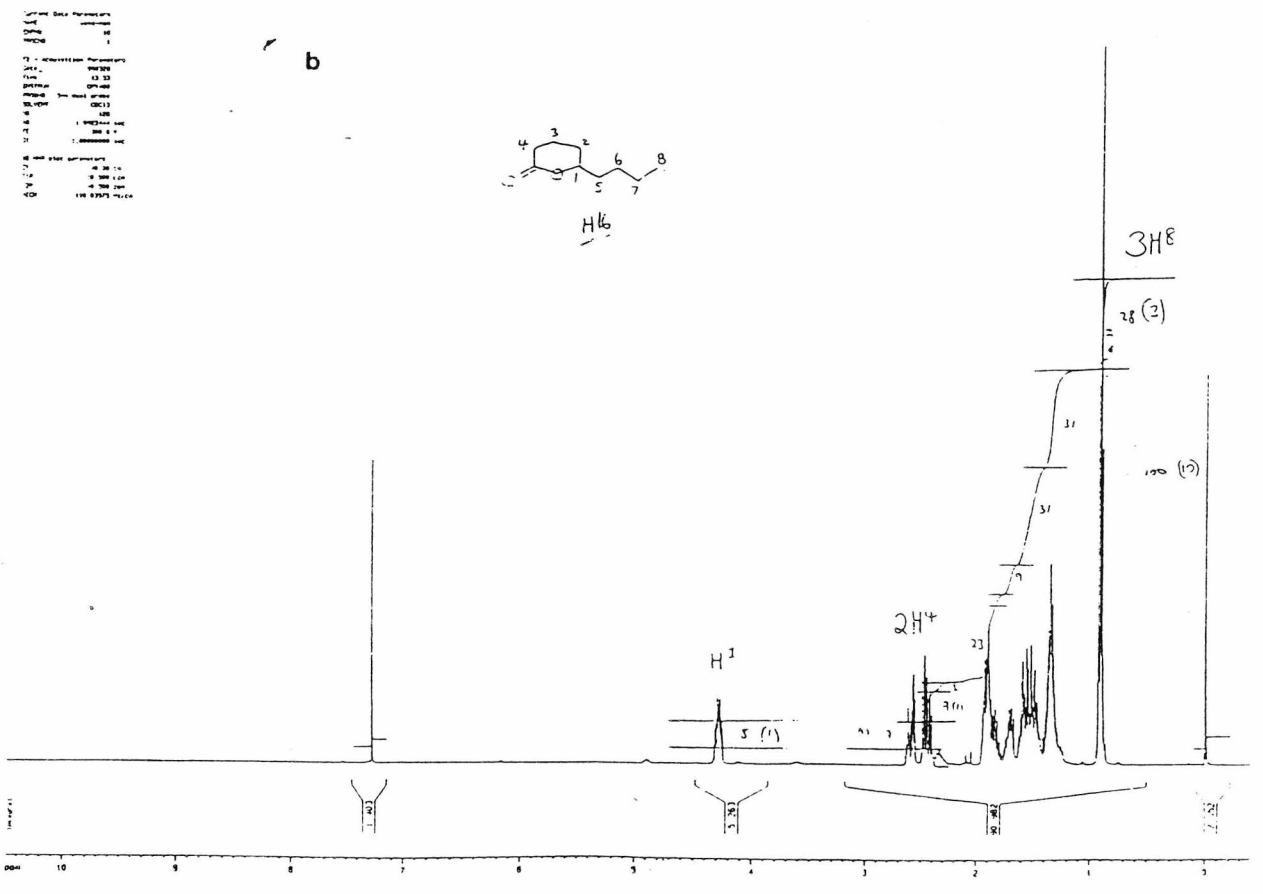
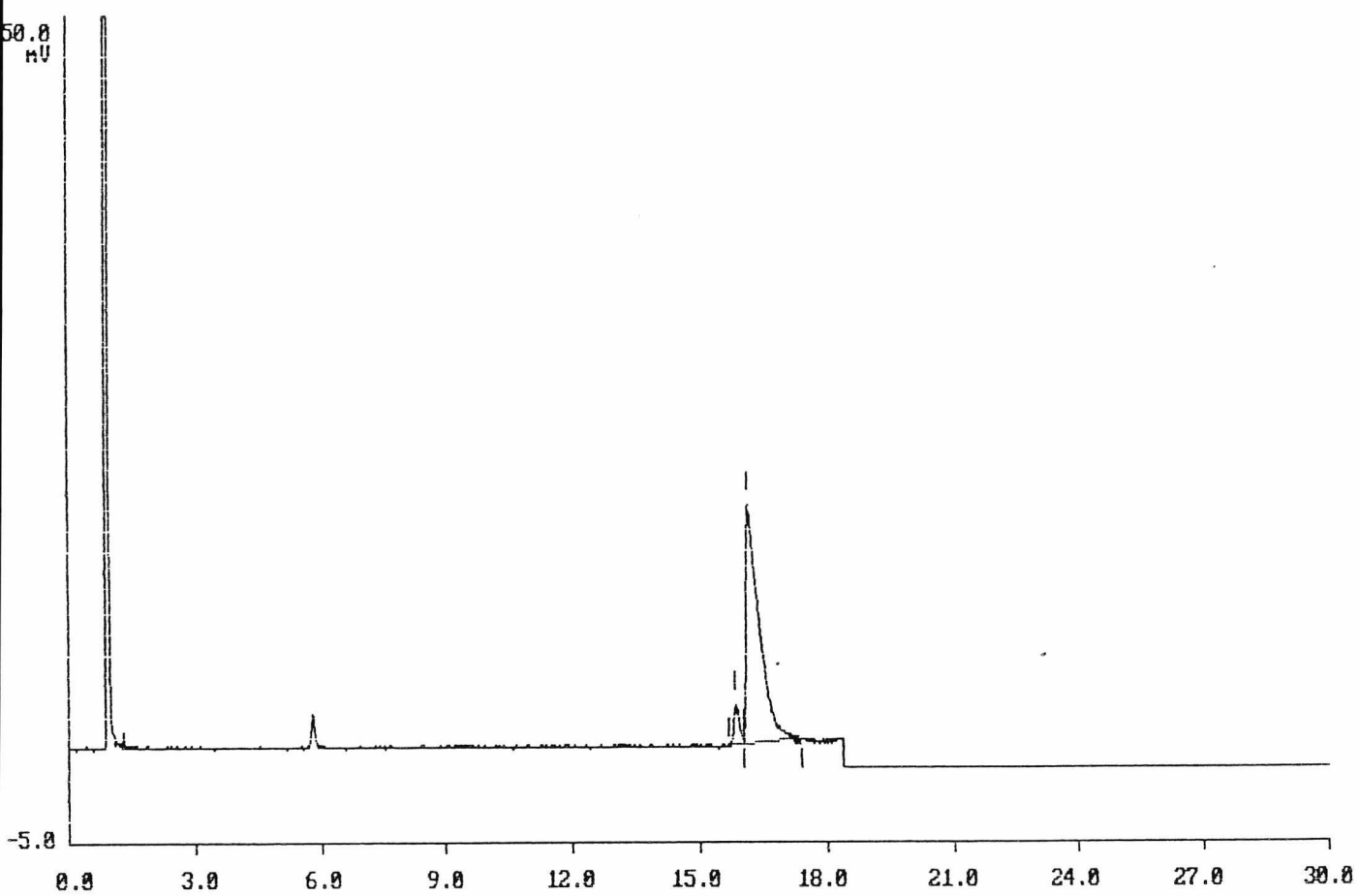

b
3H8
2H4
H1
ppm

Table 5.2: Absolute configuration of the residual enantiomers of δ-decanolactone and δ-nonalactone after 65% transformation by cell free extract derived from Pseudomonas NCIMB 9872

