THE HYDROLYSIS OF LINALYL ACETATE AND α-TERPINYL ACETATE BY YEASTS

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M.Sc. Degree in the Faculty of Natural Sciences

Department of Microbiology and Biochemistry
University of the Orange Free State

By Elias Thomas

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Supervisor Prof. M. S. Smit
Co-supervisor Prof. D. Litthauer

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Table of contents

List of figures

List of tables

List of abbreviations

A	pre-exponential factor
ACE	acetylcholine esterase
CM	carboxymethyl
CMC	carboxymethycellulose
DEAE	diethylaminoethyl
DEP	di-ethylpyrocarbonate
DFP	di-isopropylfluorophosphate
DMSO	dimethylsulfoxide
E	enantiomeric ratio
e.e.	enantiomeric excess
E/glu	glutamic acid
E_a	energy of activation
GC	gas chromatography
H/his	histidine
HCl	hydrochloric acid
HLE	horse liver esterase
KCl	potassium chloride
MgCl	magnesium chloride
NaOH	sodium hydroxide
NMR	nuclear magnetic resonance spectroscopy

PLE	porcine liver esterase
PMSF	phenylmethylsulfonylfluoride
rac	racemic
S/ser	serine
t½	half-life
YNB	yeast nitrogen base

Chapter 1 Literature review

1.1 General introduction

Since the turn of the century the utilisation of enzymes/enzyme systems as biocatalysts have posed interesting alternatives to conventional methods in organic synthesis. Enzymes are efficient catalysts typically producing reaction rates accelerated by a factor of 10^8 compared to those of non-enzymatic reactions. They are completely degradable and therefor environmentally friendly, act under mild reaction conditions i.e. pH range of 5-8 and have an optimal temperature ranging between 20°C and 40°C, thereby minimising undesired side-reactions such as decomposition, isomerisation, racemation and re-arrangement which often plague conventional chemical methods. Additionally some biocatalysts exhibit a high substrate tolerance by accepting a broad range of non-natural substrates and if required are biologically active in organic solvents. Enzymes only accelerate a reaction rate and have no impact on the position of the thermodynamic equilibrium of a reaction, thereby presenting the opportunity of enzyme-catalysed reactions being reversible, depending on the manipulation of the given environment (Faber, 1992).

There is an enzyme-catalysed process equivalent to almost every type of organic reaction. In addition enzymes can accomplish reactions impossible to emulate in organic chemistry. However, there are some major exceptions, notable being the Diels-Alder reaction. Enzymes display three major types of

selectivities i.e. chemoselectivity, regio- and stereoselectivity. The latter includes diastereoselectivity and most importantly, enantioselectivity.

In the past most bio-active compounds, in the pharmaceutical, agrochemical and the flavour and fragrance industries, were applied as racemates mainly due to economic reasons. This situation has definitely changed due to increased pressure from legislation leading to a need for enantiomerically pure compounds (Morrison, 1983). This paved the way for the utilisation of biocatalysts for selective and asymmetric exploitation. Recently, Effenberger *et al.* (1997) described the preparation of enantiomerically pure (*S*)-naproxen, which is a non-steroidal anti-inflammatory drug. They used resting cells of *Rhodococcus erythropolis* MP50 to enantioselectively hydrolyze racemic naproxen amide. Studies like these have shown the increasing importance of biocatalysts in the production of value added products. In 1996 ChiroTech produced 13 000kg of (*S*)-naproxen using a novel esterase (Stinson, 1997).

Detractors of the utilisation of biocatalysts may argue that mild reaction conditions are in actual fact narrow operating parameters and that aqueous environments are the most suitable for biocatalysts making transformations of hydrophobic organic compounds difficult. They may also argue that substrate or product inhibition can limit the efficiency of biocatalytic processes. However, with correct manipulation enzyme-catalysed reactions are increasingly becoming an efficient tool available to chemists involved in the synthesis of natural and non-natural compounds.

Biocatalysts have been an integral part of civilised man's existence for centuries especially in the manufacture of food and beverages. Up until 1998 more than 3000 enzymes were recognised by the International Union of Biochemistry with this figure estimated to be approximately 10% of the vast reservoir existing in nature. Approximately 15% of those previously recognised are available commercially (I.U.B. 1985). Enzymes have been classified into six categories according to the type of reaction they can catalyse (Table 1.1). The enzyme class, hydrolases, are the most utilised in industry and are of particular interest for the purposes of the present study.

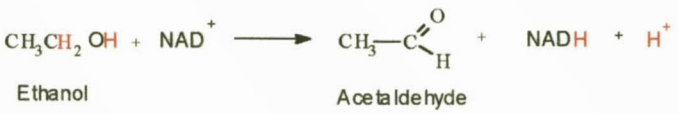
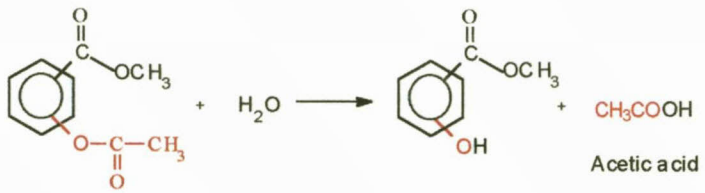
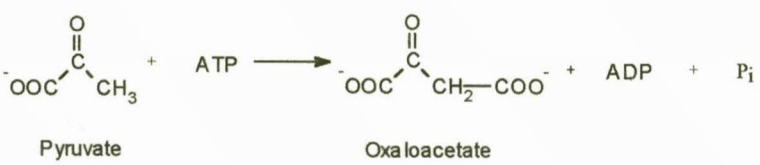
Class	Example	Reaction catalysed
1. Oxidoreductases	alcohol dehydrogenase	 Ethanol → Acetaldehyde
2. Transferases	glucokinase	 D - Glucose → D - Glucose - 6- phoshate
3. Hydrolases	an esterase	 Methyl- 4- acetoxybenzoate → Methyl- 4- hydroxybenzoate
4. Lyases	pyruvate decarboxylase	 Pyruvate → Acetaldehyde
5. Isomerases	maleate isomerase	 Maleate ⇌ Fumarate
6. Ligases	pyruvate carboxylase	 Pyruvate → Oxaloacetate

Table 1.1 Examples of each of the major enzyme classes (adapted from Matthews and van Holde, 1990).

1.2 The use of organic co-solvents in biotransformations.

The microbial transformation of organic compounds is important in nature as well as in technological applications of micro-organisms. Some of these bioprocesses involve natural hydrocarbons such as terpenes, aromatic and aliphatic hydrocarbons, with racemic linalyl acetate, a monoterpenoid, of particular interest to this study. Two major problems associated with organic compounds are the limited bioavailability of many lipophilic compounds and their toxicity to micro-organisms. To overcome these problems a second organic solvent is often used in biotechnology as well as in natural degradation of organic compounds (Axcell and Geary, 1973).

Toxicity is a very important aspect when considering the use of co-solvents. A correctly chosen co-solvent should be used to reduce the toxicity of the organic substrate or inhibitory effects of products and itself should not be toxic to the organism. The most commonly used measure of toxicity in research correlates with the degree of hydrophobicity of the co-solvent i.e. log P. Log P is defined as the logarithm of the partition coefficient in a standard 1-octanol-water two phase system. Hydrophobicity can also be quantified by the following parameters namely Hildebrand solubility parameters, Solvatochronism of dyes, dielectric constant and the dipole moment of a compound (Laane *et al.*, 1987). For the purposes of this dissertation log P values are employed.

Laane *et al.*, 1987 were able to make the following generalisations about the effect of log P on activity retention for two whole cells systems. The two systems tested were alkene epoxidation by immobilized bacterial cells in the presence of a second organic phase and gas production by anaerobic bacteria in aqueous media saturated with organic solvents. They reported that co-solvents with a log P lower than 2 are least suitable for biocatalytic systems, a log P between 2 and 4 might be suitable for some applications, but harmful effects may occur and finally co-solvents with a log P greater than 4 seem to be applicable for most biocatalytic systems. Later Herman *et al.* (1991) reported toxic effects of co-solvents on whole cells when the log P is anywhere between 1 and 5.

The mechanism of co-solvent caused toxicity is not well understood. However the toxic action can be divided into two major classes namely physical and dissolved toxicity which is essentially the difference between toxicity at the phase level and the molecular level. Dissolved toxicity is the effect of a solvent when it is present in the aqueous phase below saturation (Bar, 1987). Results from many scientists on different microbial systems using different solvents indicate that solvent toxicity in largely due to the accumulation of the solvents in the membranes, particularly the cytoplasmic membranes (Dietenbach and Keweloh, 1994). Solvent toxicity causes membrane function inhibition due to the alteration of membrane fluidity, lipid phase separations, direct solvent-protein interactions and membrane permeabilisation (Osborne *et al.*, 1990). Increased lipid membrane permeability in turn may cause enzyme inhibition, protein inactivation or a

breakdown of transport mechanisms. These finding are summarised in Table 1.2.

Table 1.2 Effects of co-solvent toxicity of biocatalysts (Osborne *et al.*, 1990).

Solvent toxicity	
Molecular toxicity Dissolved (below saturation)	**Phase toxicity** Physical (excess of saturation)
Enzyme inhibition	Extraction of nutrients
Protein denaturation	Disruption of cell wall Extraction of outer cellular components
Membrane modification: membrane expansion structure disorder permeability change etc.	Limited access to nutrients cell attraction to interface emulsion formation cell coating

Volkering *et al.* (1995) investigated the reaction rates of hydrolases in the presence of biosurfactants for the purposes of soil bioremediation. Their studies concluded that the presence of biosurfactants increase substrate biocatalyst interactions even in a hydrophobic environment thus increasing reaction rates. The above-mentioned findings indicate the importance of a similar investigation, in this current study, where the effect of organic solvents on tertiary alcohol hydrolase activity is analysed.

1.3 Hydrolases

Hydrolases are distributed in all living organisms (Olsen *et al.*, 1994) and among all the types of enzyme-catalysed reactions, hydrolytic transformations involving ester- and amide-bonds are the easiest to perform by using proteases, esterases or lipases which all form part of the hydrolase family. Various different hydrolases exhibit broad substrate specificities and with the advantage of not requiring sensitive co-factors, this group of enzymes is responsible for 66% of the total research in the field of biotransformations (Faber, 1992). Current examples of hydrolases used in large scale biocatalytic processes include the production of glucose ($10x10^6$ tonnes per annum) and acrylamide ($8x10^3$ tonnes per annum) by the hydrolases, namely amyloglucosidase and nitrile hydratase (Kelly, 1998).

Esterification, the reverse reaction of hydrolysis, has also been extensively investigated. Other types of application of hydrolytic enzymes include the formation and/or cleavage of phosphate esters, nitriles and epoxides. These reactions are generally more complicated to perform (Lortie, 1997).

1.3.1 The serine hydrolase catalytic mechanism

The general mechanism of enzymatic ester hydrolysis by hydrolases is very similar to that of chemical hydrolysis by a base (Figure 1.1). A nucleophilic group from the active site of the enzyme attacks the carbonyl carbon of the substrate ester or amide displacing the alcohol or amine and producing an

acyl-enzyme intermediate (step 1&2). This nucleophillic group can be a hydroxyl group of a serine amino acid (e.g. pig liver esterase and lipases from porcine pancreas or *Mucor.* sp) or a carboxy group of an aspartic amino acid residue (e.g. pepsin) or a thiol moiety of a cysteine amino acid residue (e.g. papain). Another nucleophile, usually water (step 3), can in turn attack the acyl-enzyme intermediate regenerating the enzyme and releasing a carboxylic acid (Fersht, 1985). When the enzyme is in an environment of low water activity any other nucleophile can compete with the water molecule for the acyl-enzyme intermediate thus leading to a number of synthetically useful transformations which include ester synthesis and interesterification reactions (Jaeger *et al.*, 1994).

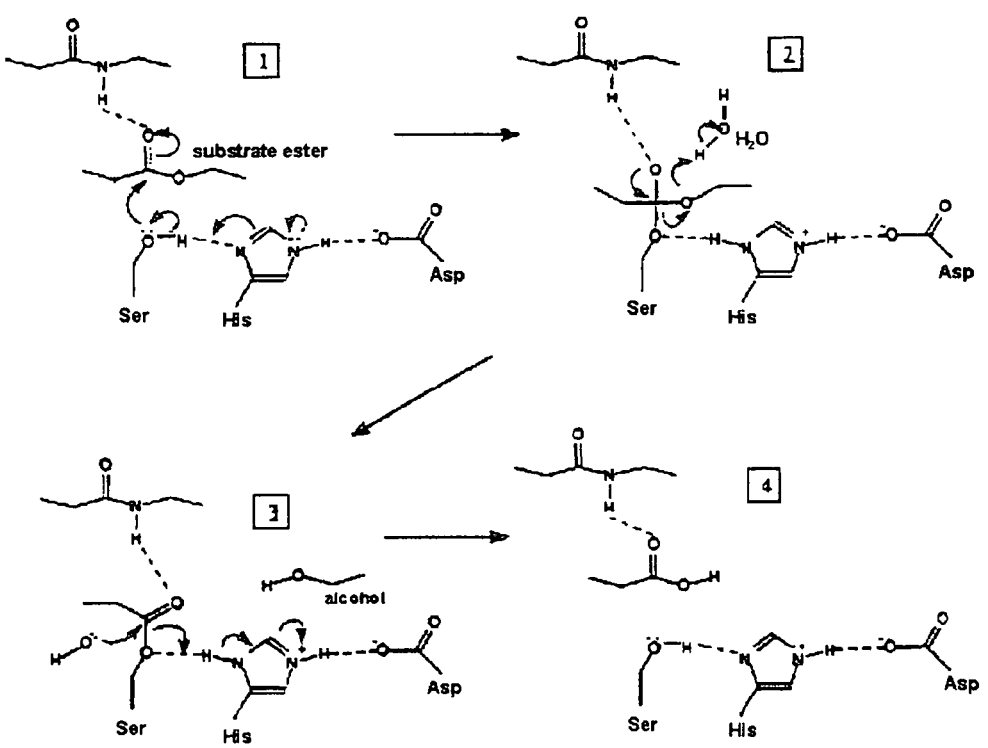


Figure 1.1 The reaction mechanism of a serine hydrolase acting upon an ester substrate (Jaeger *et al.*, 1994).

Figure 1.1 shows a more detailed explanation of the mechanism involved in serine hydrolases whereby two additional amino acid residue functionalities (i.e. an aspartic acid and histidine) located in close proximity to a serine residue form the so-called catalytic triad, thereby decreasing the pK-value of the serine hydroxyl group and facilitating the formation of the acyl-enzyme intermediate by nucleophilic attack (Cygler *et al.*, 1993).

1.3.2 Distinguishing between lipases and esterases

The phenomenon of interfacial activation has resulted in a division in the hydrolase research fraternity as to the definition of whether a hydrolase is a lipase or an esterase. In the past interfacial activation and enzyme kinetics have been used for this purpose.

The physical properties of some substrates e.g. lipids, have caused many difficulties in studying the properties of hydrolases. Triacylglycerols coexist as a complex equilibrium in various physiochemical states in aqueous media ranging from monomer (monomeric substrate), micelle, emulsion and adsorbed monolayer (super-substrates) and has led to the extensive scrutiny of the interfacial activation phenomena since first observed by Holwerda *et al.* 1936. They noted that the activity of some hydrolases, so-called lipases, was enhanced on an insoluble substrate compared with the same substrate in true monomeric physiochemical state. This led to the assumption that lipases might be a special category of esterases that are highly efficient at

hydrolyzing molecules having a carboxylic ester group and which are aggregated in water i.e. lipolysis occurs exclusively at the lipid-water interface.

Interfacial activation can be described as a function of a) the substrates of interest, triacylglygerols (which are uncharged lipids ranging from compounds with short chain fatty acids and slightly soluble in water to compounds with longer chain fatty acids esterified to glycerol and insoluble in water) and b) the preference of enzymes for aqueous media. Sarda and Desnuelle (1958) clearly demonstrated a fundamental difference between esterase and lipase activity based upon the their ability to be activated by substrate-water interfaces. Esterase activity was determined to be a function of substrate concentrations and described by Michaelis-Menten kinetics with a maximal reaction rate being reached well before the solution becomes substrate saturated i.e. the formation of a substrate-water interface does not effect reaction rate.

In contrast, lipases usually show almost no activity with the same substrate as long as it is present in the monomeric state. However, when the solubility of the substrate is exceeded, there is a sharp increase in enzyme activity as the substrate forms an emulsion i.e. a substrate-water interface (Figure 1.2).

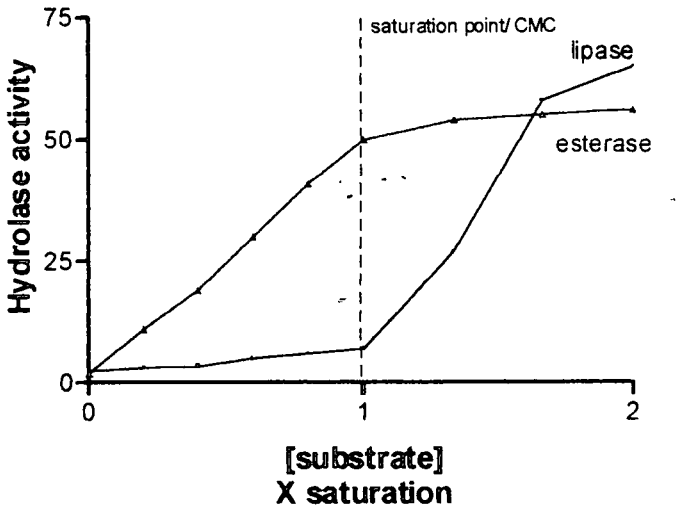


Figure 1.2 Classical activity profile of a pancreatic lipase and horse liver esterase at different substrate concentrations prior and exceeding saturation point (adapted from Sarda and Desnuelle, 1958).

