PARAMETERS INFLUENCING YEAST-MEDIATED REACTIONS IN AN ORGANIC SOLVENT

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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STATEMENT

This is to certify that the research contained in this thesis is the sole work of the candidate and contains no material previously published or written by another person except where due reference is made. The work has not been submitted for any other academic award.

C. MEDSON

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PUBLICATIONS

Parts of this work has been published in:

"The stereoselective preparation of β-hydroxy esters using a yeast reduction in an organic solvent." C. Medson, A.J. Smallridge, M.A. Trewhella, *Tetrahedron: Asymmetry*, 1997, **8**, 1049-1054.

"Bakers' yeast activity in an organic solvent system." C. Medson, A.J. Smallridge, M.A. Trewhella, *J. Mol. Cat. B: Enzymatic*, 2000, in press.

SUMMARY

The yeast-mediated reduction of a series of 3-oxobutanoate esters (methyl, ethyl, isopropyl, butyl, *sec*-butyl, *tert*-butyl and benzyl) was performed in petroleum ether at room temperature over a 24h period. The procedure was shown to be a simple and effective method for the stereoselective preparation of (*S*)-3-hydroxybutanoates. Of the seven esters tested, the best substrate was shown to be the isopropyl ester, which gave the best yield (96%) with a relatively small amount of yeast (2g/mmole substrate) and with good enantioselectivity (97% ee). Although some esters required larger amounts of yeast in order to attain complete conversion to the product, the isolated yields were generally higher than 70% and the enantioselectivity was normally greater than 97% ee. The yields and stereoselectivity obtained using this method are superior to those reported for reductions using fermenting yeast in an aqueous environment. The results suggest that substrate size and shape have an impact on the ease of binding of the substrate to the active site of the enzyme system; substrates which bind poorly require more yeast.

Time lapse ^{13}C NMR spectroscopy was used to study the yeast-mediated reduction of 3-oxobutanoates. It was shown that exposure to the solvent system at room temperature deactivates the enzyme system and slowly decreases the reaction rate until no further reduction occurs. From this information a model was constructed and used to simulate the reaction under a range of experimental conditions, including different temperatures and different esters. The ethyl, isopropyl and benzyl esters were modelled at 10, 20 and 30°C. The deactivation of the enzyme system was followed and appears to be due to a gradual disruption of the spacial arrangement of the enzyme active site making the binding of substrates less effective. The substrates which fit less well are more susceptible to these changes and therefore require more yeast for complete reduction to occur. At 10°C enzyme deactivation was not detected within the 60h period.

The yeast-mediated esterification of 2-bromopropanoic acid was investigated in an organic solvent system. It was found that only a very small amount of butyl ester was formed in the presence of butanol, and all attempts to increase the amount of product were unsuccessful. Three other organic acids were also trialled with the same result of very little or no product being formed. The main problem associated with these reactions was the loss of a substantial

proportion of the acid. Endeavors to discover why the acid was being consumed under these reaction conditions indicated that some was adsorbed onto the yeast whilst a further amount formed the carboxylate salt and remained in the aqueous layer. About half the acid remained unaccounted for.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 BACKGROUND

Humankind has utilised microorganisms for centuries, in particular yeast for bread and alcohol production. In more recent times, other microorganisms and parts thereof have been used in applications as diverse as bioremediation,[1] insulin production,[2] laundry powders[3] and the synthesis of chiral compounds.[4]

The use of microorganisms for the production of chemically and biologically useful compounds relies on the catalytic attributes of the enzymes present. With only a few exceptions, enzymes are capable of catalysing almost every type of organic reaction.[5] The structural specificities of enzymes can be exploited to effect stereoselective or regiospecific reactions on only one of a number of chemically similar functional groups in a molecule. The ease with which an enzyme catalysed reaction can be performed is often extremely difficult to duplicate non-enzymatically, especially as a single step reaction.[5] The production of chiral molecules is of particular interest to the pharmaceutical industry[6] and in the synthesis of natural products. Asymmetric synthesis mimics enzymatic transformation and therefore microorganisms with broad substrate specificity are an excellent choice for asymmetric catalysis.

Many synthetic chemists did not immediately recognise the potential of microorganisms due to difficulty in obtaining access to the microbial techniques required for handling live cells. The typical approach involved screening microbes until one was found that catalysed the necessary biotransformation, and without the necessary expertise and equipment, it was difficult to convince chemists to commit resources. Another perceived disadvantage was that the reactions needed to take place in an aqueous environment in which many organic substrates are insoluble.

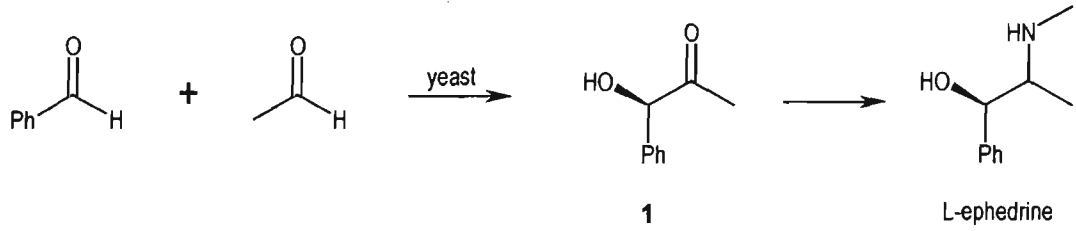
Some progress was made with isolated enzymes, as they were much easier to manipulate than live cells and were commercially available, including a significant number in immobilised form for easy reuse. Enzymes were also found to be active in organic solvents which broadened the applicability for synthetic chemistry since water is a solvent that is generally

avoided due to its high boiling point and high vapour pressure. Lipases are an excellent example of the proficiency of isolated enzymes for the resolution of racemic mixture using organic solvents - acids,[7] alcohols[8] and esters.[9] However, enzymes that catalyse reactions such as oxidation-reduction require cofactors which can be expensive or need to be immobilised to be cost effective.