Substrate	Reaction conditions % Transform.	$[\alpha]_D$ (ref) deg (c,THF)	$[\alpha]_D$ (obs) deg	ee (%)	Absolute Config.
δ-decanolactone	CFE H_20 pH 7.0 65%	-60.2 (1.74)	-60.2 (1.58)	>98%	S
δ-nonalactone	CFE H_20 pH 7.0 65%	-63.2 (2.24)	-37.9 (1.04)	>98%	S

(CFE: cell free extract)

Figure 5.7: Chiraldex G-TA chiral capillary gas-chromatographic analysis of reconstituted lactone obtained during the biotransfomation of δ-decanolactone by cell free extracts obtained from Pseudomonas NCIMB 9872. Biotransformation was terminated at 40% transformation stage, and δ-decanolactone was reconstituted from the formed hydroxy acid by chemical means. %Ee of reconstituted lactone at this stage was 88.9%



Time (min)

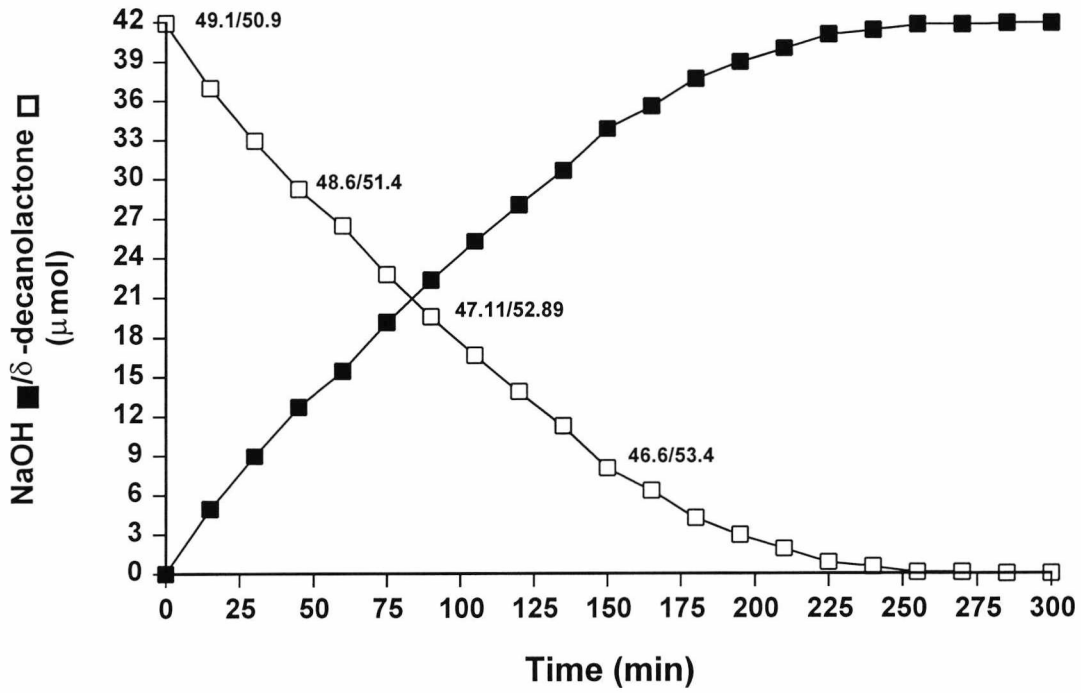
5.2.2. Biotransformations using cell free extracts derived from Acinetobacter NCIMB 9871 grown on cyclohexanol.

Figure 5.8 shows the biotransformation profile of racemic δ-decanolactone by cell free extracts derived from Acinetobacter NCIMB 9871. Unlike Pseudomonas NCIMB 9872, ε-caprolactone hydrolase did not show significant enantioselectivity towards this substrate, with the residual lactone having an enantiomeric excess of less than 6% after 60% transformation. Biotransformation of racemic δ-nonalactone was similar with the enantiomeric excess at the 60% stage for the residual lactone of about 5%. For both δ-decanolactone and δ-nonalactone, the less preferred enantiomer was of the S- configuration. Cell free extracts from Acinetobacter NCIMB 9871 showed a somewhat greater enantioselectivity towards ε-decanolactone, with the residual lactone having an enantiomeric excess of up to 35% at the 60% transformation stage (figure 5.9). By comparison of the GC elution profile to that of δ-decanolactone, the less preferred enantiomer was deduced to be of the S- configuration.

5.2.3. Biotransformations using cell free extracts from Rhodococcus coprophilus WT1

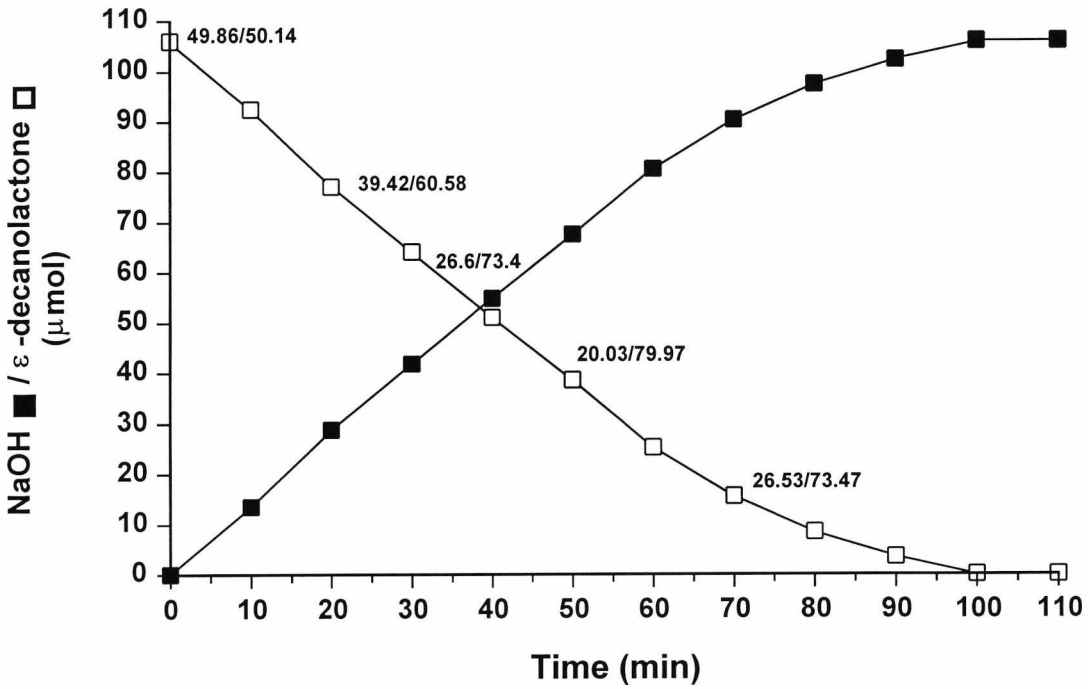
Biotransformation of δ-decanolactone to about 40% residual lactone by cell free extracts derived from R. coprophilus WT1 yielded residual lactone in enantiomeric excess of up to 15% (figure 5.10). Better enantioselectivity was shown towards racemic ε-decanolactone with an enantiomeric excess for residual lactone of 61% at the 60% stage (figure 5.11). Like the previous two microorganisms, the less preferred enantiomer was of the S- configuration.