These experiments demonstrated that lipase activity depends on the presence of an interface and led to the definition of lipases as carboxyesterases acting on emulsified substrates later substrate concentrations exceeding critical micellar concentration. This observation was further supported when the first three-dimensional structures of lipases were elucidated. It was found that the active site of lipase was covered by a lid-like polypeptide chain which rendered the active site inaccessible to substrate molecules, thereby causing the enzyme to be inactive on the monomeric substrate molecules (Winkler *et al.*, 1990). When a lipase becomes bound to a lipid interface a conformational change takes place causing the lid to move away allowing the active site of the lipase to become fully accessible (Figure 1.3). The hydrophobic side of the lid becoming exposed to the lipid phase enhances hydrophobic interactions between the enzyme and lipid interface. The above-mentioned interfacial activation phenomenon has been used to discriminate between esterases and true

lipases which show interfacial activation in the presence of long-chain triacylglycerols as substrates (Brady *et al.*, 1990).

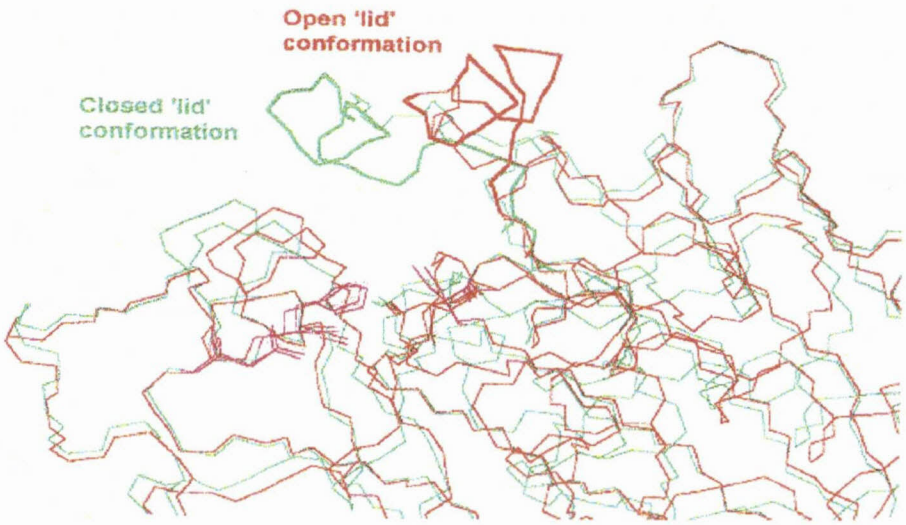


Figure 1.3 Three dimensional structure of *Rhizomucor miehei* lipase showing the open and closed lid conformations. The models were built using Hyperchem software and the co-ordinates deposited in the Brookhaven Protein Data Bank (adapted from Brady *et al.*, 1990)

Recently the classification of true lipases has been re-defined with interfacial activation and the presence of a lid domain deemed unsuitable criteria on which to catergorise a specific hydrolase as belonging to the lipase-family. Lipases are now defined as carboxy-esterases that catalyse the hydrolysis of long-chain acylglycerols (Verger, 1997).

1.3.3 Substrate specificities of lipases and esterases

In general lipases can be classified into three 3 groups according to their substrate specificity. Group 1 lipases show no positional specificity and no specificity with respect to the chemical structure of the fatty acid, the lipase obtained from *Staphylococcus hyicus* being an example of this group (van Oort *et al.*, 1989). Group 2 lipases hydrolyze primary ester bonds (at C_1 and

C_3 of glycerol) only. Examples include lipases from *Pseudomonas fragi* (Mencher and Alford, 1967) and *Pseudomonas flourescens* (Alford *et al.*, 1964). Group 3 lipases have a pronounced fatty acid preference, for example lipase B from *Geotrichum candidum*, shows specificity for fatty acids with a double bond between C_9 and C_{10} (Charton *et al.*, 1991).

The above mentioned classification using the criteria of substrate specificity is far from foolproof suggesting that specificity properties should be seen as a continuum rather than discrete categories. The stereochemical outcome of an asymmetric hydrolysis could be determined by choosing a hydrolase from a different class. The stereospecificities of lipase have also been found to be strictly dependent on the surface pressure of the substrate and on the fatty acid chain length of the substrate (Ransac *et al.*, 1991). Thus lipases and esterases alike can also be classified according to the steric properties of their respective substrates.

Relatively few true esterases are commercially available compared to lipases with a large majority of these esterase-catalysed reactions being performed using mammalian esterases especially porcine liver esterase (PLE). In comparison to lipases, the applicability of PLE and other true esterases is restricted to reactions performed in an aqueous medium. Other esterases obtained from horse (HLE) and rabbit liver possess a related and often slightly altered substrate specificity as compared to PLE while HLE is used in the resolution of small and medium sized lactones and racemic bicyclic lactones (Randrianasolo-Rokotozafy *et al.*, 1993). The applications of acetylcholine

esterase (ACE), which can hydrolyze non-natural esters, and cholesterol esterase are likewise limited with the cost of ACE prohibitive because the esterase is only commercially available from an electric eel source (Johnson *et al.*, 1993, Chenevert and Martin, 1992).

To overcome the narrow range of readily available esterases whole microbial cells have been utilised instead of isolated enzyme preparations. Fortunately, a large number of proteases can be utilised to selectively hydrolyze carboxylic acid esters and this effectively compensates for the limited number of readily available esterases (Jones, 1980). The use of microbial esterases has increased substantially over the past few years with bacterial as well as fungal esterases used at industrial level (Stinson, 1997).

1.4 Hydrolase assay systems

A number of assays to determine hydrolase activity have been developed and include spectrophotometry, titrimetry and controlled surface pressure.

1.4.1 Spectrometry

Several assays for hydrolase activity are based on spectroscopic measurements some of which are direct or indirect and make use of natural or non-natural substrates (Fossati *et al.*, 1992, van Autryve *et al.*, 1991, and McKellarn, 1986).

Esters of various chain length fatty acids of *p*-nitrophenol find common usage in lipase assay systems. Ester substrates containing 4-6 carbon atoms in the substrate backbone have readily been used for esterase assay systems with tributyrin the substrate of preference. When considering lipase activity care should be taken with short chain fatty acid esters as these substrates are also cleaved by esterases leading to the false determination of kinetic parameters in instances where non-pure enzyme preparations are investigated (Stuer *et al.*, 1989).

1.4.2 Titrimetry

The principle of the pH-stat assay for lipase activity can be explained as follows: during a lipolytic reaction an acid is released thereby decreasing the pH of the reaction medium. NaOH of known concentration is continuously administered to the reaction mixture thereby keeping pH constant and allowing an assay of enzymatic activity by titration (Desnuelle *et al.*, 1955).

1.4.3 Controlled surface pressure

Lipases act at the interface between a hydrophobic substrate and a hydrophilic water phase with changes in the surface pressure or interfacial tension used as an index of enzymatic activity (Verger *et al.*, 1976). In this assay method a mono-molecular substrate film is spread at the air-water interface which can be compressed with a surface barrier, thereby changing

the surface density of the substrate and thus the interfacial tension. The lipase injected into the water sub-phase will bind to the film and hydrolyze the substrate thereby changing the surface pressure that can be measured. The choice of substrate plays an important role as the substrate should preferentially generate water soluble hydrolysis products (e.g. trioctanoin or didecanoin). In some cases, substrates with long acyl-chains can also be utilised (Ransac *et al.*, 1991). When the substrate is hydrolyzed, it will leave the interface, thereby decreasing the surface density and surface pressure which is then compensated by compression of the film by the mobile phase barrier. The barrier movement is monitored as a function of time leading to the determination of reaction rates. The advantages of the monolayer technique include high sensitivity, the system is easily manipulated, it allows monitoring of several physiochemical parameters, facilitates the measurement of pre-steady state kinetic measurements and allows for the determination of the effects of water insoluble inhibitors.

Another method to monitor interfacial tension is the oil-drop method. The method consists of forming an oil drop in a water solution with the drop connected to a syringe containing the oil to be hydrolyzed. The shape of the drop is directly correlated to the interfacial tension of the oil-water interface. When the medium contains no detergent or fatty acid the drop is apple shaped. When a lipase is administered to the water phase, it binds to the oil-water interface and hydrolyses the substrate. The released products remain in the interface and the interfacial tension decreases with the substrate slowly becoming pear formed. Hydrolysis can be measured by a computer

controlled device called an "oil drop tensiometer" which determines the decrease in interfacial tension as the reaction progresses allowing the determination of reaction rates and kinetic parameters (Labourdenne *et al.*, 1994).

1.4.4 Other assay methods

Other assay methods include the use of high-performance liquid chromatography where β-naphthyl laurate is employed (Maurich *et al.*, 1991), nuclear magnetic resonance spectroscopy (NMR) for quantitating enzymatic activity in bi-phasic macro-emulsions (O'Connor *et al.*, 1992) and infrared spectroscopy for measuring hydrolysis in reverse micelles (Waldev *et al.*, 1989). Finally, a conductometric method has been described using the short-chain substrate triacetin (Ballot *et al.*, 1984).

1.5 Purification of hydrolases

Most of the purification procedures reported for intracellular esterases involve a series of techniques once cell lysis has occurred. Cell lysis is performed by French press, glass bead homogeniser or by using a mortar and pestle on frozen cells followed by a sonification step. Early steps prior to cell lysis include differential centrifugation (e.g. to separate membrane bound proteins from microsomal fractions), protein precipitation steps by ammonium sulfate or acetone followed by centrifugation steps. Size exclusion chromatography

(separation by molecular size) can then be performed on the active fraction. Other techniques employed include ion-exchange chromatography, hydrophobic interaction chromatography as well as affinity chromatography (molecular "fishhooks" to pick up the desired protein) which can be performed on the cell free protein extract. Within the last decade affinity chromatography, reversed-micelle and aqueous two-phase systems, ultrafiltration membranes and immunopurification have also been applied in hydrolase purification protocols (Woolley and Petersen, 1994). In general, most lipases are extracellular and therefore cell lysis is not required compared to esterases. Selected hydrolase purification protocols follow.

A crude lipase preparation from *Chromobacterium viscosum* contained more than two species of lipase that differed from each other in molecular weight and iso-electric point. Lipase A was purified by chromatography using Amberlite CG-50 and Sephadex G-75 (size exclusion chromatography). Lipase B was purified using a size exclusion column (Sephadex G-100) followed by ion exchange resins i.e. Carboxymethyl (CM)-cellulose and DEAE-Sephadex (Suguira *et al.*, 1974).

A *Lactococcus lactis ssp. lactis* strain ACA-DC 127 provided a purified esterase after chromatography steps which included anion-exchange utilising DEAE-cellulose and size exclusion via Sephadex G-100 (Taskalidou and Kalantzopoulos, 1992). Purification of an esterase from a similar bacterial strain, *Lactococcus lactis ssp. cremoris* E8, required an ammonium sulphate precipitation step followed, by hydrophobic interaction chromatography using

Alkyl Superose 5/5 column. The active fraction was subjected to a size exclusion chromatography step (HR 10/10 Superose 6) which was followed by an anion exchange step (HR 5/5 Mono Q) which led to the production of the pure esterase (Holland and Coolbear, 1996). Recently Schimmel *et al.* (1997) used Mono-Q anion exchange chromatography in the one step purification of an esterase from the fungal source, *Clonostachys compactiuscula*.

1.6 Characterisation of the catalytic site of hydrolases

The core of our knowledge about enzymes is based on experiments in which data pertaining to specificity, activity or stability have been collected. With the introduction of methods for structure determination by molecular techniques we now have access to a powerful tool for explaining the above-mentioned data in terms of structural properties at the gene level. Various methods have been employed to elucidate the important structure-function phenomena. These methods include site-specific protein modification, substrate specificity and molecular approaches (Drablos and Petersen, 1997).

1.6.1 Site-specific chemical modification of proteins

Site-specific chemical modification is strictly defined as a process which yields a stoichiometrically altered protein with the quantitative derivatization of a single, unique amino acid residue without either modification of any other amino acid residue or conformational change (Lundblad, 1995). The above-

mentioned objective is rarely obtained as several problems confound this goal. Firstly, few reagents are specific for a single functional group (Colburn, 1991) and secondly, it is unlikely that site-specific modification can take place without any conformational change (Wilson *et al.*, 1982). The modification of serine, aspartic acid and histidine amino acid residues is commonly used to elucidate the catalytic triad of enzymes that display hydrolytic activity (Tsakalidou and Kalantzopoulos, 1992). Commonly utilised reagents include phenylmethylsulfonylfluoride (PMSF) and di-isopropylfluorophosphate (DFP) which modify hydroxyl groups of serine amino acid residues, di-ethylpyrocarbonate (DEP) which modifies the imidazole ring of histidine amino acids and iodo-acetic acid which modifies the sulfhydryl functional groups of cysteine amino acid residues.

1.6.2 Substrate specificity studies

A broad range of substrate esters with structurally different side chains are used to probe the catalytic site of a hydrolase. The hydrolytic rates of the respective esters allow comparisons and observations to be made about the catalytic site of a given hydrolase (Kroon *et al.*, 1997). A similar study was performed by Toone and Jones (1990) to elucidate the active site of pig liver esterase using substituted aryl malonate substrates. Molecularly imprinted cross-linked polymers with strong esterase activity have confirmed the belief in the formation of a transition state in enzymatic catalysis and has been used as efficient synthetic catalysts in proving theories and hypotheses on enzyme research (Wulff *et al.*, 1997).

1.6.3 Genetic engineering and amino acid sequence analysis

Genetic engineering and site directed mutagenesis have further added to the structural elucidation toolkit via the determination of nucleotide sequences of hydrolase genes, their over-expression and molecular cloning into suitable hosts (Darymple *et al.*, 1996, Shaw *et al.*, 1994). Figure 1.4 shows homologies in amino acid sequences close to the active site residues in selected lipases and esterases indicating the Ser-His-(Asp/Glu) catalytic triad found in most hydrolases. The serine is in most cases well conserved in a GxSxG motif located in a turn between a β-strand and an α-helix (Drablos and Petersen, 1997).

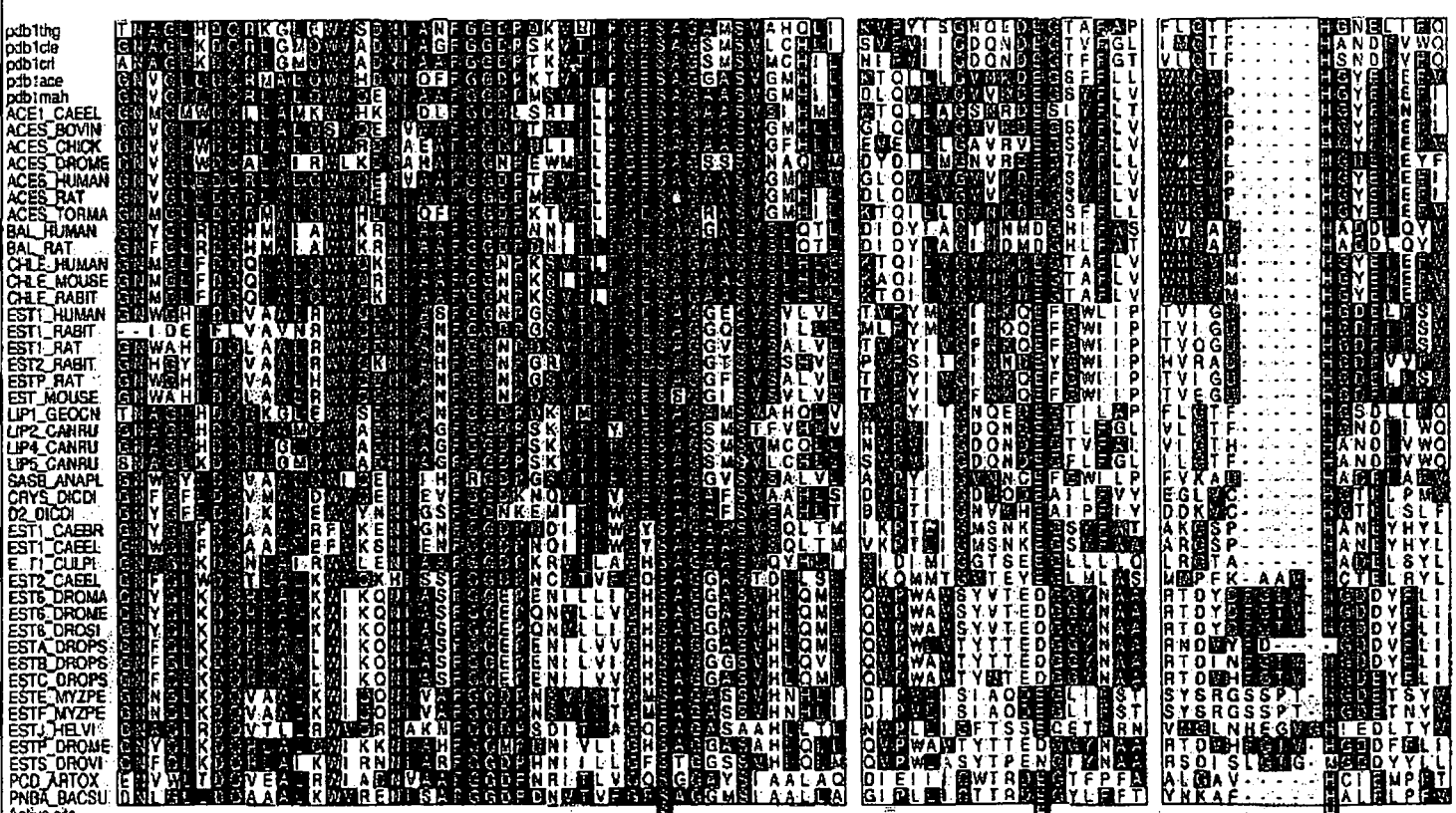


Figure 1.4 Sequence alignment for region close to active site residues of various hydrolase sources. The coloured boxes represent the structurally conserved regions as identified with Stamp. The active residues are identified below the alignment (serine-S, glutamic acid-E and histidine-H). Data obtained from Drablos and Petersen, 1997.