Figure 5.8: Biotransformation of racemic δ-decanolactone (42μmoles) by cell free extracts derived from Acinetobacter NCIMB 9871grown on cyclohexanol. The numbers accompanying the data points represents the relative percentage of the (R/S) enantiomers of the residual lactone substrate.



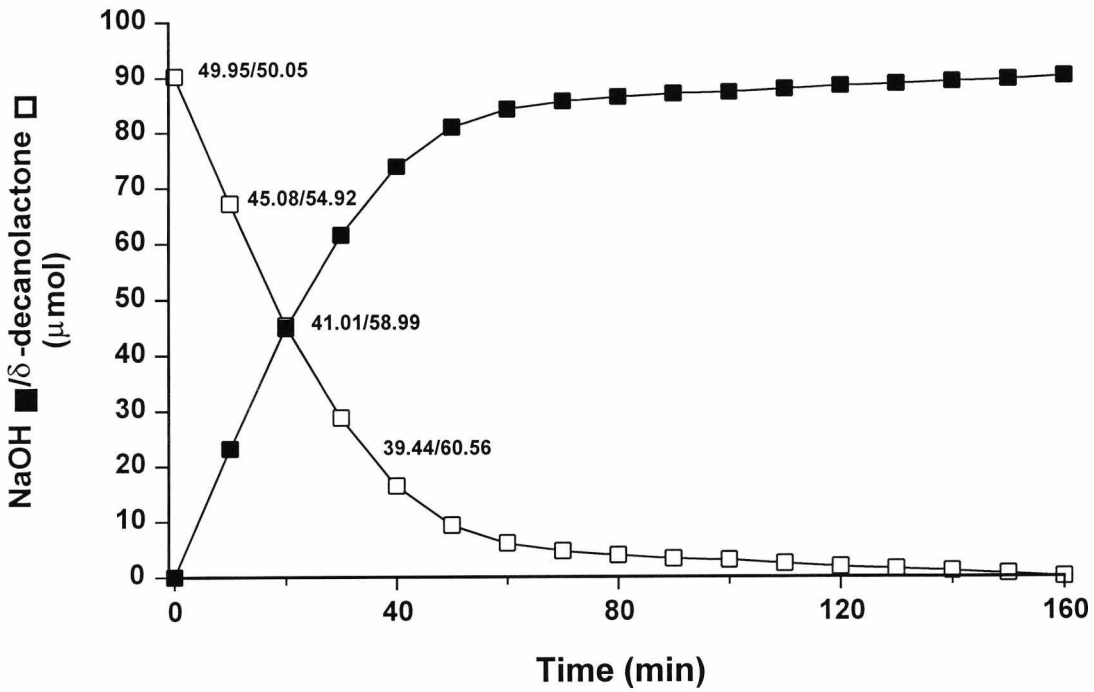
Reaction carried out in autotitrator pH stat 7.0 at 30°C.

Figure 5.9: Biotransformation of ε-decanolactone (100umoles) using cell free extracts derived from Acinetobacter NCIMB 9871 grown on cyclohexanol. The numbers accompanying the data points represent the relative percentage of the (R/S) enantiomers of the residual lactone substrate.



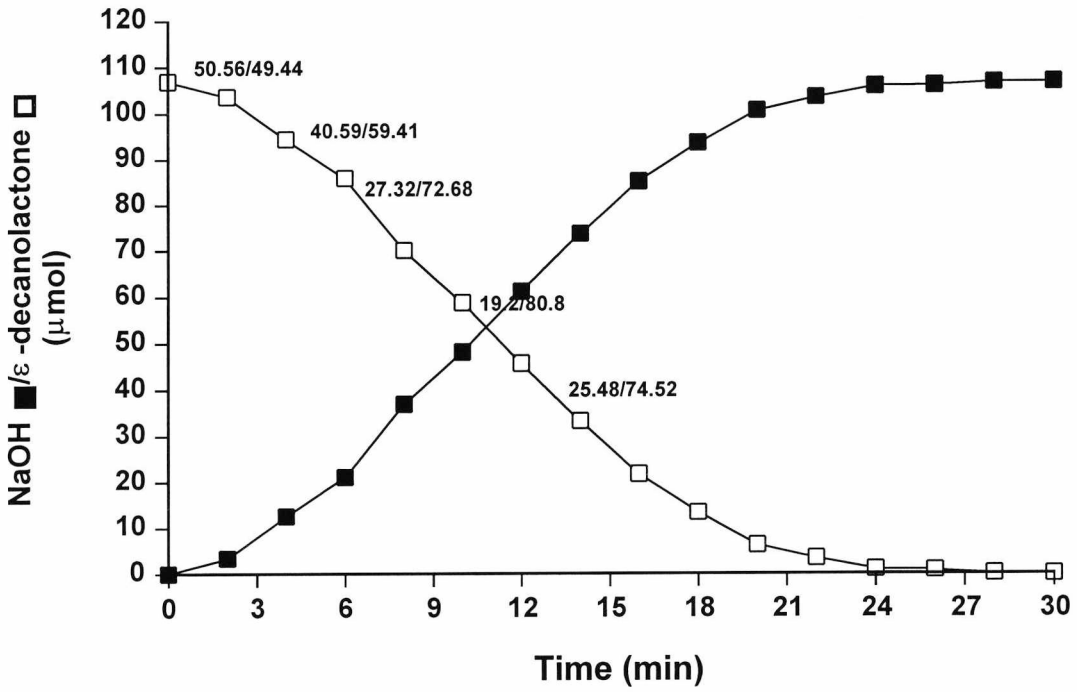
Reaction carried out in autotitrator pH stat 7.0 at 30°C.

Figure 5.10: Biotransformation of δ-decanolactone (100μmoles) by cell free extracts derived from R.coprophilus WT1 grown on cyclohexanol. The numbers accompanying the datapoints represent the relative percentage of the (R/S) enantiomers of the residual lactone substrate.