1.7 Biotechnological applications of hydrolases

Hydrolases are prime candidates as biocatalysts for biotechnological applications. The numerous advantages in conducting enzymatic hydrolysis include substrate specificity, mild reaction conditions, ease of recycling and the fact that no expensive and unstable co-factors are required for enzymatic activity (Zaks and Klibanov 1985). Parameter adjustments of the enzyme's environment and genetic manipulation has further led to the increased repertoire of hydrolases which have now found application in many fields, including the agrochemical, flavour and fragrance, paper and pulp, pharmaceutical and fine chemicals industries.

Hydrolases have a strong foothold in the dairy industry. Literature suggests the involvement of an esterase obtained from *Lactococcus* used as starter bacteria in cheese production, leading to the formation of curd during the manufacturing process and to flavour development during ripening (Holland *et al.*, 1995, Tsakalidou and Kalantzopoulos, 1992). The minimisation of soap-like flavours in butteroil due to kid-goat pre-gastric esterase has also been reported (Garcia and Hill, 1996).

Leaders in the field of pest control have taken particular notice of the isolation of an esterase conferring insecticide resistance in the mosquito *Culex tarsalis* to malathion, a commercially available pesticide, which could lead to severe repercussions in the agrochemical industry (Whyard *et al.*, 1994). Esterases isolated from bacteria and fungi have been reported to be responsible for the

degradation of hemicelluloses (e.g. xylan) and cellulosic substrates (e.g. carboxymethyl cellulose) and are a useful tool in the paper and pulp industry (McCrae *et al.*, 1994, Ralet *et al.*, 1994, Paul and Varma, 1992).

The hydrolysis/ synthesis of terpene esters (e.g. acetates of menthol, geraniol and linalool) is of importance in the flavour and fragrance industry leading to a cheaper alternative to traditional production methods (Williams *et al.*, 1990, Karra-Chaabouni *et al.*, 1996 and Osprian *et al.*, 1996).

Hydrolases find application in the pharmaceutical field with immobilised resting cells of *Rhodococcus erythropolis* MP50 responsible for the enantioselective hydrolysis of racemic naproxen amide, producing an important non-steroidal anti-inflammatory compound (Effenberger *et al.*, 1997). An esterase from *Amycolatopsis orientalis* was reported to cleave pivalic acid-containing pro-drug esters of cephalosporins which are important antibiotics worldwide (Sauber *et al.*, 1996). Another recent publication shows an application of an esterase isolated from the fungus, *Clonostachys compactiuscula*, responsible for the preparation/modification of lovastatin which is a clinically useful anti-hypercholesterolemic agent (Schimmel *et al.*, (1997).

Boland *et al.* (1991) describes the preparation of enantiomeric, diastereomeric and regiomeric compounds by enzyme catalysis for application in the fine chemical industry as starting materials for value-added products. These include the preparation of optically active acids and esters, the conversion of

aliphatic, aromatic and cyclic monohydroxy substrates, dihydroxy substrates and substrates containing sulfur, halogens, nitrogen and silicon by hydrolases from various sources including bacterial, fungal and mammalian tissue.

Chapter 2 Introduction to present study

Hydrolysis of esters by means of hydrolases such as proteases (Jones and Beck, 1976), lipases (Santiello *et al.*, 1993) and esterases (Boland *et al.*, 1991) has become a well established method for the resolution of racemic mixtures. However, one major drawback is associated with all of the commonly utilised enzymes, i.e. they are unable to accept highly substituted substrates such as esters of tertiary alcohols. To extend the applicability of these enzymes several techniques have been developed. However, all of these techniques have their limits.

Firstly, spatial separation of the chiral carbon from the location of the ester moiety, which is to be split, reduces steric hindrance however this impedes the chiral recognition process leading to low selectivities (Brackenridge *et al.*, 1993, Hof and Kellogg, 1994). Secondly in those cases where esters of tertiary alcohols were successfully hydrolyzed the reaction was facilitated by using "activated" α-haloacid moieties (O'Hagan and Zaida, 1992) which are hydrolytically labile substrates and therefore susceptible to spontaneous hydrolysis leading to a significant depletion in selectivity. Finally, the search for novel hydrolytic esterases and proteases (Schultz *et al.*, 1992) has shown limited success.

Two commercially available acetates of tertiary alcohols are *rac*-linalyl acetate and *rac*-α-terpinyl acetate. These acetates are racemates and are synthesized by conventional chemical methods. Enantiomerically pure (+)

and (-)-linalool and the enantiomers of α-terpineol are also commercially available. The enantiomerically pure monoterpenes are extracted from natural sources and are important in the flavour and fragrance industry. The (R)-(-)-enantiomer of linalool is a major constituent (≈80%) of *Cinnamonium camphora* and *Cayenne linaloe* extracts, whereas the (S)-(+)-enantiomer occurs in coriander oil. Since both enantiomers of linalool differ in odour (Ohloff, 1994) their availability in optically pure form is desirable for flavour and fragrance composition. The (R)-enantiomer has a woody/lavender note and the (S)-enantiomer a "sweet" note. Enantioselective hydrolysis by a biocatalyst might provide an alternative economically viable route to these enantiomerically pure monoterpenoids.

In the past linalool and *rac*-linalyl acetate have been substrates in several studies. Linalool was a non-substrate for several microbial lipases in esterification reactions. However *rac*-linalyl acetate could be hydrolyzed with varying success using whole microbial cells without detectable enantioselectivity. In the best above-mentioned case optical purities of linalool and *rac*-linalyl acetate did not exceed 17%. Recently Osprian *et al.* (1996) reported the discovery of novel bacterial isolates capable of hydrolyzing *rac*-linalyl acetate preferentially producing S-(+)-linalool. The best bacterial isolate was *Rhodococcus ruber* SM 1792 with a conversion of 29.8%, an enantiomeric excess (e.e.) of 55% and enantiomeric ratio (E) of 4.8. Later a poster presentation by Strauss *et al.*, (1998) reported the separation of two tertiary alcohol hydrolases with opposite stereopreferences from *Rhodococcus ruber* SM 1792. However the poster did not indicate

whether the enzymes were purified to homogeneity and whether characterisation experiments had been performed.

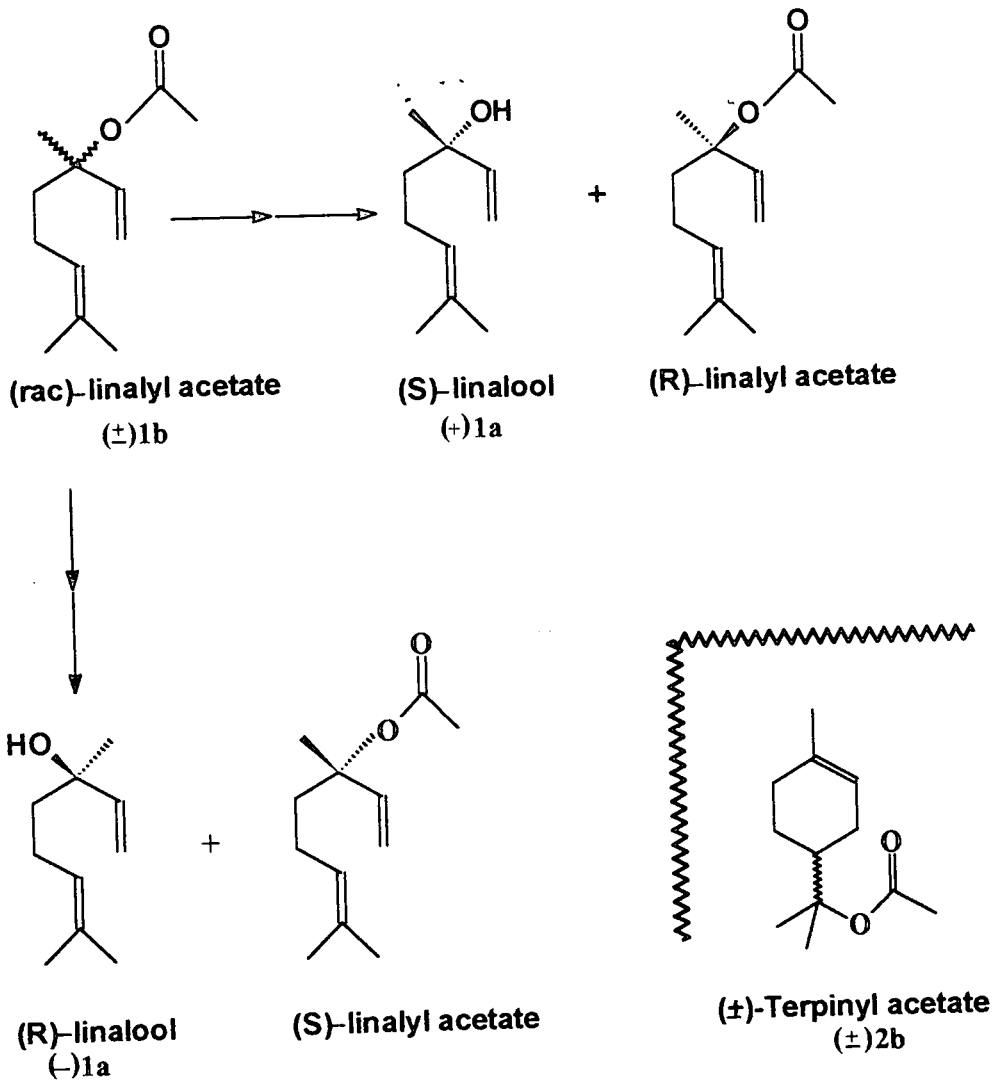


Figure 2.1 Schematic resolution of *rac*-linalyl acetate

During recent years, we have been exploring the use of yeasts for the biotransformation of monoterpenes and monoterpenoids. We found that yeasts can hydroxylate monoterpenes and monoterpenoids (Van Rensburg *et al.*, 1997) and that carvone and geraniol are reduced by a large number of yeasts (Van Dyk *et al.*, 1995, Van Dyk *et al.*, 1998). The aim of the present

study was to screen the yeast culture collection of the University of the Orange Free State for yeast isolates which can be used for the enantioselective hydrolysis of *rac*-linalyl acetate and *rac*-α-terpinyl acetate respectively. We screened 74 yeast strains from 17 genera as well as 29 unclassified isolates with enzyme purification and characterisation attempted on one of the hydrolases of interest.

Chapter 3 Materials and methods

This section has been divided into four parts namely: 1.) the screening of yeast isolates from the UOFS culture collection for organisms capable of hydrolysing *rac*-linalyl acetate and *rac*-α-terpinyl acetate, 2.) whole cell experiments utilising *Trichosporon* sp. UOFS Y-0117 for the hydrolysis of *rac*-linalyl acetate, 3.) the preparation of a crude tertiary alcohol esterase and finally 4.) the partial characterisation of a crude enzyme preparation obtained from *Trichosporon* sp. UOFS Y-0117 for the hydrolysis of *rac*-linalyl acetate.

Yeasts were obtained from the Yeast Culture Collection of the University of the Orange Free State. (±)-Linalyl acetate (**1b**), (±)-α-terpinyl acetate (**2b**), (*R*)-linalool ((*R*)-(-)-**1a**), (*S*)-α-terpineol ((*S*)-(-)-**2a**) and (*R*)-α-terpineol ((*R*)-(+)-**2a**) (Chapter 2) were obtained from Fluka. All chemicals used were of analytical grade and obtained from Merck. A 50mM sodium phosphate buffer (pH 7.5) was used throughout, unless otherwise stated. Reactions were monitored on a Hewlett-Packard 6890 gas chromatograph equipped with an FID detector and a β-DEX 120 column (Supelco Inc., 30 m x 0.25 mm, 0.25μm film) with N_2 as carrier gas at 100°C for the determination of conversion and enantioselectivity. Retention times were **1b** = 22.5 min, (*S*)-(+)-**1a** = 16.6 min, (*R*)-(-)-**1a** = 16.2 min, **2b** = 48.9 min, (*S*)-(-)-α-**2a** = 41.8 min, (*R*)-(+)-**2a** = 43.2 min. In experiments where only conversion was monitored a Hewlett-Packard 4890A gas chromatograph equipped with an FID detector and a polar column (Excel wax, 30 m x 0.5 mm inner diameter) with N_2 as carrier gas at 100°C with a flow rate of 1ml/ min was used.

Retention times were **1b** = 8.2 min, **1a** = 7.6 min. Enantiomeric ratio or E values are defined by Stecher and Faber as: $E = \ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$ (Stecher and Faber, 1997).

Graphpad Prism software was employed for statistical analysis. All experiments were performed in triplicate with error bars showing the 95% confidence interval unless indicated otherwise in materials and methods.

3.1 Screening of yeast isolates from the UOFS culture collection for hydrolysis of *rac*-linalyl acetate and *rac*-α-terpinyl acetate.

3.1.1 Growth conditions

Yeasts were grown at 30°C for 48 hours in shake-flask (100ml) cultures in a yeast extract-malt extract (YM) medium (20ml). The YM medium contained peptone (10g/l), yeast extract (3g/l), glucose (10g/l) and malt extract (20g/l).

3.1.2 Screening

Aliquots of the whole cultures (200µl) were placed in 1.5 ml micro-centrifuge tubes. Racemic linalyl and α-terpinyl acetate were separately dissolved in pristane (10% v/v) and 20µl of this mixture was administered to the whole cells. The reaction mixtures were incubated on a rotary shaker at 30°C for 48 hours. Ethyl acetate (200µl) was used to quench the reaction and extract the monoterpenoids present after vortexing for 1 min and centrifuging at 4000rpm

for 5 min. The organic layer was analysed with TLC (silica gel Merck 60 F_{254}) using hexane/ethyl acetate (20:3) as mobile phase. Monoterpenoids present were visualized by spraying with a vanillin/conc. H_2SO_4 (5g/l) mixture. Extracts containing monoterpenoids were subjected to GC-analysis. (Refer to Table 4.1 for results).

3.1.3 Biotransformations for chiral analysis

The preparation of the biocatalyst was the same as the above method. Whole cell culture (1ml) was centrifuged at 10000 x g for 5 min. The pellet was re-suspended in 200 µl phosphate buffer (50mM, pH 7.5). The substrate [2µl of a racemic monoterpenyl acetate/ethanol mixture (1:1 v/v)] was then added and the reaction mixture vortexed (1 min). The reaction mixture was incubated with shaking at 30°C for 3 or 6 h. The reaction was quenched and extracted with ethyl acetate (200µl) after which chiral GC analysis of the organic layer followed. Refer to Table 4.2 for results. The above-mentioned experiments were performed on a single run basis only.

3.2 Whole cell experiments using *Trichosporon* sp. UOFS Y-0117 for the hydrolysis of *rac*-linalyl acetate.

3.2.1 Induction experiments using *rac*-linalyl acetate as co-carbon source.

Yeasts were grown in shake flasks (250ml) YNB broth (50ml) supplemented with 1% glucose and different concentrations of *rac*-linalyl acetate (0-0.5mM).

Growth was monitored for 48 hours using a Klett apparatus at 640nm with YNB-broth containing 1% glucose as blank. Aliquots (4ml) of whole culture were removed after 3, 9, 18, and 36 hours, centrifuged at 4000 rpm for 10 min and re-suspended in phosphate buffer (pH 7.5) at a concentration of 10% (wet mass/volume), upon which enzyme assays were performed on supernatant as well as the re-suspended pellet (Figure 4.1).

The enzyme was assayed by adding racemic linalyl acetate (final concentration 30mM) to 2ml of the above mentioned respective aliquots and incubating at 30°C for 2 hours. Aliquots (100µl) were removed after selected time intervals (30, 60, 90, and 120 min) and extracted with ethyl acetate (50µl), vortexed for 1 min and centrifuged at 4000rpm for 5 min followed by GC analysis of the organic layer. From this data reaction rates (V_0) and enantiomeric ratios (E) were calculated.

3.2.2 The effect of carbon sources on hydrolase activity.

Carbon sources investigated were glucose, glycerol, ribose, maltose, lactic acid, succinic acid, birchwood xylan, oats spelt xylan and carboxymethylcellulose (CMC). The respective carbon sources were each prepared in 100ml conical flasks to concentrations of 1% and 4% w/v or v/v respectively to which *Trichosporon* sp. UOFS Y-0117 was inoculated. Growth was monitored for 48 hours for glucose, glycerol, ribose, maltose, lactic acid and succinic acid, and 96 hours for birchwood xylan, oats spelt xylan and carboxymethycellulose (CMC) upon which whole cultures, where growth had

occurred, were harvested by centrifugation at 4000rpm for 10 min. Enzyme assays were performed as described in the previous section on 4ml aliquots of the supernatant as well as the re-suspended pellet (phosphate buffer, pH 7.5) at a concentration of 10% wet mass/volume (Figure 4.2).

3.2.3 The effect of culture age on hydrolase activity.

A pre-inoculum (6 x 25ml) was prepared by inoculating yeast cells to YNB-broth with 1% maltose as carbon source and incubated on a rotary shaker for 48 hours at 30°C. Pre-inoculum (50ml) was transferred to 3 x 500ml side arm conical flasks containing the above-mentioned broth and carbon source. Growth was monitored for 48 hours using a Klett apparatus at 640nm with YNB-broth containing 1% maltose solution as blank. Aliquots (2ml) were removed every 3 hours after 9 hours had elapsed. Cells were harvested by centrifugation (4000rpm for 5 min) and the cell concentration for each aliquot was adjusted to 5 % (wet mass/ vol) by addition of phosphate buffer (pH 7.5). Racemic linalyl acetate (final concentration 30mM) was administered to the cell mixture and incubated on a rotary shaker for 3 hours. Aliquots (100µl) were extracted and analysed by gas chromatography (Figure 4.3).