Reaction carried out in autotitrator pH stat 7.0 at 30°C.

Figure 5.11: Biotransformation of ε-decanolactone (100μmoles) by cell free extracts derived from R.coprophilus WT1 grown on cyclohexanol. The numbers accompanying the datapoints represent the relative percentage of the (R/S) enantiomers of the residual lactone substrate



Reaction carried out in autotitrator pH stat 7.0 at 30°C.

CHAPTER 6

DISCUSSION

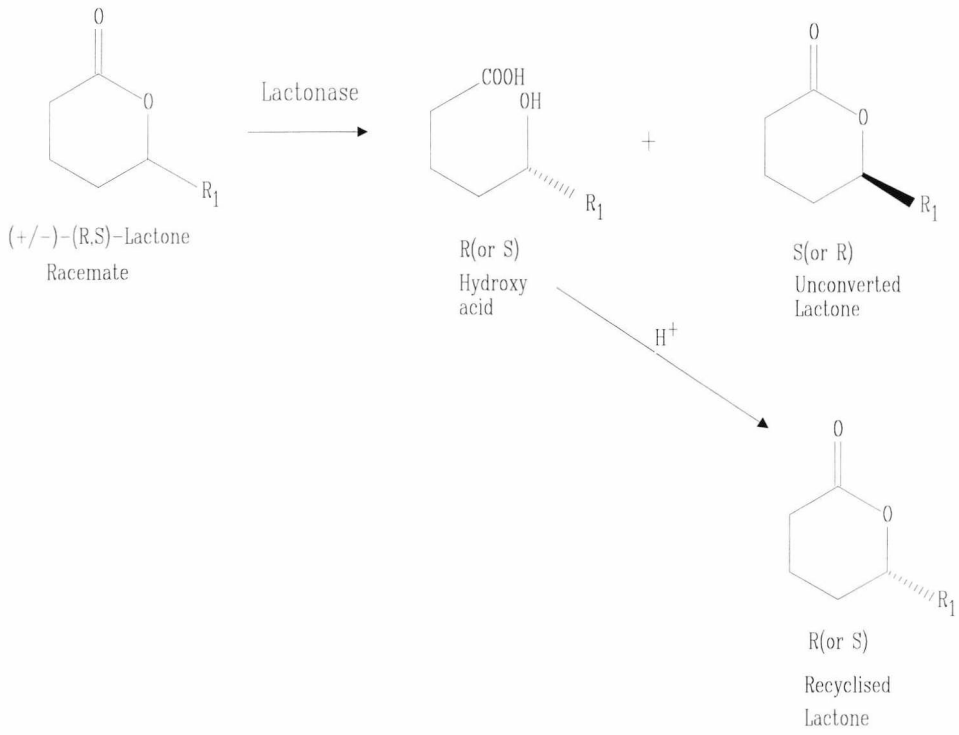
6.1. **The Microorganisms**

As detailed in the Introduction, Baeyer-Villiger like lactonisation capabilities of some microorganisms have been recognised (Conrad, Dubus and Gunsalus, 1961; Norris and Trudgill, 1971; Donoghue and Trudgill, 1975; Griffin and Trudgill 1972). The most studied and best understood microbial Baeyer-Villiger system is that of the Gram-negative bacterium Acinetobacter NCIMB 9871 (Donoghue, Norris and Trudgill, 1976; Chen, Peoples and Walsh, 1988; Schwab, Li and Thomas, 1983). The enzyme responsible for this reaction is cyclohexanone monooxygenase (see section 1.7). This enzyme has been shown not only to accept a broad spectrum of ketone substrates, but also to be both regioselective and enantiospecific in its oxidation of cyclic ketones to the corresponding lactones.

Unfortunately, this microorganism is not ideally suited for use in biotransformations, being graded as a Hazard Group 2 Pathogen capable of causing respiratory diseases. In addition, the obligate requirement for the expensive co-factor NADPH, has highlighted the need for alternative, safer and more economic biocatalysts. It is thus not surprising that there has been an intensive search for other microorganisms bearing such unique catalytic machinery, which has led to the isolation and characterisation of a range of taxonomically diverse microorganisms capable of Baeyer-Villiger like oxidations. Most of these microorganisms were discovered due to their ability to grow on simple cyclic alcohols or ketones. Elucidation of the pathways by which these substrates were metabolised has revealed the involvement of a highly integrated network of enzymes, all of which have an important role to play in cyclic alcohol, or cyclic ketone metabolism.

The main objective of this project was to develop an efficient, potentially economic, and safe method for chiral lactone production. I aimed to achieve this via an indirect route, whereby lactonase enzymes derived from cyclic alcohol/ketone metabolising microorganisms were used for the resolution of racemic lactones, as illustrated in scheme 6.1.

Scheme 6.1: Potential route for the resolution of racemic δ-lactones by bacterial lactonases



Enantioselective resolutions could be achieved if the lactonase enzyme in question was absolutely specific for one enantiomer of a racemic lactone substrate, thus leaving the other lactone enantiomer untouched. In such a case the maximum product yield obtainable would be 50% and this would be in 100% enantiomeric purity. On the other hand the lactonase

enzyme may show a preference for one enantiomer of the lactone substrate thus hydrolysing it at a faster rate than the other enantiomer. Such kinetic resolutions may give 100% product yield, however the enantiomeric purity of the resulting products would vary depending on the extent of the reaction. Other parameters to consider during kinetic and absolute resolutions are ring size, structure and the properties of the substituent side chain.

In addition to Acinetobacter NCIMB 9871, the cyclopentanol metabolising Gram-negative Pseudomonas NCIMB 9872, and, the Gram-positive cyclohexanol metabolising R. coprophilus WT1 isolated in our laboratory by Lenn (1992), were proposed for study. In each of the bacteria, the appropriate lactone did not serve as a good substrate for growth. This was consistent with the observations of Trudgill and Griffin (1972) and Trudgill and Donoghue (1975), who reported slow rates of oxidation of δ-valerolactone and ε-caprolactone by whole cells of Pseudomonas NCIMB 9872 and Acinetobacter NCIMB 9871 respectively. This observation may reflect the inability of these polar substrates to rapidly penetrate the cellular membrane of these bacteria, thus leading to poor accessibility of the lactone substrates to the intracellular lactonase enzyme. The bacteria were therefore grown on the appropriate cyclic alcohol as discussed below.

6.2. Growth of the bacteria and induction of enzymes responsible for lactone hydrolysis

The main impetus of the growth and enzyme induction studies was to establish optimum growth and harvesting conditions for the maximal recovery of lactone hydrolase activity. Growth on cyclohexanol and cyclohexanone appeared to illicit a similar growth response in Acinetobacter NCIMB 9871, suggesting that switching between either substrate as a carbon and energy source would not confer any further advantages as far as biomass cultivation and

possibly enzyme induction were concerned. Similar observations were made for Pseudomonas NCIMB 9872 grown on cyclopentanol or cyclopentanone. However, growth of R. coprophilus WT1 on cyclohexanol resulted in the accumulation of cyclohexanone in the growth medium during the early stages of growth. This phenomenum did not occur with either of the other microorganisms studied, and may indicate a high level of cyclohexanol dehydrogenase activity or a low level of cyclohexanone monooxygenase activity during the early stages of growth.