3.2.4 The effect of different co-solvents at different concentrations as well as different substrate concentrations on the reaction rate and enantioselectivity.

In the co-solvent experiments the effect of pristane, 1-octanol, dimethylsulfoxide (DMSO) and ethanol on the hydrolysis of linalyl acetate was

investigated. A pre-inoculum (6 x 50ml) was prepared by inoculating yeast cells to YNB-growth media with 1% glucose as carbon source and incubated on a rotary shaker for 48 hours at 30°C. Pre-inoculum (100ml) was transferred to 3 x 1L conical flasks containing the above-mentioned broth and carbon source. Cells were harvested after 24 hours by centrifugation at 4000rpm for 10 min. The cells were re-suspended in phosphate buffer (pH 7.5) to a concentration of 10% wet mass/volume. Aliquots (4ml) of the cell mixture were removed to which *rac*-linalyl acetate (final concentration 30mM) was administered in the presence of different co-solvents of final concentration 2.4% v/v reaction mixture (final volume of reaction mixture 5ml). Aliquots (100µl) of the reaction mixture were extracted at time intervals and conversion of substrate determined. In a follow up experiment reaction rates were determined in the presence of different concentrations of ethanol and DMSO. Later a similar experiment in which *rac*-linalyl acetate concentration was varied (5-200mM) was performed in the presence of 2.4% v/v ethanol (Figures 4.4-4.6).

3.2.5 Effect of digitonin.

In this experimental series the effect of digitonin, a cell permeabilising agent, on the rate of hydrolysis of linalyl acetate was investigated. The same procedure was performed as in 3.2.4 for the preparation of the biocatalyst. Aliquots (4ml) of the prepared cell mixture were removed to which *rac*-linalyl acetate (final concentration 30mM) was administered in the absence and presence of the recommended concentration of digitonin (0.01% w/v reaction

mixture). Similarly the effect of 2.4% v/v ethanol on the hydrolysis of linalyl acetate was also investigated in the presence and absence of digitonin. Enzyme assays were performed on 100µl aliquots at specific time intervals (Figure 4.7).

3.2.6 Determination of optimum pH.

Yeast cells were grown and prepared as in 3.2.4 until after the centrifugation step. A buffer cocktail containing 50mM of each of sodium acetate, di-sodium monohydrogenphosphate and sodium dihydrogenphosphate respectively was used to re-suspended the above pellet to a concentration of 10% wet mass/volume. The cell mixture was then divided into 5ml stock suspensions which were equilibrated at different pH's (pH5-pH9) with adjustments performed with 1M HCl or NaOH. Aliquots (4ml) of the cell mixture were removed from the stock solutions to which *rac*-linalyl acetate (final concentration 30mM) was administered in the presence of ethanol (2.4% v/v reaction mixture) with the final volume of reaction mixture being 5ml. Aliquots (100µl) of the reaction mixture (5ml) were extracted and analysed at specific time intervals. The above-mentioned experiments and assays were performed at the pH specified (Figure 4.8).

3.2.7 Determination of optimum temperature.

Yeast cells were harvested and prepared as in 3.2.4. The pellet was re-suspended in a 50mM phosphate buffer (pH 7.5). The cell mixture was then divided into 5ml stock suspensions which were equilibrated at different

temperatures (4°C-45°C) on rotary shaker. Aliquots (4ml) of the cell mixture were removed to which *rac*-linalyl acetate (final concentration 30mM) was administered in the presence of ethanol (2.4% v/v reaction mixture) with the final volume of reaction mixture being 5ml. The reaction mixtures were incubated at the specified temperatures with aliquots (100µl) of the reaction mixtures removed, monoterpenoids extracted and enzyme assays performed at specific time intervals (Figure 4.9).

3.2.8 Re-use of whole cells.

In this experimental series the effect of the re-use of whole cells of *Trichosporon* sp. UOFS Y-0117 on the rate of hydrolysis of *rac*-linalyl acetate was investigated. The same procedure was performed as in 3.2.4 for the preparation of the biocatalyst. Aliquots (4ml) of the prepared cell mixture were equilibrated at 30°C for 30 min to which *rac*-linalyl acetate (final concentration 30mM) was administered. The reaction mixture was incubated on a rotary shaker at 30°C with 100µl aliquots extracted and analysed at specific time intervals (15 – 90 min). After 2 hours the reaction mixture was removed from the rotary shaker and the reaction quenched by centrifugation at 4000rpm for 10 min. The supernatant was discarded and the pellet re-suspended in phosphate buffer to the same cell concentration as previously used. Racemic linalyl acetate was then administered and hydrolase activity determined as before. The above mentioned procedure was repeated a further three times on the same batch of cells (Figure 4.10). Experiments explained in Section 3.2.1 to 3.2.8 were performed in triplicate.

3.3 Purification of the tertiary alcohol ester hydrolase.

3.3.1 Evaluation of purification methods for a tertiary alcohol ester hydrolase from *Trichosporon* sp. UOFS Y-0117.

A pre-inoculum (6 x 50ml) was prepared by inoculating yeast cells to YNB-broth containing 1% maltose as carbon source and incubating on a rotary shaker for 48 hours at 30°C. Pre-inoculum (100ml) was transferred to 3 x 1L conical flasks containing the above-mentioned growth media and carbon source. Cells were harvested after 24 hours by centrifugation at 4000rpm for 10 min. The cells were re-suspended in phosphate buffer (pH 7.5) to a density of 50% wet mass/volume. The cell paste was subjected to lysis using a glass bead blender (1g of 100 μm beads /ml cell paste) for 15 x 2 minute cycles with 15 second intervals between cycles. The resulting homogenate was subsequently subjected to centrifugation at 18 000rpm for 20 minutes. The supernatant was then filtered through a Whatman No.1 paper filter and the volume was adjusted to 20ml with phosphate buffer (pH 7.5). Aliquots (2ml) were then loaded onto small-scale columns containing different chromatographic resins (5ml) following the respective manufacturer's specifications. The effectiveness of hydrophobic (butyl and phenyl Toyopearl, ion-exchange (DEAE- carboxymethyl and sulfopropyl Toyopearl) columns and a PIKSI™ kit (AX6L affinity matrices) at separating the hydrolase from other proteins was investigated (Table 4.3 and 4.4).

3.3.2 Preparation of a partially purified tertiary alcohol ester hydrolase.

The following protocol was used in further experiments to obtain partially purified hydrolase. The cell free homogenate obtained, after the glass bead blender cell lysis step, was loaded onto a DEAE Toyopearl column and bound protein eluted using a salt gradient (0-2M KCl) with 4ml fractions collected at 5 minute intervals. The collected fractions where analysed for protein content at A_{280nm} and assayed for tertiary alcohol ester hydrolase activity. The assay procedure was as follows: 30mM *rac*-linalyl acetate was administered to 500µl from each fraction, hand mixed and incubated on a rotary shaker at 30°C for 1 hour. Monoterpenes were extracted using ethyl acetate (100µl) by vortexing for 1 min followed by centrifugation at 4000rpm for 5 min. Conversion was then determined using GC analysis. Peaks in enzyme activity were pooled (Figure 4.11 and Table 4.5). Protein determination of the active fraction was performed using the method of Bradford (1976) with bovine serum albumin as standard.

3.4 Characterisation of the partially purified tertiary alcohol ester hydrolase from *Trichosporon* sp. UOFS Y-0117.

3.4.1 Determination of kinetic constants in the presence of different ethanol concentrations.

The experimental series was conducted using aliquots (3ml) of the active fraction from the DEAE column step in the presence of three different ethanol concentrations (0, 2.4 and 8.0% vol/ reaction volume). Various concentrations of *rac*-linalyl acetate (0-200mM) were then administered to the enzyme

preparation with the final volume of the reaction mixture being 4ml. Aliquots (100µl) were removed at time intervals (15, 30, 60, 90 and 120 min) and subjected to ethyl acetate extraction (50µl). Progress curves and reaction rates (V_0) were determined using GC analysis (Figure 4.12). In subsequent experiments ethanol 2.4% v/v of the final reaction volume was administered in conjunction with the *rac*-linalyl acetate.

3.4.2 Effect of inhibitors on hydrolase activity.

The effect of the serine modification reagent namely PMSF (serine), DEP (histidine) and iodo-acetic acid (cysteine) were investigated. Aliquots (3ml) of the active fraction, from the DEAE column step, were subjected to various concentrations of inhibitors (PMSF 2.5-20mM, DEP 20mM and iodo-acetic acid 10-100mM). The PMSF was administered from a stock solution (200mM in propanol with pH adjusted to 7.5), DEP directly (pH of reaction mixture adjusted to 7.5) and the iodo-acetic acid was administered from a stock solution (500mM in phosphate buffer, pH 7.5). The final reaction volume was 4ml. The enzyme mixtures were incubated on a rotary shaker at 30°C for 1 hour in the case of the PMSF and iodo-acetic acid. Remaining activity was determined by the removal of 100µl aliquots at time intervals which were assayed for enzyme activity as in section 3.4.1. The DEP experiment was followed spectrophotometrically throughout at A_{240nm}. Once the absorbance values had peaked indicating the modification of histidine residues present, a 3ml aliquot was removed and assayed for enzyme activity followed by the

addition of 1M Tris buffer (pH8.5) to a final volume of 4ml to restore enzyme activity (Figure 4.13-4.15).

3.4.3 Effect of EDTA and metal cations on hydrolase activity.

Aliquots (3ml) of the active fraction, from the DEAE column step, were subjected to various concentrations of a metal chelating reagent ethyldiaminetetra-acetic acid (EDTA) (10-100mM) (Figure 4.16). The EDTA was administered from a stock solution (500mM in phosphate buffer (pH 7.5)). The effect of various metal chlorides namely Ag^{+}, Ba^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} and Sn^{2+} at two concentrations (10 and 100mM) was also investigated (Figure 4.17). The final reaction volume was 4ml. The reaction mixture was incubated on a rotary shaker at 30°C for 1 hour. Remaining activity was determined by the removal at time intervals of 100µl aliquots which were assayed for enzyme activity as in section 3.4.1. Experiments explained in Section 3.4.1 to 3.4.3 were performed in triplicate.

3.4.4 pH-stability of hydrolase activity in whole cells and as crude enzyme.

Whole cells of *Trichosporon* sp. UOFS Y-0117 were prepared and harvested as previously described. However before cell lysis and the preparation of the active DEAE fraction the cell paste was split in two allowing a comparative study between whole cells and the crude enzyme preparation. The same procedure was performed as in 3.3.2 to prepare the active fraction obtained from the DEAE step. Protein determination was performed on the crude

enzyme fraction. The cell suspension and crude enzyme were diluted (buffer cocktail containing 50mM of sodium acetate, di-sodium monohydrogenphosphate and sodium dihydrogenphosphate and then divided into 10ml stock solutions which were equilibrated at different pH's (pH5-pH9). pH adjustments were performed with 1M HCl or NaOH. At various time intervals (3, 6, 9, 24 and 72 hours) a 2ml aliquot was removed from the stock solutions and the pH was adjusted to pH 7.5 using a concentrated (2M phosphate buffer) making the final reaction mixture volume 3ml. *Rac*-linalyl acetate (final concentration 30mM) was administered to each aliquot in the presence of ethanol 2.4% v/v. The reaction mixtures were then incubated on a rotary shaker at 30°C with enzyme assays performed on 100µl aliquots at specific time intervals (3.4.1). Refer to Figure 4.18 and 4.19.

3.4.5 Temperature stability of the hydrolase in whole cells and as crude enzyme.

In this experimental series the effect of temperature on enzyme stability was determined. The same procedure was performed as in 3.4.4 for the preparation of the cell mixture and the crude enzyme preparation except that this experiment was carried out in a phosphate buffer (pH 7.5). Protein determination was performed on the crude enzyme fraction. Stock suspensions (10ml) of the prepared cell mixture and crude enzyme preparation were equilibrated at different temperatures i.e. 4°C, 15°C, 25°C, 30°C, 37°C and 45°C on a temperature gradient shaker. Aliquots (2ml) were removed from the stock solutions at various time intervals (3, 6, 9, 24 and 72 hours). *Rac*-linalyl acetate (final concentration 30mM) in the presence of

2.4% v/v ethanol was administered to the aliquots and the reaction mixtures were incubated on a rotary shaker at 30°C with enzyme assays performed on 100µl aliquots at specific time intervals (3.4.1). The subsequent manipulation of the data allowed the calculation of half life values (Figures 4.20-4.24). Experiments explained in Section 3.4.4 and 3.4.5 were performed on a single run basis only.

Chapter 4 Results and discussion

4.1 Screening of yeast isolates from the UOFS culture collection for hydrolysis of *rac*-linalyl acetate and *rac*-α-terpinyl acetate.

The results obtained from the screening experiments are tabulated in Table 4.1 and 4.2.

Table 4.1 A summary of species and strains from different yeast genera tested for the hydrolysis of *rac*-linalyl acetate (**1b**) and *rac*-α-terpinyl acetate **(2b)**. The number of isolates that hydrolyzed **1b** and **2b** are given.

Genera	No. of Species	No. of Strains	No. which hydrolyzed 1b	No. which hydrolyzed 2b
Candida	15	19		
Cryptococcus	3	3		
Debaryomyces	3	3		
Dekkera	1	1		
Galactomyces	1	1		
Geotrichum	8	12	5	
Kloeckera	1	1		
Kluyveromyces	1	1		
Metchnikowia	1	1		
Pichia	7	7	2	1
Rhodospiridium	3	3	1	
Rhodotorula	6	7	2	
Sporobolomyces	2	2		
Trichosporon	2	7	1	
Wickerhamia	1	1		
Wingea	1	1		
Yarrowia	1	4	1	
Unclassified		29		

Table 4.2 Enantioselective hydrolysis of **1b** and **2b** by selected yeasts.

Yeast	Substrate	Preferred enantiomer	RT[*1] (h)	c[*2] (%)	e.e$_p$[*3] (%)	E[*4]
***Geotrichum capitatum* CBS 0572.82**	1b	S	6	33	50	3.9[*5]
***Geotrichum klebahnii* CBS 0625.85**	1b	S	6	15	51	3.3
***Geotrichum spicifer* CBS 0758.85**	1b	S	6	6	47	2.9
***Geotrichum sericeum* CBS 0192.55**	1b	S	6	8	44	2.7
***Rhodotorula ferulica* CBS 7416**	1b	S	6	31	37	2.6
***Trichosporon sp.* UOFS Y-0117**	1b	S	3	32	26	1.9
***Geotrichum fragrans* SAS 44**	1b	S	6	5	8	1.2
***Yarrowia lipolytica* CBS 6114**	1b	S	6	6	7	1.1
***Rhodosporidium sphaerocarpum* CBS 6985**	1b	S	6	1	1	1.0
***Pichia pini* CBS 0744 T**	1b	R	6	2	14	0.8
***Pichia holstii* UOFS Y-0140**	1b	R	3	48	12	1.4
***Pichia holstii* UOFS Y-0140**	2b	S	3	42	32	2.4

[*1] RT = reaction time, [*2] c = conversion, [*3] e.e$_p$ = enantiomeric excess of product. [*4] E = enantiomeric ratio calculated as E = ln[1-c(1+ee$_p$)]/ln[1-c(1- ee$_p$)] (Stecher and Faber, 1997). [*5] An E value of 4.7 (RT = 6h, c =32%, e.e$_p$ = 56%) was obtained with this yeast during further experiments using lower substrate concentrations (2 µl/ml of 1:1 v/v **1b**/ethanol ca ~ 5mM *rac*-linalyl acetate). Cell density ca ~ 2mg/ml.

The presented data indicates that *rac*-linalyl acetate is more readily accepted than α-terpinyl acetate (Table 4.1) with only one yeast (*Pichia holstii* UOFS Y-0140) being able to hydrolyze α-terpinyl acetate. *Pichia holstii* UOFS Y-0140 is also the only yeast which accepted both *rac*-linalyl acetate and α-terpinyl acetate as substrates (Table 4.2).

Strains from the genus *Pichia* were also the only yeasts which preferentially hydrolyzed the (R)-linalyl acetate to yield (R)-(-)-linalool. All the other yeasts screened preferred (S)-linalyl acetate. This possible trend should be investigated further in a later study. We selected *Trichosporon sp.* UOFS Y-0117 to investigate further as it displayed a relatively high conversion rate of 32% in 3 hours (Table 4.2) and formed a solid pellet. *Geotrichum capitatum* on the other hand had a superior E-value of 3.9 (later 4.7 in the presence of ethanol (1:1 v/v) and at lower *rac*-linalyl acetate concentrations) but formed an exudate which made handling of this microbe technically difficult. The relatively low enantiomeric ratio (E-value) did not influence our choice since we thought that two tertiary alcohol hydrolases with opposite enantioselectivities might be present causing the observed low E-values. This had been the case in the purification of a similar hydrolase obtained from bacteria (Strauss *et al.*, 1998). High activity (short conversion times) was considered more important, since this would influence analytical procedures during purification and characterisation of the enzyme thus in subsequent experiments characteristics of *Trichosporon sp.* UOFS Y-0117 whole cells and cell free extract are described.

4.2 Whole cell experiments using *Trichosporon sp.* UOFS Y-0117 for the hydrolysis of *rac*-linalyl acetate.

The following set of experiments were performed on whole cell cultures of *Trichosporon sp.* UOFS Y-0117 to optimize conditions conducive to superior conversion rates and enantioselectivities for *rac*-linalyl acetate.