High speed supernatant fractions derived from the microorganisms were used for analysis of enzymes in the cyclic alcohol or ketone catabolic pathway to avoid ambiguities that may be introduced by membrane bound NADH dependent dehydrogenses usually present in crude cell extracts. Cell breakage was by sonication, and an optimal sonication time was deemed to have been achieved when the specific activity of the lactonase enzyme did not increase further with sonication. Enzyme induction profiles for Acinetobacter NCIMB 9871 grown on cyclohexanol and Pseudomonas NCIMB 9872 grown on cyclopentanol indicated that the activities of the three key enzymes required for cyclic alcohol metabolism increased during the early exponential phase of growth, but started to decline as they reached the late exponential phase, when the concentration of substrate in the medium was low, indicating that these enzymes are induced during growth. Cyclohexanol dehydrogenase activity was not demonstrable in high speed supernatants derived from R. coprophilus WT1 grown on cyclohexanol or cyclohexanone. This was unexpected, especially when considering the ability of this microorganism to utilise cyclohexanol as a growth substrate. However, this

observation is not unprecedented; Norris and Trudgill (1971) were unable to demonstrate cyclohexanol dehydrogenase activity in cell extracts derived from the taxonomically related cyclohexanol grown Nocardia globerula CL1 which also possesses a cyclohexanone monooxygenase. Enzyme instability has been postulated as a plausible cause for this observation (Davey and Trudgill, 1967). Other explanations may lie in the requirement for the supply of external growth factors to the growth medium necessary for cyclohexanol dehydrogenase activity, as exemplified by the methanol dehydrogenase from Rhodopseudomonas acidophilia which requires methylamine hydrochloride for activity and couples to phenazine methosulphate as the electron acceptor (Bamforth and Quayle, 1978). Anthony and Zatman (1967) have also highlighted the requirement for ammonium ions for alcohol dehydrogenase activity. Enzyme activity data could not be obtained during the very early stages of growth of these microorganisms because not enough protein could be obtained for analysis from the low levels of biomass retrieved.

At all stages during growth, the lactonase activity was 10-20 times greater than the activity due to either the monooxygenase or the dehydrogenase. This is consistent with the observations of previous workers (Trudgill and Griffin, 1972; Trudgill and Donoghue, 1975) who were unable to isolate lactones as products from metabolic studies on these bacteria, and thus had to speculate about the possible involvement of a lactone hydrolase in the metabolism of cyclic alcohols and cyclic ketones. It is possible that this large differential in activity exists in order to ensure that the overall direction is in the favour of catabolism.

In order to elucidate further the potential inducibilty of these enzymes, the microorganisms were grown on a substrate whose catabolism did not involve any of the key enzymes associated with cyclohexanol or cyclohexanone metabolism. As mentioned in the Results

section, none of the conventional growth substrates: glucose, citrate, acetate or glycerol, supported growth of these microorganisms. However, growth of these microorganisms on p-hydroxybenzoate was rapid. It is unlikely that the lactonase activity observed in cell extracts derived from bacteria grown on the cyclic alcohol or cyclic ketone are the result of miscellaneous esterases that may occur constitutively within the cell, since p-hydroxybenzoate grown bacteria did not exhibit lactonase activity or any of the other key enzyme activities required for cyclic alcohol metabolism.

A greater than 600-fold increase in activity was observed for cyclohexanol dehydrogenase, cyclohexanone monooxygenase and ε-caprolactone hydrolase in cyclohexanol grown Acinetobacter NCIMB 9871 compared to bacteria grown on p-hydroxybenzoate. Interestingly, in cyclohexanone grown cultures, cyclohexanol dehydrogenase activity was low, at about one-tenth the activity observed in cyclohexanol grown cultures. However, cyclohexanone monooxygenase and ε-caprolactone hydrolase activity were comparable to activities observed in cyclohexanol grown cultures. Furthermore, ε-caprolactone grown cultures only showed significant ε-caprolactone hydrolase activity, while cyclohexanone monooxygenase activity was almost non-existent. Cyclohexanol dehydrogenase activity however was similar to that observed in cyclohexanone grown cultures. Similar data were recorded for Pseudomonas NCIMB 9872.

It thus appears that the enzymes responsible for cyclic alcohol metabolism in these bacteria are induced independently of one another and hence may have different mechanisms controlling their synthesis. Sequential induction of the enzymes of the pathway for cyclohexanol utilisation may occur.

More detailed experiments would need to be conducted to comprehensively elucidate the molecular principles underlying the induction of these enzymes, However, it is interesting to note that these bacteria only selectively induce those enzymes directly implicated in the metabolism of a particular substrate in the cyclic-alcohol metabolic pathway.

The accumulation of cyclohexanone during the early stages of growth of R. coprophilus WT1 on cyclohexanol may be indicative of a different regulatory mechanism controlling enzyme induction for the catabolic pathway in this microorganism. Assuming that cyclohexanol was an inducer of cyclohexanol dehydrogenase but not cyclohexanone monooxygenase (the latter enzyme being inducible by cyclohexanone), then growth on cyclohexanol would lead to cyclohexanone accumulation in the medium until cyclohexanone monooxygenase is induced to a sufficient level to utilise this substrate effectively. Although basal enzyme studies revealed high levels of cyclohexanone monooxygenase and ε-caprolactone hydrolase activity in cells of this bacteria grown on cyclohexanol and cyclohexanone, the inability to demonstrate cyclohexanol dehydrogenase activity in cell extracts from this microorganism, makes it difficult to determine conclusively the induction mechanisms that may be occuring.

Optimisation of lactonase yield in the high speed supernatant fraction was a compromise between growth to yield high biomass and the level of induction of the enzyme. It was apparent that growth on cyclohexanol or cyclohexanone resulted in recovery of similar levels of biomass with the lactonase enzyme induced to similar levels in both Acinetobacter NCIMB 9871 and R. coprophilus WT1. The same applied for growth of Pseudomonas NCIMB 9872 on either cyclopentanol or cyclopentanone. The typically high specific

activities exhibited by the lactonase enzymes from all three microorganisms permitted a degree of flexiblility in the time of harvesting the cultures. For most studies bacteria were harvested at the mid or late exponential phase of growth, when the biomass concentrations were high, and lactonase enzymes were sufficiently induced to give a specific activity of between 10 - 15μmols/min/mg protein. Owing to the good stability of these enzymes at -20°C, they could be stored for several months without losing significant amounts of activity.

6.3. Biotransformations

Performing biotransformations with the aid of the autotitrator offered an important advantage in that the extent of the reaction could be estimated directly by assaying the appearance of product in real-time, without having to rely on subsequent analysis by GC and HPLC.

Previous assays for lactonase activity in cell extracts from these microorganisms have relied on a discontinuous alkaline hydroxamate colorimetric assay for residual lactone, first introduced by Cain (1961). This assay, which measures the concentration of residual lactone in reaction mixtures, suffers from a number of drawbacks as discovered during the course of this project: (1) it is a sampling method and does not give a continuous, true indication of the enzyme kinetics (i.e initial reaction rate); (2) it is not sufficiently sensitive to assay very low enzyme activities; and (3) there is a high tendency for experimental error due to the many chemical steps involved in the analysis of the residual substrate. Perhaps of greatest importance is the fact that this method does not show the appearance of the

hydroxyacid product, hence, casting a shadow over the link between enzyme activity and substrate loss; i.e substrate loss cannot be proved to be solely due to enzyme activity.

For these reasons autotitration of the hydroxyacid (formed from lactone hydrolysis) with NaOH was considered a more reliable procedure for assaying lactonase activity. Preliminary tests were carried out to confirm that there was hydroxyacid production from lactone hydrolysis. The high speed supernatant fraction derived from Acinetobacter NCIMB 9871 grown on cyclohexanol, was incubated with ε-caprolactone in the autotitrator at pH7. Progress of the reaction was followed by gas chromatography until no more ε-caprolactone could be detected. Comparison of NMR and IR spectra of the extracted biotransformation product with that of chemically synthesized 6-hydroxyhexanoate confirmed that this was the product of ε-caprolactone hydrolysis. Since an equal number of moles of NaOH was required for neutralization (by manual titration) of a known amount of 6-hydroxyhexanoic acid, an equimolar addition of NaOH to reaction media containing ε-caprolactone and high speed supernatant was indicative of a unitary stoichiometry of hydroxyacid production (Table 6.1)

Table 6.1: Quantitative assessment of acid production by high speed supernatant derived from Acinetobacter NCIMB 9871 incubated with ε-caprolactone as substrate

ε-caprolactone added μmol (A)	NaOH consumed μmol (B)	Ratio B/A
5	5.6	1.12
20	21.4	1.07
40	42.5	1.06
100	103.6	1.036

6.3.1. Substrate specificity

It was assumed that δ-valerolactone hydrolase derived from Pseudomonas NCIMB 9872 grown on the five-membered ring, cyclopentanol, might show significant activity towards γ-butyrolactone and some of the other substituted 5-membered ring γ-lactones (see table 5.1 for structures of the lactones), since ε-caprolactone hydrolase derived from either Acinetobacter NCIMB 9871 or R. coprophilus WT1 grown on the 6-membered ring cyclohexanol, showed activity not only towards ε-caprolactone (a seven membered ring lactone), but also towards lactones of a lower ring size. However, this was not the case, and all the lactonases exhibited relatively similar substrate specificities.

Purified δ-valerolactone hydrolase and the high speed supernatant derived from Pseudomonas NCIMB 9872 showed identical substrate specificities, with maximal activity occurring towards the natural substrate, δ-valerolactone. ε-Caprolactone hydrolase present in high speed

supernatants derived from Acinetobacter NCIMB 9871 and R. coprophilus WT1, showed similar activities; the maximal activity for both of these microorganisms also occurred with their natural substrate, ε-caprolactone.

There is a possibility that steric factors may play an important part in the activity of these enzymes, since substrates with bulky side chains such as δ-dodecanolactone failed to stimulate significant activity. However, the observation that ε-decanolactone served as a substrate for ε-caprolactone hydrolase but not δ-valerolactone hydrolase suggests that the active site of these enzymes may have significant structural dissimilarities. This possibility is reinforced when when considering the enantioselectivity of the lactonases. δ-Valerolactone hydrolase from Pseudomonas NCIMB 9872 was very enantioselective in its activity, showing a greater preference for the R-enantiomer of the racemic δ-lactones tested. However, this enzyme cannot be absolutely enantioselective for the R-enantiomer of its lactone substrate, since the transformation of this substrate went past the 50% stage, when it would be expected that all the R-enantiomer would have been exhausted. A kinetic profile for the enzymatic hydrolysis of δ-decanolactone revealed that this reaction slowed down significantly as it approached the 40% transformation stage. Since only slight (if any) inactivation of this enzyme was encountered during this reaction, this implies that the enzyme is kinetically enantioselective towards the R-enantiomer. The general theory of enzyme-catalysed kinetic resolutions (Chen et al., 1982), suggests that the optical purity of the resulting hydroxyacid would decrease with an increase in the degree of transformation of the racemic δ-lactone, whilst the optical purity of the remaining lactone would increase. Accordingly, to maximize the production of the optically active R-configuration hydroxyacid, the reaction was terminated after about 40% transformation, whereas in order to optimize the production of the optically active S-

configuration lactone, the reaction was allowed to proceed to up to 65% transformation before being terminated.

Isolation of the hydroxyacid was difficult to achieve due to spontaneous relactonization upon extraction into organic solvent. Therefore, the hydroxyacid was analysed by reconverting it back to the lactone by acidic treatment. Analysis of biotransformation products showed that for the reaction terminated after 40% transformation, the R- enantiomer of the hydroxyacid (reconverted back to lactone by acidic treatment), was in enantiomeric excess of up to 90%. For the 65% transformation, the S- enantiomer of the residual substrate, δ-decanolactone, was in enantiomeric excess of >99.9%. This is an improvement on the results reported by Blanco et al. (1988), who achieved enantiomeric excesses of only about 80% for the S-enantiomer of δ-decanolactone using horse liver esterase as biocatalyst in an aqueous biotransformation system.

Even though substrate specificity data of ε-caprolactone hydrolase from the Gram-negative Acinetobacter NCIMB 9871, and the Gram-positive R. coprophilus WT1 suggested that the lactonase enzymes from these two microorganisms are very similar, enantioselectivity studies indicate that this may not be the case. Transformation to about 40% residual lactone yielded residual δ-decanolactone in only about 6% enantiomeric excess, and residual ε-decanolactone in about 35% enantiomeric excess, for the ε-caprolactone hydrolase from Acinetobacter NCIMB 9871. In contrast, when ε-caprolactone hydrolase from R. coprophilus was the catalyst, δ-decanolactone was obtained in about 15% enantiomeric excess whereas with ε-decanolactone, the residual lactone was in about 60% enantiomeric excess. In both cases, the less preferred enantiomer for hydrolysis was of the S- configuration.

6.4. Purification and Characterisation of δ-valerolactone hydrolase from *Pseudomonas* NCIMB 9872.

The pronounced enantioselectivity of the δ-valerolactone hydrolase of Pseudomonas NCIMB 9872 towards its lactone substrates warranted further investigation into its physical characteristics. In particular it was desirable to understand the underlying factors that were responsible for the high enantioselectivity that was exhibited by this enzyme in comparison to the much lower enantioselectivity shown by the ε-caprolactone hydrolase enzymes from Acinetobacter NCIMB 9871 and R. coprophilus WT1.