4.2.1 Induction experiments using *rac*-linalyl acetate as co-carbon source.

Firstly, we investigated the use of *rac*-linalyl acetate as co-carbon source to induce increased hydrolase activity. Literature suggests that the presence of the substrate of interest may sometimes increase hydrolase production (Schimmel *et al.*, 1997). The results from this experiment indicate that the presence of *rac*-linalyl acetate has no significant effect on hydrolase production. Almost no hydrolase activity was observed in the supernatant, confirming that the hydrolase of interest is cell associated. The low levels of hydrolase activity detected in the supernatant is probably a result of residual linalool present in the growth broth (see Figure 4.1). Later experiments using differential centrifugation on the cell free fraction showed the hydrolase of interest to be in the cytosolic fraction (results not shown).

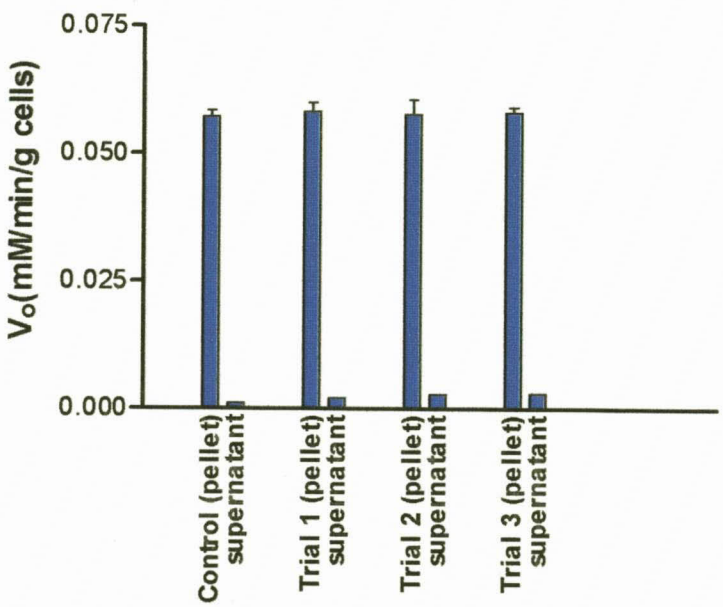


Figure 4.1 The effect of *rac*-linalyl acetate as inducer. Control = 0mM LA present in growth broth, Trial 1 = 0.05mM, Trial 2 = 0.25mM and Trial 3 = 0.50mM. Enzyme assays after 36 hours incubation under *rac*-linalyl acetate induced growth conditions.

4.2.2 The effect of carbon sources on hydrolase activity.

Polymers and carbon sources entering the main catabolic pathway at different positions were investigated. The polymers (e.g. carboxymethylcellulose) are responsible for the induction of ρ-coumaryl and ferulic acid esterases which form part of the xylan degrading enzyme complex. These esterases function upon sterically hindered substrates and thus these compounds were chosen (Moore *et al.*, 1996). *Trichosporon sp.* UOFS Y-0117 grew on a number of carbon sources namely glucose, glycerol, maltose and succinate as seen in Figure 4.2. No growth was observed on ribose, lactic acid, oats spelt xylan, birchwood xylan and carboxymethylcellulose after 48 hours. *Trichosporon sp.* UOFS Y-0117 cells grown on 1% maltose as sole carbon source gave a superior reaction rate (0.105mM/min/g cells) compared to the other carbon sources investigated. Maltose (1%) as carbon source was employed in subsequent experiments.

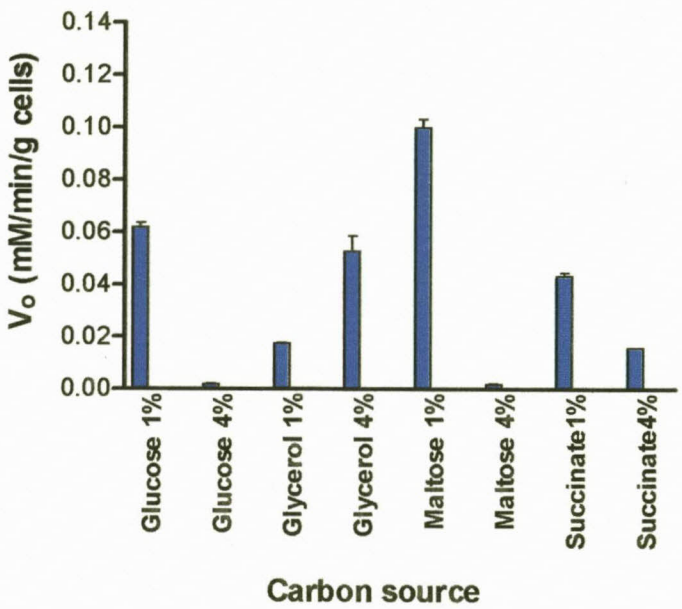


Figure 4.2 The effect of carbon sources on hydrolase activity.

The superior reaction rate compared to glucose is most likely due to the presence of a hydrolase suppression pathway (McCrae *et al.*, 1994), present within the yeast, triggered by glucose. High concentrations of glucose, maltose and succinate also suppressed hydrolase activity.

4.2.3 The effect of culture age on hydrolase activity.

Trichosporon sp. UOFS Y-0117 grows rapidly and culture age has no significant effect on hydrolase activity (Figure 4.3). Subsequently the cell mass of *Trichosporon sp.* UOFS Y-0117 for the remainder of the project was harvested after 24 hours (early stationary phase). Enzymatic activity in this case was defined as the conversion of *rac*-linalyl acetate after 3 hours by 1g of wet cells.

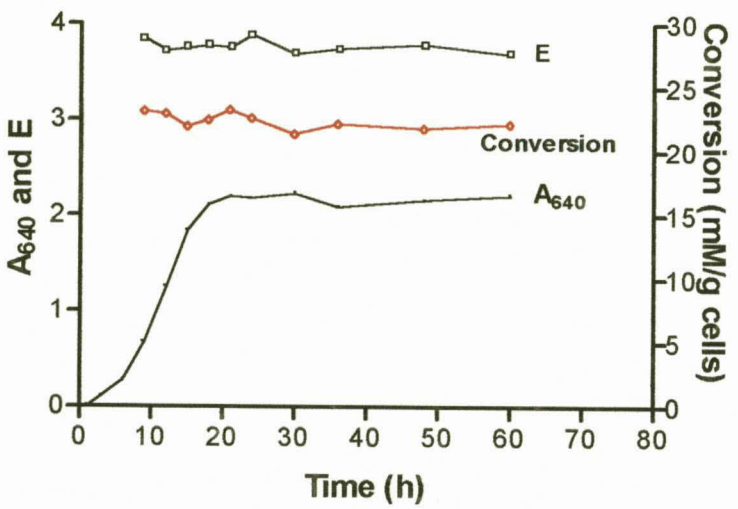


Figure 4.3 The effect of culture age of *Trichosporon sp.* UOFS Y-0117 on hydrolase activity. Enzyme activity in this case was defined as amount of *rac*-linalyl acetate converted after 3 hours by 1g of wet cells. A_{640} refers to the measurement of cell density, E refers to enantiomeric ratio with $E = \ln[1-c(1+ee_p)]/\ln[1-c(1- ee_p)]$ (Stecher and Faber, 1997).

4.2.4 The effect of different co-solvents at different concentrations as well as different substrate concentrations on the reaction rate and enantioselectivity.

Herman *et al.* (1991) reported toxic effects of co-solvents on whole cells when the log P is anywhere between 1 and 5 mainly due to modification of cell membranes. Bearing this in mind the effect of organic co-solvents with varying degrees of hydrophobicity were investigated. A preliminary experiment (Figure 4.4) showed the effect of four organic solvents, at a constant concentration of 2.4% v/v, on the hydrolysis of *rac*-linalyl acetate. The data indicates that ethanol facilitates hydrolase activity because of a low log P-value of -0.24 (Axcell *et al.*, 1973). This hydrophilic organic solvent improves solvation and enhances biocatalyst/substrate interactions and producing improved conversion values. Conversely pristane has a high log P-value of ~10 (Axcell *et al.*, 1973) and is a highly hydrophobic organic solvent and facilitates the formation of a two-phase system removing substrate from the biocatalyst which remains in the aqueous phase. n-Hexane has an intermediate log P-value of +3.5 (Axcell *et al.*, 1973) and is highly toxic to the yeast and hence the low conversion values. This toxicity is, as mentioned above, largely due to interference with the cell membrane. However the tertiary alcohol ester hydrolase is a cytosolic enzyme. The reduced conversion rate in the presence of n-hexane and pristane is probably due to the solvation of the substrate in a hydrophobic second phase. Although dimethylsulfoxide (DMSO) with a low log P-value of -1.3 (Axcell *et al.*, 1973) should have no toxic effect on hydrolase activity it produces conversion values similar to the control in which no co-solvent is present.

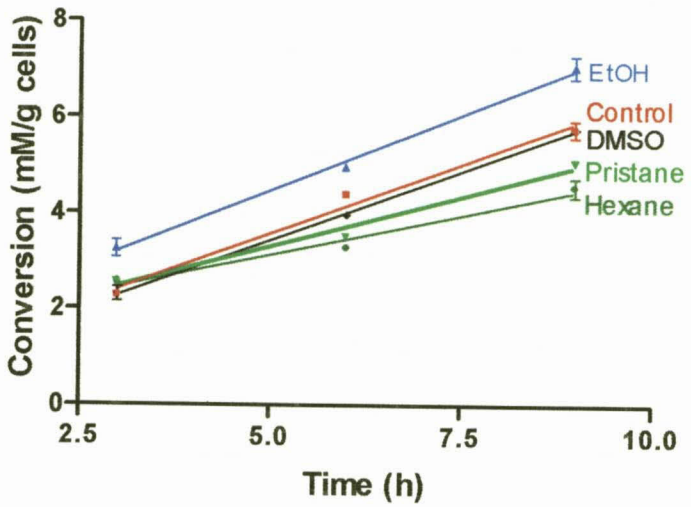


Figure 4.4 The effect of various organic solvents *Trichosporon sp.* UOFS Y-0117 on hydrolase activity. Enzyme activity in this case was defined as the amount of *rac*-linalyl acetate converted after 1 hour by 1g of wet cells.

The effect of various concentrations of ethanol and DMSO were then investigated (Figure 4.5). This data confirms that hydrolase activity (reaction rates – V_0) are enhanced by low concentrations (2.4% v/v reaction mixture) of ethanol compared to the control in which no organic solvent is present (0.05 vs 0.07 mM/min/g cells). The effect of DMSO was not significant compared to ethanol and we decided to employ ethanol (2.4% v/v) in all further experiments, unless otherwise stated.

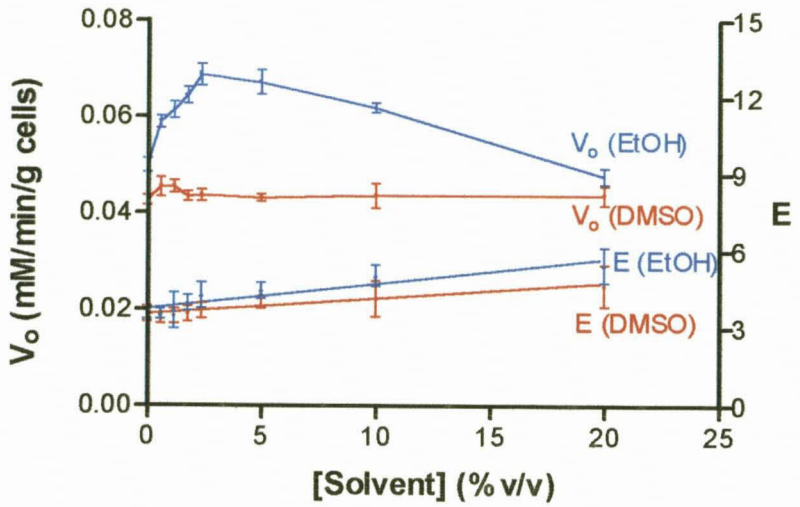


Figure 4.5 The effect of various concentrations of ethanol and dimethylsulfoxide (DMSO) on reaction rates and enantioselectivity of *rac*-linalyl acetate hydrolysis for whole cells of *Trichosporon sp.* UOFS Y-0117.
Enantiomeric ratio defined as $E = \ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$ (Stecher and Faber, 1997).

In the follow up experiment (Figure 4.6) *rac*-linalyl acetate concentrations were varied in the presence of 2.4% v/v ethanol which produced a maximum reaction rate (V_0) of 0.04 mM/min/g cells.

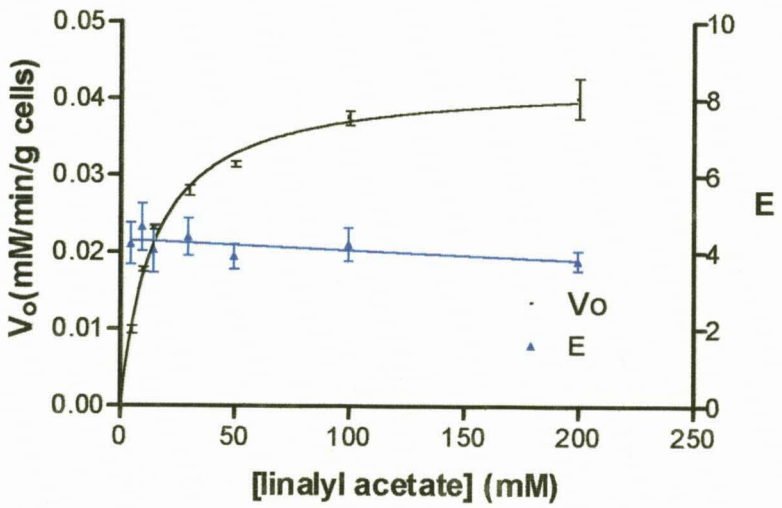


Figure 4.6 The effect of various concentrations of *rac*-linalyl acetate on reaction rates for whole cells of *Trichosporon sp.* UOFS Y-0117 in the presence of 2.4% v/v ethanol.
Enantiomeric ratio defined as $E = \ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$ (Stecher and Faber, 1997).

It is notable that although linalyl acetate and linalool are very toxic to cells with calculated log P-values of +3.7 and +3.4 respectively, high substrate concentrations (200mM) had no adverse effect on hydrolase activity.

In the above experiments a statistical F-test was performed on the regression line through the enantiomeric ratio (E-values) to determine whether any of the above-mentioned conditions increase/decrease the enantioselectivity of the hydrolase of interest. The hypothesis testing at the 95% confidence level indicates that the regression lines have a gradient of zero and therefore E-values are not influenced by changes in substrate or co-solvent.

4.2.5 Effect of digitonin on hydrolase activity.

The data presented in Figure 4.7 indicates that the cell permeabilising reagent digitonin does not facilitate enzyme/substrate interactions with the conclusion that the transport of substrate across the cell membrane is not a rate determining step. It is not clear why digitonin, which affects cell membranes, had an adverse effect on enzyme activity since the enzyme is cytosolic therefore not membrane bound and should not be affected by the membrane damaging reagent, digitonin.

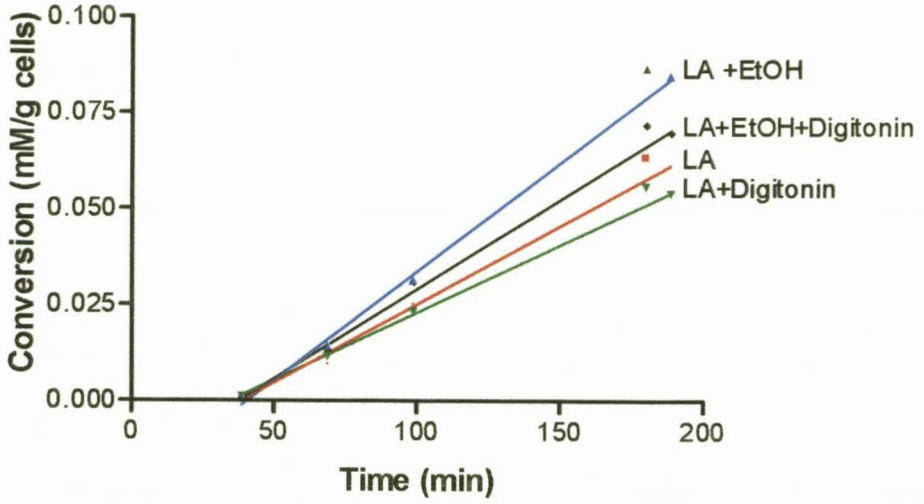


Figure 4.7 The effect of digitonin on the conversion of *rac*-linalyl acetate by whole cells of *Trichosporon sp.* UOFS Y-0117 in the presence and absence of 2.4% v/v ethanol. Enzyme activity in this case was defined as the amount of *rac*-linalyl acetate converted after 1 hour by 1g of wet cells.

4.2.6 Determination of optimum pH.

Figure 4.8 shows the effect of pH on reaction rate and enantioselectivity. The optimum reaction rate was observed at pH 7.5 (0.03mM/min/g cells). The profile favours neutral and alkaline pH's which is consistent with members of the serine hydrolase family (Holland and Coolbear, 1996). pH also has no effect on enantioselectivity which remained constant at an approximate value of 4. In further experiments we decided to use pH 7.5, except when otherwise stated.

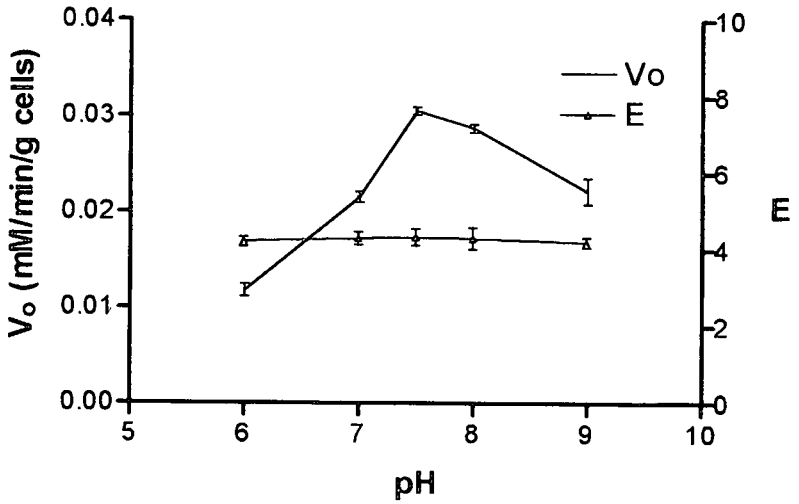


Figure 4.8 The effect of pH on reaction rates and enantioselectivity for whole cells of *Trichosporon sp.* UOFS Y-0117 in the presence of 2.4% v/v ethanol.
Enantiomeric ratio defined as $E = \ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$ (Stecher and Faber, 1997).