As a prerequisite to purifying this enzyme, large quantities of Pseudomonas NCIMB 9872 biomass which contained lactonase activity induced at a high level had to be generated. As discussed earlier, the enzyme induction profile for this microorganism grown on cyclopentanol shows that the lactonase specific activity was lower towards the end of exponential growth, when the substrate had almost been exhausted from the medium. It thus appeared that the level of expression of δ-valerolactone hydrolase during growth was a function of the concentration of cyclopentanol in the medium. Hence a fed-batch fermentation was developed for the cultivation of this bacteria, in which further pulses of cyclopentanol were added during growth to prevent its depletion from the medium. Accumulation of cyclopentanone in the medium occurred under these conditions.

Purification of the enzyme was straightfoward, by a two-step protein purification procedure. This involved an anion exchange chromatography step, followed by a hydrophobic interaction chromatography step, yielding up to 110-fold purification of δ-valerolactone hydrolase from cell free extracts derived from Pseudomonas NCIMB 9872. This is interesting since most of

the protein purification procedures that have been reported for hydrolases have involved up to six different steps.

The remarkable selectivity obtained with the lactonase is attributable to the hydrophobic interaction chromatography (HIC) step that was incorporated into the protein purification procedure. The principle for protein adsorption to HIC media is complementary to ion exchange chromatography and gel filtration, and is favoured by a large degree of hydrophobicity in the protein of interest. Thus the high selectivity observed for δ-valerolactone hydrolase is indicative of the presence of a large number of hydrophobic residues in the amino acid backbone of the protein. A similar selectivity was observed during the purification of the nitrilase enzyme from a strain of Rhodococcus rhodochrous by Hoyle (1996) (personal communication). Perhaps a universal feature of hydrolase enzymes is the presence of a large number of hydrophobic amino acid residues, in which case hydrophobic interaction chromatography would be a sensible step to incorporate into the purification procedures for these enzymes.

The yield for δ-valerolactone hydrolase was high (about 27%) when compared to the data reported for the purification of ε-caprolactone hydrolase (3%) from Acinetobacter NCIMB 9871 (Bennett et al., 1988). The yield differential obtained may be a reflection of the number of purification steps employed rather than any dissimilarity between the enzyme systems within the two microorganisms.

There was an obvious discrepancy beween the molecular weight of δ-valerolactone hydrolase as determined by gel filtration (21kda) and SDS-PAGE (28kda). Molecular weight determination by gel filtration is not only a function of the molecular size of the protein in

question, but also a function of the conformational shape. Hence for a series of compounds with similar molecular shape and density, a sigmoidal relationship would exist between their K_{av} values and the logarithms of their molecular weights. Calibration curves constructed for a particular gel type have a linear relationship between K_{av} and logMW over a considerable range. Gel filtration was carried out in this instance with the assumption that δ-valerolactone hydrolase was of the same conformational shape as the globular molecular weight standards that were employed for the calibration of the standard curve. However, if this were not the case, or if the protein had been partly denatured due to storage, then estimation of molecular weights by this method may not be appropriate, and may need to be validated by other means. These shortcomings to molecular weight estimation by gel filtration are not applicable to estimations by SDS-PAGE. Molecular weight estimations by this method are thus expected to be more reliable and appropriate for use in this thesis.

It appears that the δ-valerolactone hydrolase from Pseudomonas NCIMB 9872 is much smaller than the dimeric ε-caprolactone hydrolase from Acinetobacter NCIMB 9871 and Norcadia globerula CL1, which were reported to have molecular weights of 58kda and 62kda respectively (Bennett et al., 1988). Both enzymes are believed to be formed from two electrophoretically indistinguishable subunits. δ-Valerolactone hydrolase from Pseudomonas NCIMB 9872 on the other hand is a monomeric protein with molecular weight of approximately 28kda, and may bear more similarity in size to the 2-pyrone-4,6-dicarboxylate hydrolase purified from Pseudomonas ochraceae (Maruyama, 1982). This enzyme, which catalyses specifically the interconversion between 2-pyrone-4,6-pyrone dicarboxylate and 4-oxalmesaconate, was reported to have a molecular weight of 31kda by gel filtration and 33kda by SDS-PAGE.

Conformational analysis of δ-valerolactone hydrolase by native gel electrophoresis, suggests that it exists in solution in a single conformational form. Proteins in solution may exist as a mixture of interconverting conformations which may be distinguished by native gel electrophoresis depending on the rate of conformational interconversion. A prerequisite for separation is that the half-time for interconversion between conformations must be greater than one-tenth the duration of the electrophoresis. The rates of interconversion are not easily determinable and as such the data presented here can only be used as a guide. More detailed information about conformation would best be investigated by spectral methods such as circular dichroism.

The data thus far reported suggests the presence of a single enzyme that is responsible for the remarkable enantioselectivity exhibited by HSS derived from Pseudomonas NCIMB 9871. The inability to match the amino acid sequence for the NH_2 terminus of this enzyme (see section 4.2.5) with sequences from other lactone hydrolases suggests that this enzyme may be novel. Despite this, physical and kinetic properties such as the isoelectric point, the pH optimum and temperature dependence of this enzyme fall within a range that can be assumed to be typical of lactone hydrolases and esterases in general. In addition, this enzyme exhibited classical Michaelis-Menten enzyme kinetics, had a high affinity for its natural substrate δ-valerolactone (Km = 1mM) and a very high V_{max} (~800μmol/min/mg).

Aldridge (1953) proposed a classification of mammalian esterases based on their activity towards organophosphorus compounds such as diethyl p-nitrophenyl phosphate. Thus, A-esterases are not inhibited by organophosphorus compounds, but hydrolyze them as substrates, and B-esterases exhibit a wide substrate specificity and are inhibited stoichiometrically by

organophosphates without hydrolysing them, whereas C-esterases are neither inhibited by organophosphates nor do they hydrolyse them.

Although only a limited number of lactone hydrolases from other bacteria and animal sources have been studied, they fall into two recognizable groups; those which have a sensitivity towards thiol-reactive inhibitors (Kersten et al., 1982; Maruyama, 1983) or those which have a divalent cation requirement for activity (Fishbein and Bessman, 1966a,b). The observation by Bennett et al. (1988) that the ε-caprolactone hydrolase from Acinetobacter NCIMB 9871 and Nocardia globerula CL1 had more in common with the group B organophosphate-sensitive mammalian esterases, and less in common with either of the recognised groups of lactone hydrolases, inspired the incentive to test the δ-valerolactone hydrolase from Pseudomonas NCIMB 9872 with a range of potential inhibitors. The organophosphate inhibitors diethyl p-nitrophenyl phosphate (paraoxon; E 600) and diisopropyl phosphofluoridate were not available for testing. The observation by Myers and Kemp (1954) that the symptoms of acute poisoning of rats by methanesulfonyl fluoride were comparable to that of diisopropyl phosphofluoridate made this a suitable replacement for this organophosphate inhibitor in this study. This reagent which is recognised as a serine protease inhibitor had no obvious effects on δ-valerolactone hydrolase activity. On the basis of this observation, it is suffice to disqualify δ-valerolactone hydrolase from being related to the group B esterases which are sensitive to the serine-protease organophosphate inhibitors. Furthermore, observation that p-chloromercuribenzoate inhibited δ-valerolactone hydrolase activity serves to distinguish this enzyme from the C-esterases which are markedly stimulated by this reagent. On the basis of this information it is interesting to note that δ-valerolactone hydrolase from Pseudomonas NCIMB 9872 grown on cyclopentanol bears very little resemblance to the ε-caprolactone

hydrolases from Acinetobacter NCIMB 9871 and Nocardia globerula CL1 grown on the structurally related cyclohexanol.

The sensitivity of δ-valerolactone hydrolase to metal chelators such as EDTA and citrate, suggests that this enzyme has an obligate requirement for metal ions for its activity, further distinguishing it from the ε-caprolactone hydrolases. Perhaps this enzyme has more in common with the Ca^{2+} dependent γ-lactonases of human blood and rat liver microsomes described by Fishbein and Bessman (1966b). However the metal ion effects reported by these authors are opposite to those reported in this thesis. Thus, Ca^{2+} has an inhibitory effect on δ-valerolactone hydrolase, Mg^{2+} does not appear to affect the activity, while Mn^{2+} markedly increases the activity of this enzyme.

Future considerations

The data reported in this thesis highlights the potential for use of δ-valerolactone hydrolase from Pseudomonas NCIMB 9872 as an enantioselective biocatalyst for the production of chiral lactones. Although this enzyme showed remarkable enantioselectivity towards the racemic δ-lactones tested, the substrate specificity exhibited requires further investigation. Presently this enzyme is known to be able to hydrolyse 6- and 7-membered ring δ- and ε-lactones but shows little activity towards the 5-membered ring γ- lactones. δ-Valerolactone hydrolase activity towards bicyclic lactones would be a plausible sequel to this remarkable story, since these lactones have been implicated in prostaglandin and steroid synthesis.

The observation that the ε-caprolactone hydrolases from Acinetobacter NCIMB 9871 and R. coprophilus WT1 showed somewhat better enantioselectivity towards ε-decanolactone than δ-decanolactone is in contrast with the enantioselectivity exhibited by δ-valerolactone hydrolase. This warrants further investigations into substrate-activity relationships for the three enzymes. In particular, probing the active site of these lactonases with engineered substrates as has recently been done with the Baeyer-Villiger cyclohexanone monooxygenase enzyme Acinetobacter NCIMB 9871 and Pseudomonas NCIMB 10007 (Kelly et al., 1995a,b) would be a plausible way foward.

Perhaps of greater interest is the observation that δ-valerolactone hydrolase shows a closer similarity to the divalent cation dependent γ-lactonases of human blood and rat liver microsomes. Unlike the ε-caprolactonases from Acinetobacter NCIMB 9871 and Nocardia globerula CL1, these enzymes catalyse reversible lactone hydrolysis in aqueous solution with pH-dependent reaction equilibria. Thus, the potential for the formation of chiral lactones from

chiral and racemic hydroxyacids in aqueous solution using δ-valerolactone hydrolase would be a worthwhile consideration. Alternatively, these lactonisations can be performed in low water systems.

Robinson et al. (1994) reported a number of factors that affect the enzymatic intramolecular lactonisation of 16-hydroxyhexadecanoic acid in microaqueous systems. Of the many factors investigated, the choice of solvent, water content, concentration of the substrate and the pH were reported to have the greatest influence. The choice of solvent has also been shown to influence the regioselectivity (Rubio, Fernandez-Mayorales and Klibanov, 1991) and enantioselectivity (Sakurai et al., 1988) of enzyme catalysed reactions. Tawaki and Klibanov (1992) reported a complete reversal of enzyme enantioselectivity upon a change in the solvent, as part of their kinetic investigations of transesterification reactions catalysed by Aspergillus oryzae protease in different solvents. In the light of this, investigating the effect of different solvents on the activities of δ-valerolactone hydrolase and ε-caprolactone hydrolase could help in understanding the differential enantioselectivities exhibited by these enzymes.

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Sterochemical control yeast reduction 1. Asymmetric synthesis of L-carnitine.
Journal of the American Chemical Society 105 5925-5926

Appendix

(i) Recipe for Gel Preparation

(a) Stock solutions

Stock Solutions	Components	Final volume in Distilled H_20	Storage
Acrylamide	29.1g Acrylamide 0.9g NN'-Methylene bisacrylamide 60ml dH_2O	100ml	2weeks at 4^oC
(1.875) Tris Hcl Buffer pH 8.8	56.8g Tris 150ml dH_2O Adjust pH to 8.8 with 5N HCl	250ml	4weeks at 4^oC
(1.25M) Tris Hcl Buffer pH 6.8	37.8g Tris 150ml dH_2O Adjust pH to 6.8 with 5N HCl	250ml	4weeks at 4^oC
10% Sodium Dodecyl Sulphate (SDS)	10.0g SDS 85ml dH_2O	100ml	4months at room temp.
10% Ammonium Persulphate	0.5g Ammonium persulphate 4ml dH_2O	5ml	1week at 4^oC
Tris-Glycine Electrode Buffer (X10)*	144.2g Glycine 30.3g Tris 10g SDS 800ml dH_2O	1000ml	2months at room temp. (pH~8.3)
Loading Buffer	2.5ml 1.25M Tris-HCl pH 6.8 1.0g SDS 2.5ml 2-Mercaptoethanol 5.8ml Glycerol 5mg Bromophenol blue 35ml dH_2O	50ml	5ml aliquots stored for 4-6months at -20^oC

* Dilute electrode buffer 1 in 10 before use: e.g 400ml in 3600ml dH_2O

(b) SDS-PAGE recipe for one 1.5mm or two 0.75mm thick 10% acrylamide gels

	Resolving gel	Stacking gel
Acrylamide (ml)	5.0	0.8
Distilled H_2O (ml)	6.8	3.6
1.875M Tris-HCl pH 8.8 (ml)	3.0	
1.25M Tris-HCl pH 6.8 (ml)		0.5
Degas the solution, then add		
10 % SDS (µl)	150	50
Temed (µl)	7.5	5
10% Ammonium Persulfate (µl)	50	17

(c) Destaining solution (Coomassie R-250)

Dissolve 0.25g of coomassie brilliant blue R-250 in 90ml of methanol:water (1:1 v/v) and 10ml of glacial acetic acid. Filter the solution through a whatman number 1 filter to remove any particulate matter.

Immerse the gel in about 5 volumes of staining solution and place on a platform rotating slowly for about 4 hours at room temperature.

Bands can be observed by soaking stained gels in methanol:water solution (1:1) (without any coomassie dye) for about three hours.

(ii) 0.2M Buffers used for pH studies

BUFFER	pH
Succinate	5
Succinate	5.5
Phosphate	6
Phosphate	6.5
Phosphate	7
Tris(hydroxymethyl) aminomethane (Tris)	7.5
Tris	8
Tris	8.5
Glycine NaOH	9
Glycine-NaOH	9