4.2.7 Determination of optimum temperature.

Figure 4.9 shows the temperature profile for reaction rates and enantioselectivity. A maximum reaction rate of 0.033 mM/min/g cells at an optimum temperature of 30°C indicates a temperature sensitivity of the hydrolase of interest once the temperature of 30°C is exceeded. The decrease in E-values as temperature increases is probably a result of autohydrolysis (a control experiment was performed at 45°C). The observation was confirmed in later experiments when half-life values for the hydrolase in whole cells and as crude enzyme were determined and compared.

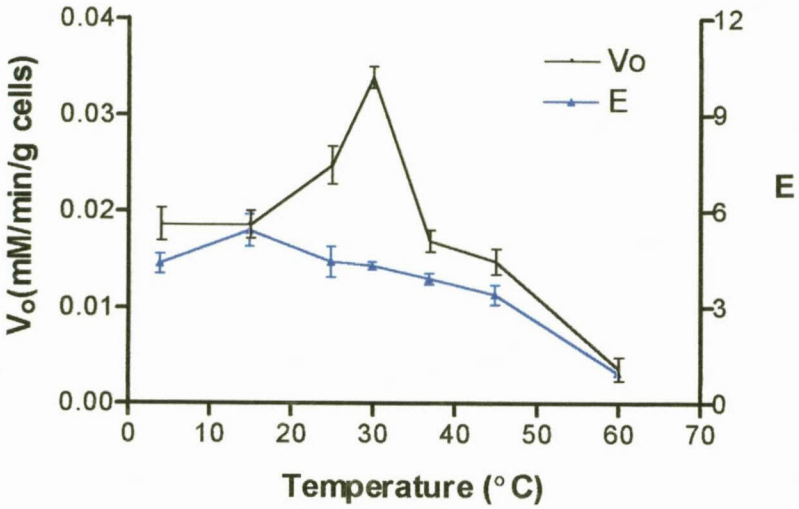


Figure 4.9 The effect of temperature on reaction rates and enantioselectivity for whole cells of *Trichosporon sp.* UOFS Y-0117 in the presence of 2.4% v/v ethanol.
Enantiomeric ratio defined as $E = \ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$ (Stecher and Faber, 1997).

If the biocatalyst was used in a large scale continuous process a temperature of 30°C and a pH of 7.5 would give a maximum conversion rate without a significant loss in enantioselectivity (E-value).

4.2.8 Re-use of whole cells.

Figure 4.10 indicates that the re-usability of *Trichosporon sp.* UOFS Y-0117 is limited to one cycle. A rapid decrease in activity was observed with further recycling. Subsequent cycles was technically difficult due to the formation of a looser pellet even at 18 000rpm. However, when the cells were viewed under a microscope the cells were still intact.

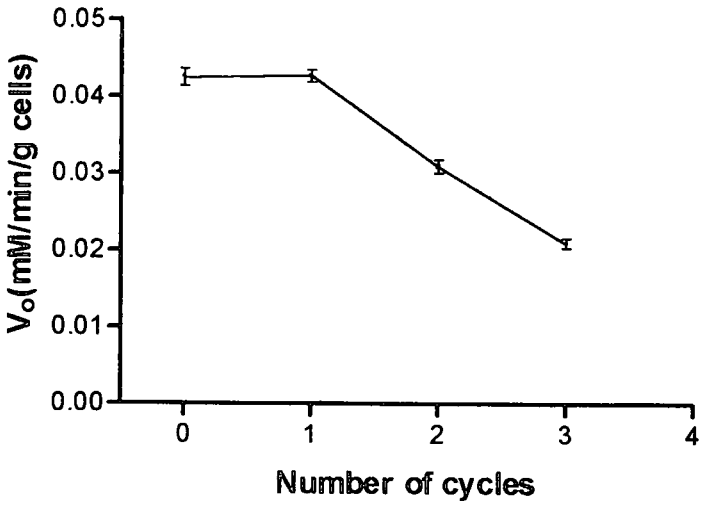


Figure 4.10 The effect of re-use of *Trichosporon sp.* UOFS Y-0117 on the rate of hydrolysis of *rac*-linalyl acetate.

The interactions between ethanol and to a greater extent *rac*-linalyl acetate, which has a log P-value of +3.7 as well as the product linalool (+3.4), upon the biocatalyst is marked due to the significant loss of activity with each new exposure to substrate and product. We hypothesize that the nature of the substrate, *rac*-linalyl acetate, plays a major role in damaging the integrity of the yeast's cell membranes (Osborne *et al.*, 1990) leading to the observed decrease in activity. It is possible that prolonged exposure to ethanol, and in particular linalyl acetate and linalool caused the yeast's cell membranes to lose their integrity. This might have resulted in the enzyme leaking out of the cells thus explaining the loss of activity during further cycles.

4.3. Purification of the tertiary alcohol ester hydrolase

4.3.1 Evaluation of purification methods for a tertiary alcohol ester hydrolase from *Trichosporon sp.* UOFS Y-0117.

Once experiments had been conducted with whole cells of *Trichosporon sp.* UOFS Y-0117 the enzyme/ enzyme system of interest capable of hydrolyzing *rac*-linalyl acetate was investigated on a physiochemical level.

4.3.2 The preparation of a crude tertiary alcohol ester hydrolase from *Trichosporon sp.* UOFS Y-0117.

The binding of the hydrolase of interest to various chromatographic resins was evaluated on a small scale. Tables 4.3 and 4.4 show protein content (A_{280}), tertiary alcohol hydrolase activity and activity/protein values for fractions eluted from different chromatographic resins.

Table 4.3 A_{280}-values, activity and activity/A_{280}-values of selected preparative scale chromatography resins.

Column type: Hydrophobic interaction							
	Butyl Toyopearl			Phenyl Toyopearl			
Fraction no.	A_{280}	Activity (mM/h)	Activity/A_{280}	A_{280}	Activity (mM/h)	Activity/A_{280}	**[KCl] M**
1	0.85	0.16	0.18	1.05	0.26	0.25	**2***
2	0.35	0	0	0.26	0	0	**1**
3	0.44	0	0	0.43	0	0	**0**

Table 4.3 continued.

Column type: Ion exchange							
	Carboxymethyl Toyopearl			Sulphopropyl Toyopearl			
Fraction no.	A_{280}	Activity (mM/h)	Activity/A_{280}	A_{280}	Activity (mM/h)	Activity/A_{280}	**[KCl] M**
1	1.73	0.35	0.20	0.76	0.40	0.53	**0***
2	0.52	0.96	1.85	0.52	0.14	0.27	**1**
3	0.05	0	0	0.09	0	0	**2**

Table 4.3 continued.

Column type: Ion exchange				
	DEAE Toyopearl			
Fraction No.	A_{280}	Activity (mM/h)	Activity/A_{280}	**[KCl] M**
1	0.8	3.05	3.87	**0***
2	1.51	0	0	**1**
3	0.08	0	0	**2**

* Potassium chloride (KCl) concentration at which protein samples were loaded

The data for hydrophobic and ion exchange columns indicate that most of the tertiary alcohol hydrolase activity was found in the non-binding fraction and eluted with the majority of other proteins loaded onto the columns. In the case of carboxymethyl toyopearl some protein did bind to the column, however when the experiment was conducted on a larger scale the protein of interest bound but could only be removed by increasing (stepwise) polyethyleneglycol (PEG) concentrations. A 90% decrease in activity was also observed after the active fractions were assayed. Our attention then shifted to the DEAE column which had an activity/A_{280}-value of 3.87 and seemed a logical first step to remove cell debris and some "other proteins" in a rapid manner (flow rate of

48ml/hour) in preparation for subsequent steps. The active DEAE fraction (0M KCl) was also ideally suited to be directly loaded onto affinity binding MIMETIC™ ligand A6XL PIKSI™ mini columns with a decrease in pH (8.5 to 6.8), an increase in magnesium chloride (MgCl) concentration (10mM) and no buffer or desalting steps required.

Table 4.4 A_{280}, activity and activity/A_{280}-values of affinity binding PIKSI™ A6XL mini columns.

	Mimetic Orange 2			**Mimetic Orange 3**			
Fraction No.	A_{280}	Activity (mM/h)	Activity/A_{280}	A_{280}	Activity (mM/h)	Activity/A_{280}	**[KCl] M**
1	0.17	0.16	0.92	0.13	0.08	0.64	**0***
2	0.54	1.06	1.96	0.50	1.27	2.51	**0**
3	0.07	0.13	1.77	0.06	0.24	3.75	**0**
4	0.03	0.18	6.96	0.02	0.17	7.44	**0**
5	0.05	0.14	2.61	0.03	0.12	4.00	**1**
6	0.02	0.06	3.23	0.02	0.12	8.05	**1**
7	0.04	0.01	1.28	0.01	0.07	8.07	**1**
8	0.01	0	0	0.01	0.01	1.02	**2**
9	0.01	0	0	0.01	.0	0	**2**
10	0.01	0	0	0.01	0	0	**2**

Table 4.4 continued.

	Mimetic Yellow 1			**Mimetic Yellow 2**			
Fraction No.	A_{280}	Activity (mM/h)	Activity/A_{280}	A_{280}	Activity (mM/h)	Activity/A_{280}	**[KCl] M**
1	0.13	0.12	0.93	0.11	0.05	0.49	**0***
2	0.53	0.90	1.71	0.58	0.84	1.44	**0**
3	0.12	0.29	2.41	0.11	0.22	1.93	**0**
4	0.06	0.18	2.73	0.05	0.20	3.64	**0**
5	0.04	0.15	3.42	0.06	0.13	2.63	**1**
6	0.02	0.07	3.80	0.02	0.07	3.00	**1**
7	0.01	0.07	7.40	0.01	0.07	2.40	**1**
8	0.01	0	0	0.01	0	0	**2**
9	0.01	0	0	0.01	0	0	**2**
10	0.01	0	0	0.01	0	0	**2**

Table 4.4 continued.

	Mimetic Blue 1			Mimetic Blue 2			
Fraction No.	A_{280}	Activity (mM/h)	Activity/A_{280}	A_{280}	Activity (mM/h)	Activity/A_{280}	**[KCl] M**
1	0.21	0.14	0.66	0.12	0.11	0.91	**0***
2	0.46	1.10	2.34	0.55	0.55	1.16	**0**
3	0.06	0.25	3.76	0.17	0.17	1.66	**0**
4	0.03	0.15	5.13	0.14	0.14	5.12	**0**
5	0.07	0.17	2.36	0.16	0.16	2.00	**1**
6	0.09	0.07	0.79	0.06	0.06	1.76	**1**
7	0.02	0.10	6.20	0.08	0.08	1.10	**1**
8	0.02	0	0	0.01	0	0	**2**
9	0.02	0	0	0.01	0	0	**2**
10	0.01	0	0	0.01	0	0	**2**

*Potassium chloride (KCl) concentration at which protein samples were loaded.
[MgCl] = 10mM
Only columns to which enzyme of interest bound shown in above Table 4.4.

The absorption capacity of affinity binding MIMETIC™ ligand A6XL PIKSI™ mini columns is well documented and even though the enzyme of interest did bind to some of the columns, notably specific activity values of 8.05 and 8.07 for Mimetic Orange 3, in general most of the activity was found in the void fraction with a significant amount of unbound protein. Gel filtration chromatographic resins were also attempted on the active DEAE fraction, with a salt concentration of up to 0.3M KCl to prevent protein aggregation. Elution profiles (not shown) of these resins indicated protein aggregation i.e. one major protein peak coinciding with the activity peak, and they were deemed not useful in further enzyme purification steps.

The above mentioned observations and time constraints encouraged the use of the DEAE resin to remove "other" proteins and cell debris thereby producing a relatively stable crude enzyme preparation to be used in further

experiments. A typical elution profile for DEAE anion exchange chromatography is depicted in Figure 4.11 illustrating the non-binding of the hydrolase of interest eluting with the unbound fraction.

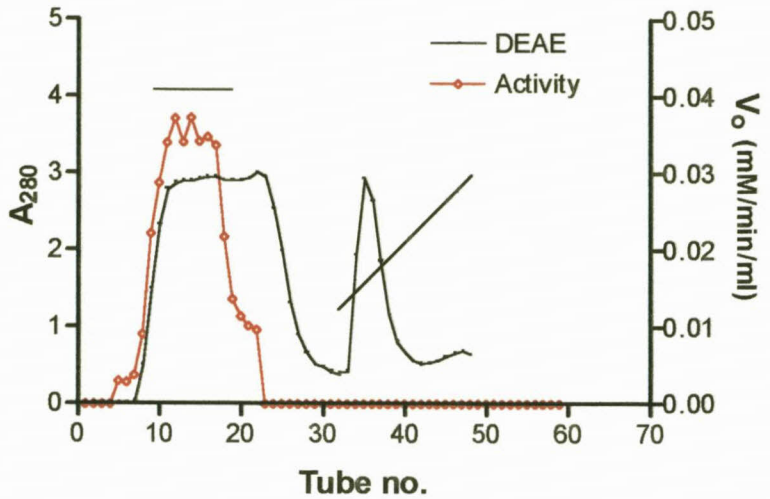


Figure 4.11 Elution profile for DEAE anion exchange chromatography for the partial purification of a tertiary alcohol hydrolase from *Trichosporon sp.* UOFS Y-0117.

The figure also shows the active fractions that were pooled (-) and utilised in further experiments and the potassium chloride (KCl) salt gradient that elutes the bound protein fraction containing no tertiary alcohol hydrolase activity.

This observation is confirmed by Table 4.5 in which a relatively small fraction of protein is removed by the column step. Even though the hydrolase of interest does not bind to the column the yield of 248% indicates that this column step facilitates interactions between the enzyme and substrate by removing a probable inhibitor to the enzyme that is present in the crude protein homogenate. This phenomenon produces a superior reaction rate (0.133μM/ml enzyme/minute) and a marginally higher purification factor (3.23). E-values remained the same throughout (data not shown).

Table 4.5 Purification table of a partially purified tertiary alcohol hydrolase obtained from *Trichosporon sp.* UOFS Y-0117.

Step	Vol (ml)	Activity (U/ml)	Total Activity (U)	Yield (%)	[Prot] (μg/ml)	Specific activity (U/mg)	Purification factor
Crude	40	0.033	1.32	100	300	4.4	1
DEAE	25	0.133	3.27	248	230	14.23	3.23

U = 0.033μmol of linalyl acetate/ minute/ ml enzyme

4.4 Characterisation of the partially purified tertiary alcohol ester hydrolase from *Trichosporon sp.* UOFS Y-0117.

4.4.1 Determination of kinetic constants in the presence of different ethanol concentrations.

The results presented in Figure 4.12 indicates that the presence of ethanol in a low concentration (2.4%v/v) produces a conversion far superior to conditions where ethanol is absent or where a higher concentration of ethanol (8%v/v) is present. This observation is similar to that observed for whole cells. Unfortunately, due to the hydrophobic nature of *rac*-linalyl acetate and resultant weak solubility in aqueous solvents thereby preventing the formation of a single phase reaction mixture, none of the classical substrate inhibition models could fit the data.

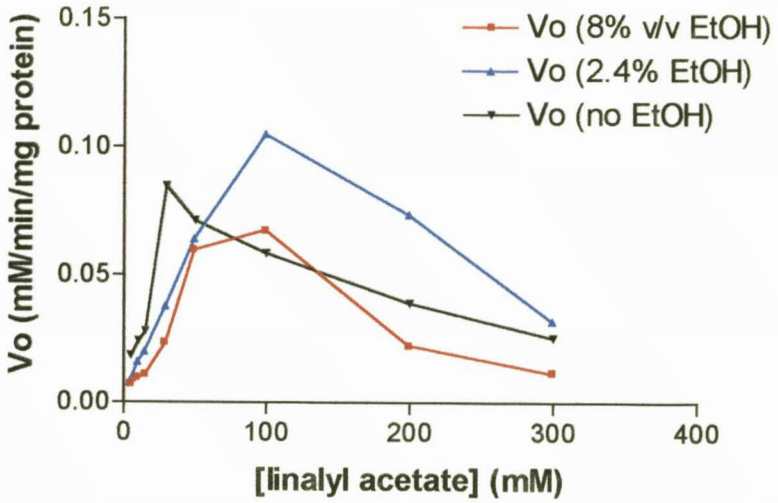


Figure 4.12 Relationship between reaction rate and substrate concentration for the hydrolysis of *rac*-linalyl acetate by a crude enzyme preparation obtained from *Trichosporon sp.* UOFS Y-0117.

The hydrophobic toxic effects of the substrate could also explain the decrease in activity due to increased molecular and later phase toxicity of the increasing concentrations of substrate and product (with intermediate log P-values) present causing protein denaturation and enzyme inhibition (Osborne *et al.*, 1990).

4.4.2 Effect of inhibitors on hydrolase activity.

Literature indicates that hydrolytic enzymes have either a carboxylate, serine or a cysteine present in the enzyme's catalytic centre that acts as a nucleophile in the reaction mechanism (Fersht, 1985). This has prompted experiments where the crude enzyme is incubated with PMSF (a serine hydrolase inhibitor) and iodo-acetic acid (a cysteine hydrolase inhibitor) to determine which amino acid residue is responsible for hydrolysis of *rac*-linalyl acetate. The data obtained from Figure 4.13 shows that a concentration of 20mM of PMSF caused a complete inactivation of hydrolase activity. The presence of iodo-acetic acid (a cysteine hydrolase inhibitor) had no significant effect on hydrolase activity (Figure 4.14) indicating that the hydrolase responsible for the hydrolysis of *rac*-linalyl acetate belongs to the serine hydrolase family.

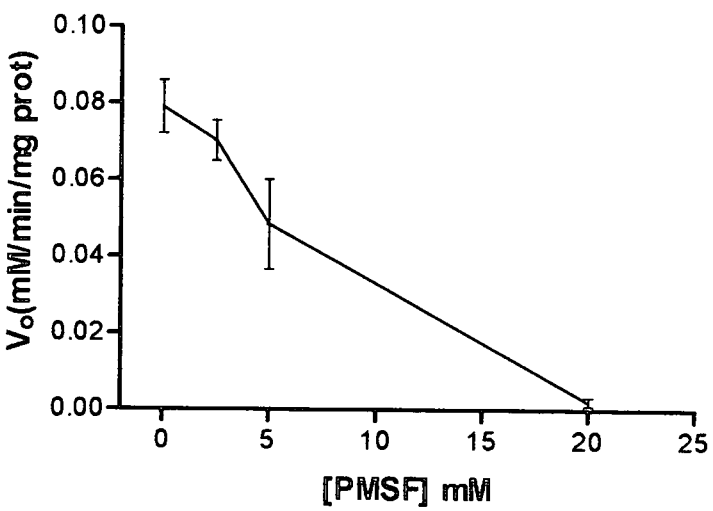


Figure 4.13 The effect of PMSF on the rate of hydrolysis of *rac*-linalyl acetate by a crude enzyme preparation obtained from *Trichosporon sp.* UOFS Y-0117.

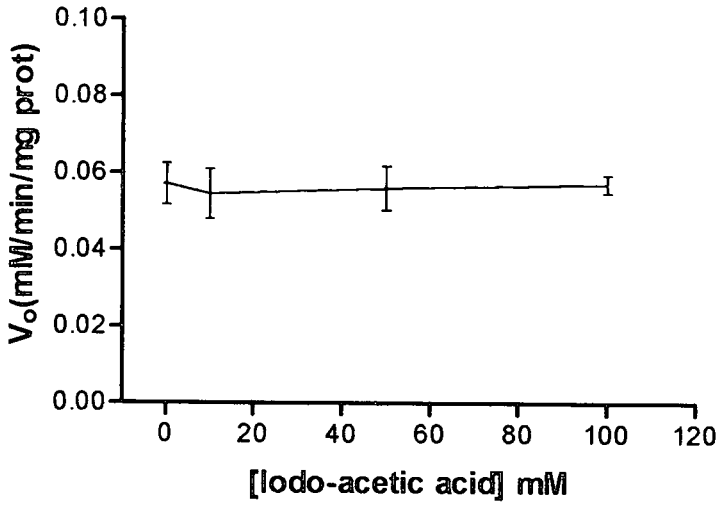


Figure 4.14 The effect of iodo-acetic acid on the rate of hydrolysis of *rac*-linalyl acetate by a crude enzyme preparation obtained from *Trichosporon sp.* UOFS Y-0117.

Similarly Figure 4.15 shows that DEP, which is responsible for histidine modification, is responsible for a complete loss of hydrolase activity once all histidine amino acid residues have been modified. The modification of histidine residues is denoted by the increase in A_{240} values as the modification process can be followed spectrophotometrically. In the figure below A_{240} values increase as histidine residues are modified with a corresponding decrease in hydrolase activity. The addition of Tris buffer (↓), which literature suggests reverses the modification process, was unable to restore hydrolase activity in this case. The inability for Tris buffer to reactivate hydrolase activity has previously occurred in literature (Lundbland, 1995).

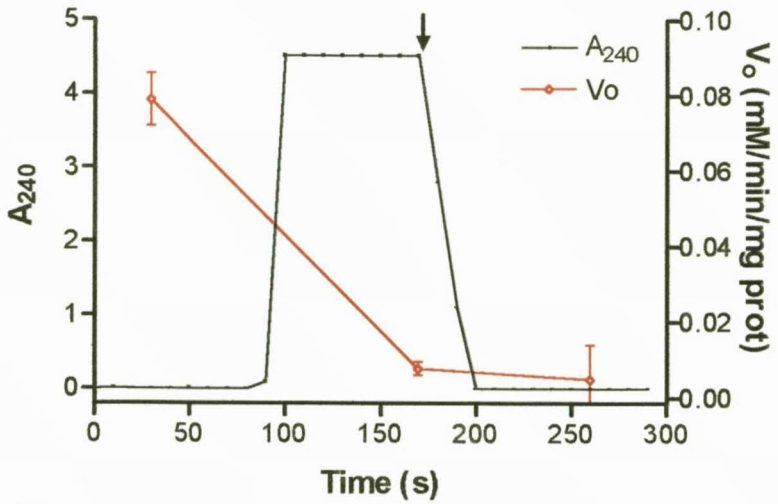


Figure 4.15 The effect of DEP on the rate of hydrolysis of *rac*-linalyl acetate by a crude enzyme preparation obtained from *Trichosporon sp.* UOFS Y-0117. Addition of 1M Tris-buffer (pH 8.5) shown by (↓).

The previous experiments have thus proved the presence of a serine and histidine amino acid residue in the catalytic site, thus we have established the positive identification of two out of three amino acid residues which are present in the catalytic triad of serine hydrolases of which lipases and true lipases form a part (Drablos and Petersen, 1997).

4.4.3 Effect of EDTA and metal cations on hydrolase activity.

The following two experiments complement each other to determine whether a metal cation has any effect on hydrolytic activity. The metal chelating reagent ethyldiamine tetra-acetic acid (EDTA), ranging in concentration up to 100mM, has no significant effect on hydrolase activity (Figure 4.16) indicating

the absence of a metal cation required for hydrolytic activity. The presence of metal cations (Figure 4.17) has no significant effect on hydrolase activity.

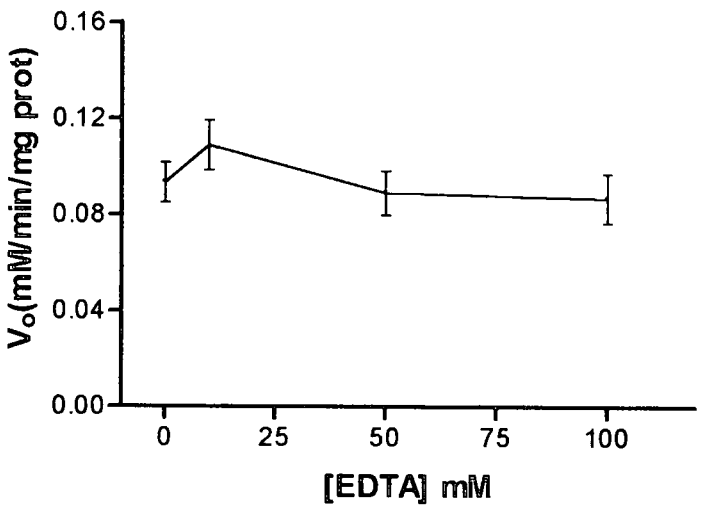


Figure 4.16 The effect of EDTA on the rate of hydrolysis of *rac*-linalyl acetate by a crude enzyme preparation obtained from *Trichosporon sp.* UOFS Y-0117.

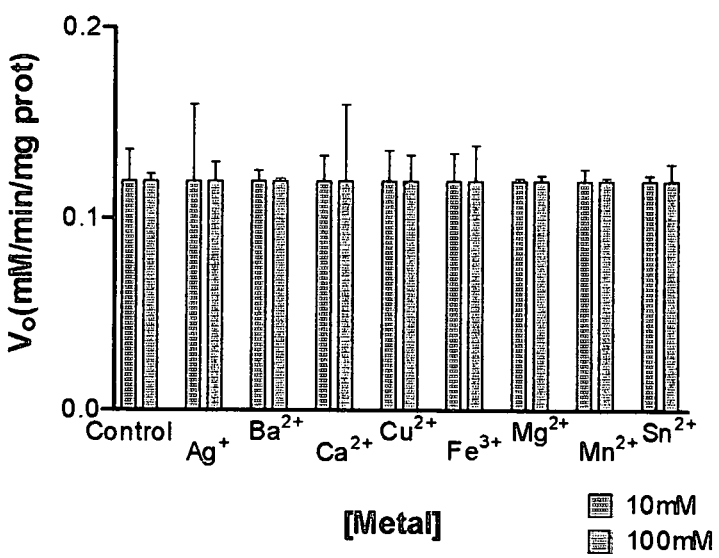


Figure 4.17 The effect of metal cations on the rate of hydrolysis of *rac*-linalyl acetate by a crude enzyme preparation obtained from *Trichosporon sp.* UOFS Y-0117.

4.4.4 pH-stability of hydrolase activity in whole cells and as crude enzyme.

Figure 4.18 and Figure 4.19 show that the crude enzyme preparation is less pH stable than the hydrolase in the whole cell culture especially at lower pH-values. At pH 5 the crude enzyme preparation was severely inactivated from the onset producing extremely low reaction rates.

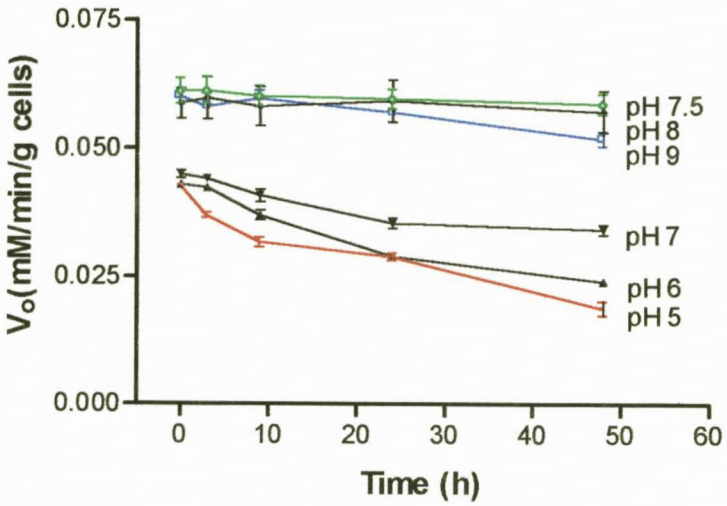


Figure 4.18 pH Stability of a tertiary alcohol hydrolase in whole cells of *Trichosporon sp.* UOFS Y-0117.

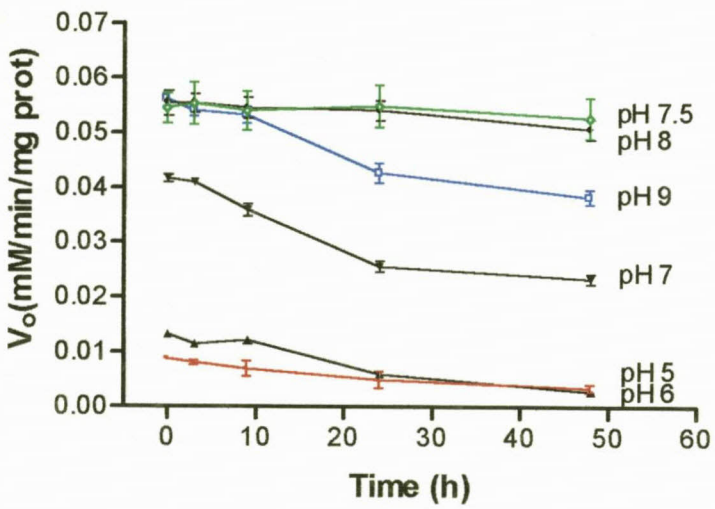


Figure 4.19 pH Stability of a crude tertiary alcohol hydrolase obtained from *Trichosporon sp.* UOFS Y-0117.

The relative stability of the enzyme in the whole cells can be ascribed to the presence of the cell wall and other physiological factors that regulate the internal environment in which the hydrolase is present. Conversely the crude enzyme preparation is wholly exposed to the extreme environment. The pH optimum for both whole cells and crude enzyme preparation can be observed between pH 7.5 and pH 8 which compares favourably with another esterase which has a slightly higher pH optimum of 9.2 (Schimmel *et al.*, 1997). Some hydrolases however have lower pH optimums depending on their source e.g. pre-gastric esterase of kid goat has a pH optimum of 6.0 (Garcia and Hill, 1996).

4.4.5 Temperature stability of the hydrolase in whole cells and as crude enzyme.

Figure 4.20 and Figure 4.21 describe the data obtained from the thermostability experiments. Figure 4.20 shows how the hydrolase present in whole cells is less susceptible to high temperatures (45°C) with a 50% loss of activity after 72 hours.

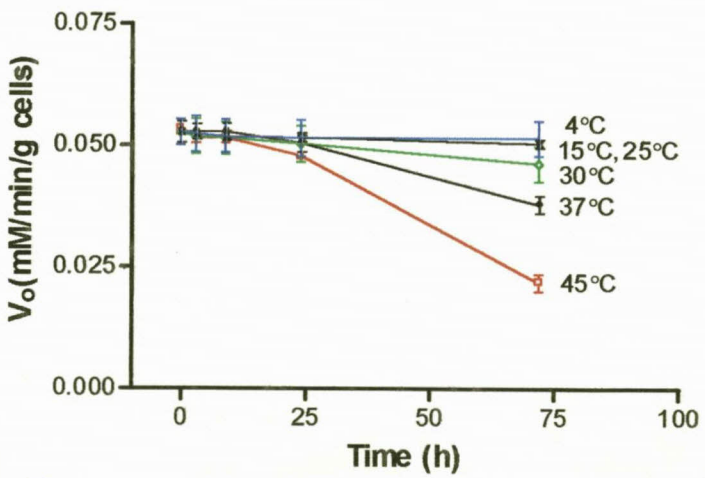


Figure 4.20 Enzyme activity remaining at time intervals for whole cells from *Trichosporon sp.* UOFS Y-0117 for the hydrolysis of *rac*-linalyl acetate.

Conversely Figure 4.21 clearly shows the susceptibility of the crude enzyme preparation to relatively high temperatures (45°C) with a two-thirds loss in hydrolase activity in less than 10 hours

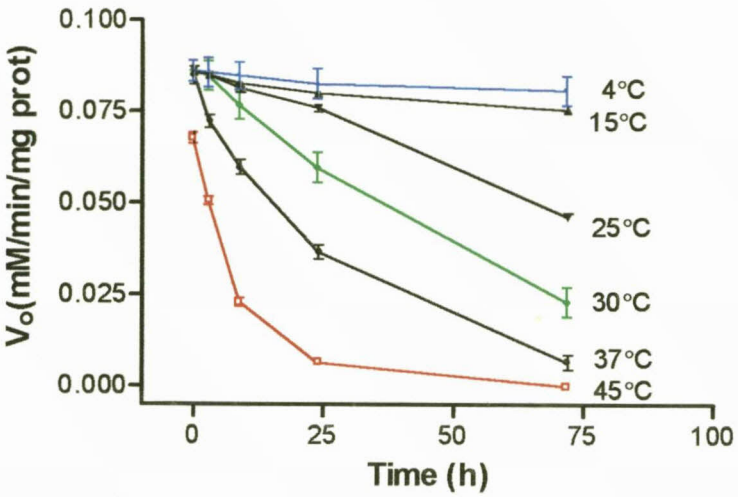


Figure 4.21 Enzyme activity remaining at time intervals for a crude enzyme from *Trichosporon sp.* UOFS Y-0117 for the hydrolysis of *rac*-linalyl acetate.

Assuming that the inactivation follows first order kinetics the rate constants for inactivation can be determined from a graph of log(%remaining activity) vs time (Figure 4.22 and Figure 4.23). Once the graphs are prepared the gradients determine the rate constant (k) for each temperature for whole cells and crude enzyme preparation.

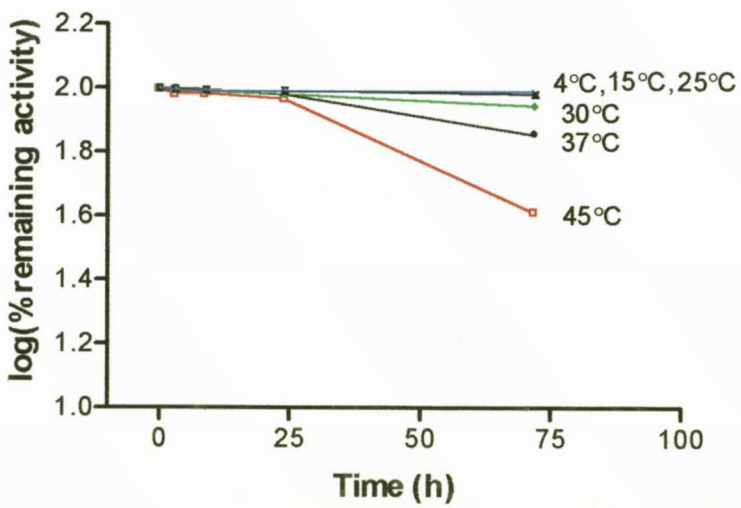


Figure 4.22 Plot of log(%remaining activity) vs time for whole cells of *Trichosporon sp.* UOFS Y-0117 for the hydrolysis of *rac*-linalyl acetate.

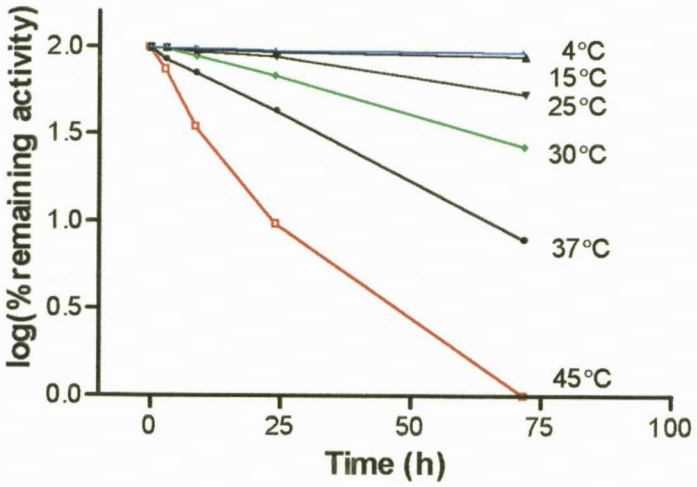


Figure 4.23 Plot of log(%remaining activity) vs time for a crude enzyme preparation obtained from Trichosporon *sp.* UOFS Y-0117 for the hydrolysis of *rac*-linalyl acetate.

Once rate constants (k) are determined they can be used to calculate half-life values (Table 4.6). We can observe from Table 4.6 that the whole cells have a half-life approximately four times larger than the crude enzyme preparation at 4°C. At 45°C whole cells have a half-life fifty-four times larger than the crude enzyme preparation. Eventhough half–life may be relatively high, one should take into account that we start off an experiment with extremely low hydrolase activity and coupled with long assay times "detectable" activity is sometimes limited to an extremely short period of time.

Table 4.6 Rate constants (k) and half-life ($t_{½}$) values for whole cells and crude enzyme preparation obtained from *Trichosporon sp.* UOFS Y-0117 for the hydrolysis of *rac*-linalyl acetate.

	Whole cells		**Crude enzyme**	
Temp (°C)	**k (hrs^{-1}) ($x10^{-4}$)**	**$t_{½}$ (hrs)**	**k (hrs^{-1}) ($x10^{-4}$)**	**$t_{½}$ (hrs)**
4	2.19	3468	8.61	800
15	53.80	1287	16.65	416
25	53.71	1291	86.12	80
30	168.62	410	185.64	37
37	215.95	321	350.22	19
45	258.44	268	1332.73	5

The rate constants (k) can be used to produce Figure 4.24 which is based on the Arrhenius equation which makes calculation of the activation energy and the pre-exponential or frequency factor (A) possible.

$$k = Ae^{-E_a/RT}$$

The activation energy (E_a) is calculated from the gradient and the pre-exponential factor from the y-intercept of the Arrhenius plots. The E_a-value is the activation energy required for inactivation of the enzyme i.e. the energy "threshold" required for the inactivation process to occur. The tertiary alcohol hydrolase present in whole cells required 47.43 $kJ.mol^{-1}$ of energy to cross the above-mentioned threshold compared to the crude enzyme preparation (91.77 $kJ.mol^{-1}$).

The pre-exponential factor (A) which is derived from collision theory, describes the probability that a molecule will have sufficient energy to be inactivated.

For the hydrolase present in the whole cells the value (1.807 $X10^5$ h^{-1}) is very low compared to the $1.175X10^{14}$ h^{-1} of the crude enzyme preparation.

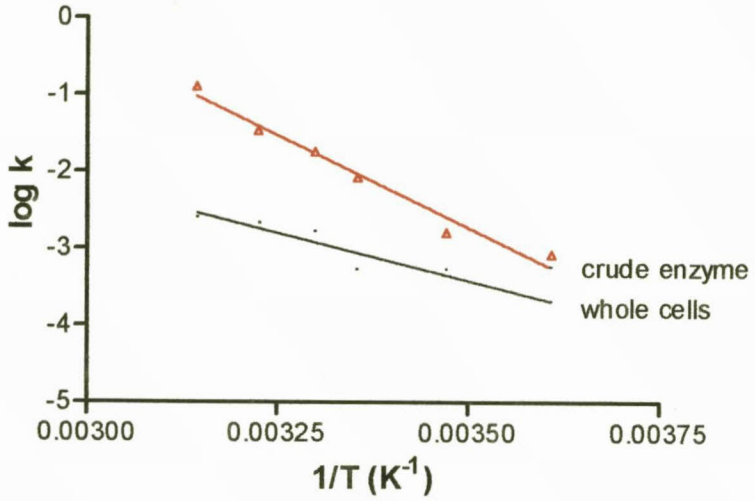


Figure 4.24 Plot of log k vs 1/Temperature for *Trichosporon sp.* UOFS Y-0117 for the hydrolysis of *rac*-linalyl acetate: whole cells and crude enzyme preparation.

By dividing the A-value of the crude enzyme preparation by that of the whole cells we obtained a value of $6x10^8$ which is a quantitative value of the degree of thermostability of the tertiary alcohol hydrolase in whole cells above the crude enzyme. The hydrolase present in a whole cell is thus $6x10^8$ more stable than the hydrolase present in the crude enzyme preparation. A similar trend was seen by Pronk and co-workers (1992) upon immobilisation of *Candida cylindracea* lipase. No change in activation energy was observed on immobilisation of their enzyme but an approximately fourfold reduction in the value of the frequency factors was observed upon immobilisation. The protecting effect of "immobilising" the enzyme in its natural environment (the cell) is however much more dramatic.

The above mentioned data suggests that the whole cells should be employed as a biocatalyst due to the relatively low pH and thermostability of the crude enzyme preparation. The conditions investigated in this study confirms that the crude enzyme preparation does not produce a significantly higher reaction rate compared to whole cell cultures.

A possible follow up experiment would involve the effect of temperature on the enantioselectivity of the tertiary alcohol hydrolase present as Phillips (1996) suggests that temperature modulation can effect the stereochemistry of enzymatic catalysis. Immobilisation effects, using artificial matrixes, on the biocatalyst should also be investigated to improve enantiomeric yield.

Chapter 5 Conclusions

The increased use of biocatalysts in industry has prompted further studies in their possible applications to complement conventional synthesis methods. Hydrolases, particularly esterases and lipases, are the biocatalysts most widely used in organic synthesis. Many enzymes with high activity for esters of primary and secondary alcohols are available (Kelly, 1998). However, very few are available for the synthesis or hydrolysis of esters of tertiary alcohols. This was demonstrated again recently by Osprian *et al.*, (1996) when they screened commercially available hydrolases and proteases for the resolution of *rac*-linalyl acetate. They found no commercially available enzymes that could hydrolyze this tertiary alcohol ester. A limited number of novel bacterial isolates could hydrolyze this ester, but demonstrated relatively low enantiomeric ratios. The most promising activity was found in *Rhodococcus ruber* SM 1792 with an enantiomeric ratio of 4.8.

During recent years the Biotransformation group at the UOFS have been exploring the use of yeasts for the hydroxylation and reduction of monoterpenes and monoterpenoids (Van Rensburg *et al.*, 1997, Van Dyk *et al.*, 1995, Van Dyk *et al.*, 1998). It was decided to extend this study to the resolution of *rac*-linalyl acetate and α-terpinyl acetate. Out of the 104 yeasts screened 12 showed hydrolase activity towards *rac*-linalyl acetate while only one hydrolyzed α-terpinyl acetate. Activities and selectivities were relatively low, but in the same order as observed for the bacteria. The two strains with the highest activity were *Trichosporon sp.* UOFS Y-0117 and *Pichia holstii*

UOFS Y-0140. With the exception of the *Pichia* strains all the positive yeasts preferentially hydrolyzed the (*S*)-enantiomer of the substrate. Enantiomeric ratios varied between 1 and 4. *Pichia holstii* UOFS Y-0140 was also the only yeast which hydrolyzed α-terpinyl acetate (preferentially the (*S*)-enantiomer, E = 2.4). The enzyme activity in *Pichia holstii* UOFS Y-0140 seemed quite unique but it was decided to first study the enzyme system in *Trichosporon sp.* UOFS Y-0117 in more detail, since it was considered more representative of linalyl acetate hydrolases in yeasts.

In whole cell experiments we explored the effects of growth media, effect of co-solvents, substrate concentration (substrate toxicity), optimal pH and temperature, and a cell membrane permeabilising reagent. Finally the re-usability of whole cells of *Trichosporon sp.* UOFS Y-0117 was also investigated. The highest enzyme activity was observed when the yeast was grown in a defined medium (i.e. YNB) supplemented with 1% maltose. Enzyme activity was significantly lower when a rich complex medium such as YM medium was used or when the carbon source was present in high concentrations (i.e. YNB medium with 4% glucose, maltose or succinate). Enzyme activity and enantioselectivity remained constant from mid-exponential to late stationary phase. Optimal pH (7.5) and temperature (30°C) coincided with physiological conditions and values reported for other esterases (Holland and Coolbear, 1996). Low concentrations of ethanol (i.e. 2.4% v/v) improved initial reaction rates, probably because it enhanced the solubility of the hydrophobic substrate. This might indicate that the enzyme is not subject to interfacial activation (i.e. a lipase) but requires the substrate to

be dissolved. High substrate concentrations (up to 200 mM) had no adverse effects on initial reaction rates, even though linalyl acetate has a log P of 3.7. Log P values between 1 and 5 is normally associated with disruption of membranes, protein denaturation and enzyme inhibition (Osborne *et al.*, 1990). The re-usability experiment, however, showed a significant decrease in hydrolase activity as the same batch of cells was repeatedly exposed to substrate and product over a longer period.

Efforts to purify the linalyl acetate hydrolase from *Trichosporon sp.* UOFS Y-0117 were unsuccessful because the hydrolase of interest did not adhere to most of the conventional resins tested. This contrasts with the results obtained by Strauss *et al.*, (1998) who succeeded in separating two linalyl acetate hydrolases with opposite enantioselectivities from *Rhodococcus ruber* SM1792. Experiments on the enzyme in cell free extracts using specific amino acid modification reagents indicated that this linalyl acetate hydrolase is probably a member of the serine hydrolase family, since it contains a serine and a histidine in the active site. Thermostability experiments demonstrated that the hydrolase in cell free extracts was relatively stable, but that the enzyme in whole cells was even more stable. pH stability experiments showed that the enzyme in whole cells was stable over a wider pH range. The enzyme in cell free extracts also gave lower initial reaction rates when exposed to substrate concentrations exceeding 100mM.

Low hydrolase activity coupled with long assay times and time consuming GC analysis made the study of the linalyl acetate hydrolase in *Trichosporon sp.*

UOFS Y-0117 technically difficult. It also appears that this biocatalyst will have limited applicability in preparative processes using either continuos flow or batch systems, because the low reaction rates will require prolonged exposure of the enzyme to the substrate, which in this case appears to destroy enzyme activity. The linalyl acetate hydrolases from the other yeasts should also be investigated in more detail to establish whether they are subject to the same limitations. Screening efforts should be maintained in order to find yeasts containing enzymes with higher activity and improved enantioselectivity and to explore to a larger extent the novel hydrolases present in yeasts. Once some of these enzymes have been purified and/or the genes sequenced it will be possible to employ overexpression and mutagenesis techniques to establish structure function relationships. It will also allow comparative studies between genera and species and perhaps allow for preliminary screening on a genetic level.

Chapter 6 Summary

Hydrolysis of esters by means of hydrolases such as proteases (Jones and Beck, 1976), lipases (Santiello *et al.*, 1993) and esterases (Boland *et al.*, 1991) has become a well established method for the resolution of racemic mixtures.

The first aim of the present study was to screen the yeast culture collection of the University of the Orange Free State for yeast isolates which can be used for the enantioselective hydrolysis of *rac*-linalyl acetate and *rac*-α-terpinyl acetate, which are tertiary alcohol ester terpenes, respectively. We screened 74 yeast strains from 17 genera as well as 29 unclassified isolates. Approximately 16% of the strains screened contained tertiary alcohol hydrolase activity. Whole cell experiments, enzyme purification and characterisation were attempted on one of the hydrolases of interest obtained from *Trichosporon sp.* UOFS Y-0117.

Whole cell experiments on reported optimal hydrolase activity in the presence of 1% maltose in a defined media (YNB). The effect of various co-solvents was also documented with a low concentration of ethanol (2.4% v/v) producing superior hydrolase activity. No toxicity, to the microbe, was observed by *rac*-linalyl acetate (up to 200mM) due to the cell membrane present. The use of digitonin proved that substrate transport across the cell membrane is not a reaction rate determining step. The re-usability

experiment showed a significant decrease in hydrolase activity (ca 30% after 1 cycle) as the same batch of cells was exposed to substrate and product. The results also show an optimal pH of 7.5 and temperature of 30°C which coincides with physiological conditions and literature.

Protein purification was attempted on a cell free extract once we determined that the hydrolase was intracellular. Small scale evaluations of different chromatographic resins ranging from ion exchange, hydrophobic interaction and affinity chromatography followed. Large scale experiments with gel filtration resins were also attempted. Purification steps were largely unsuccessful and we decided to continue with a DEAE fraction which produced a superior yield (244%).

Characterisation experiments, using the DEAE active fraction, followed in which we explored the effect of *rac*-linalyl acetate concentrations. Enzyme inhibition and protein denaturation at low *rac*-linalyl acetate concentrations (detected at ca 65-100mM) is significant compared to whole cells (not detected at 200mM). This hydrolase is also an esterase. Specific amino acid modification reagents results indicate the presence of a serine and histidine amino acid present in the catalytic centre i.e. the hydrolase belongs to the serine hydrolase family. A metal chelating reagent EDTA and various metal cations had no effect on hydrolase activity. pH-stability experiments indicate a pH of 7.5 to be optimal for the retention of hydrolase activity in whole cells and crude enzyme preparation. Thermostability experiments show whole cells are four times more stable than the crude enzyme preparation at 4°C. The

energy of inactivation required for activity loss is lower in whole cells (47.43kJ) compared to crude enzyme preparation (91.77kJ). The probability for an event to occur which causes inactivation is relatively low (1.807^{5} events/h). The converse applies to the crude enzyme preparation (1.175^{14} events/h). Thus the crude enzyme is $6x10^{8}$ less stable than the hydrolase present in the whole cell.

Keywords:

Hydrolase, yeasts, tertiary alcohol ester, linalyl acetate, linalool, esterase, thermostability, characterisation.

Opsomming

Die hidrolise van ester bindings deur middel van proteases (Jones and Beck, 1976), lipases (Santiello *et al.*, 1993) en esterases (Boland *et al.*, 1991) word dikwels gebruik om rasemiese mengsels te skei.

Die eerste doel van hierdie studie was om organismes wat positief vir tersiêre alkohol ester hidrolase aktieweteit toets, te vind deur middel van 'n siftingsproses. Eksperimente is gedoen op giste van die gisversameling van die Universiteit van die Oranje Vrystaat vir die omskakeling van rasemiese mengsels van linaliel asetaat en terpiniel asetaat as substrate. Ons het 74 gisisolate van 17 genera en 29 onklassifiseerde isolate getoets vir die bogenoemde aktiviteite. Sowat 16% van die isolate het positief getoets vir hierdie hidrolase aktiwiteit. Eksperimente met heel selle is eers doen, gevolg deur gedeeltelike ensiemsuiwering en -karakterisering van een van hierdie hidrolases in die gis *Trichosporon sp.* UOVS Y-0117 gevind.

Heel sel eksperimente het getoon dat maksimale hidrolase aktiwiteit in die teenwoordigheid van 1% maltose in 'n gis-stikstof medium geïnduseer word. Verskillende mede-oplosmiddels het getoon dat lae konsentrasies ethanol (2.4% v/v) die hoogste hidrolase aktiwiteit bewerkstellig. Geen newe-effekte op die gis is gesien nie, selfs by hoe konsentrasies linaliel asetaat (200mM), waarskynlik as gevolg van die beskermende teenwoordigheid van die selmembraan. Digitonien, 'n middel wat selmembrane deurlaatbaar maak, het ook geen effek op die reaksie spoed gehad nie. Proewe om die moontlike

hergebruik van heel selle het getoon dat 'n noemenswaardige verlies in hidrolase aktiwiteit plaasvind na een siklus (ca 30% afname) wanneer dieselfde selle aan substraat en produk blootgestel word. 'n Optimale pH van 7.5 en 'n optimale temperatuur van 30°C was ook gevind.

Eksperimente is op klein skaal gedoen op 'n selvrye ekstrak om te bepaal watter chromatografieharse (ioonuitruiling, hidrofobiese en affiniteit) vir die suiwering van die hidrolase gebruik kon word en uitsluitingschromatografie media is ook getoets. Suiweringstappe was meestal onsuksesvol en ons het besluit om met 'n aktiewe fraksie na DEAE ioonuitruilingschromatografie voort te gaan.

Karakterisering van die aktiewe DEAE fraksie, het getoon dat ensiem inhibisie en protein inaktivering plaasvind by lae konsentrasies linaliel asetaat (65-100mM) in vergelyking met heelselle (geen effek tot by 200mM). Spesifieke aminosuur modifikasie reagense het getoon dat 'n serien en histidien in die katalitiese setel teenwoordig is en dus is hierdie hidrolase deel van die serine hidrolase familie. EDTA en verskillende metaalkatione het geen beduidende effek op hidrolase aktiwiteit gehad nie. pH eksperimente dui daarop dat 'n pH van 7.5 optimaal was om hidrolase aktiwiteit te behou in heel selle en selvrye ensiem ekstrak. Thermiese stabiliteit eksperimente dui daarop dat die ensiem in die heel selle baie meer stabiel is as die selvrye ensiem ekstrak. Die aktiveringsenergie vir deaktivering van die ensiem is laer in heelselle (47.43kJ) in vergelyking met selvrye ensiem ekstrak (91.77kJ). Die pre-eksponensiële faktor vir inaktivering in heelselle was $1.807X10^5$ uur^{-1}. Vir die

selvrye ensiem ekstrak is hierdie waarde $1.175X10^{14}$ uur^{-1}. Dus is die selvrye ensiem ekstrak $6x10^{8}$ keer minder stabiel in vergelyking met die hidrolase in die heel selle.

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