One way to overcome this problem is to use whole cells. Bakers' yeast is a suitable microorganism; it is a cheap and readily available enzyme source with all the necessary cofactors present; there is no requirement to immobilise the cells for reuse, as they are cheap enough to replace; the growth conditions are well documented and easily achievable in an laboratory; a wide range of substrates can be accommodated and many types of reactions can be performed.

1.2 YEAST IN ORGANIC SYNTHESIS

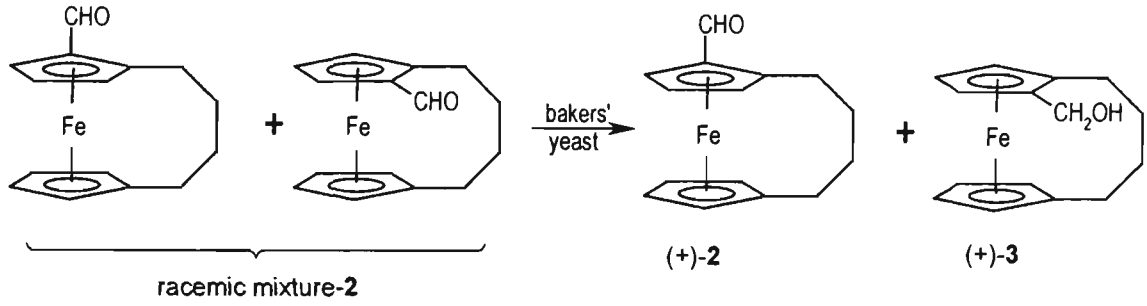
The use of yeast for organic synthesis has been seriously examined over the last two decades, with a systematic investigation of both the yield and, more particularly, the stereochemistry of the resultant product. Several hundred different enzymes have been isolated from yeast.[10] The yeast-mediated condensation of benzaldehyde and acetaldehyde to give optically active L-1-hydroxy-1-phenyl-2-propanone, **1**, which is then chemically converted into L-ephedrine, represents one of the first industrial exploitations of a microbial transformation (Scheme 1-1).[2]



1 L-ephedrine

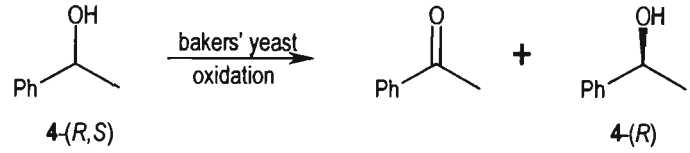
Scheme 1-1

Yeast has been reported to catalyse many asymmetric reactions with quite a diverse range of substrates. A more unusual class of substrates are organometallic compounds containing carbonyl groups.[11,12] One example is the selective reduction, and therefore resolution, of (±)-6-formyl-[4]-ferrocenophanes **2** (Scheme 1-2).[13]



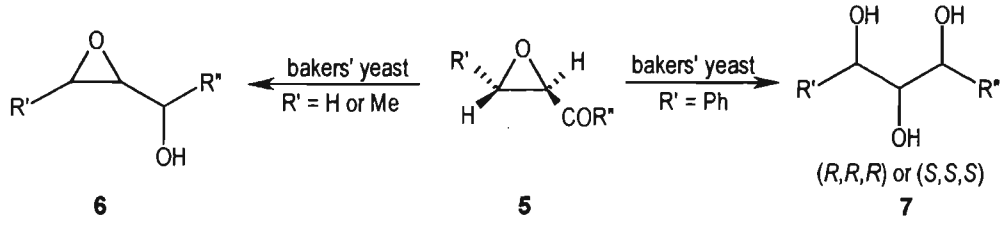
Scheme 1-2

By changing the reaction conditions, the type of yeast transformation can be manipulated. Reduction is the most common type of yeast reaction but there are also reports of yeast-mediated oxidation reactions (Scheme 1-3). Good resolution of phenylethanol **4** was reported to occur when ammonium acetate was added to the reaction (41% yield, 100% ee).[14]



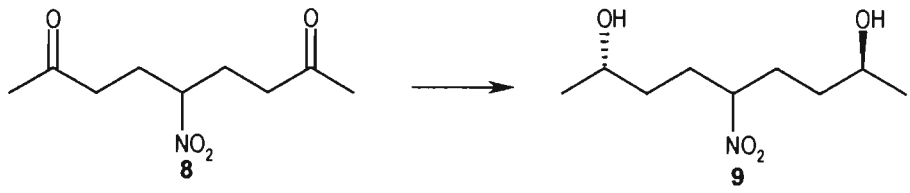
Scheme 1-3

Yeast-mediated reductions have been reported to produce plural asymmetric centres (Schemes 1-4, 1-5). The yeast-mediated transformation of the epoxide **5** gives two distinctly different results, depending on the substituent attached to the epoxide ring. Small groups such as hydrogen or methyl attached at the epoxide ring protect it from attack, resulting in the formation of **6**. With a 2-phenyl substituent, the sole product is the 1,2,3-triol **7** as a single racemic diastereoisomer derived by a reduction/hydrolysis sequence involving a *syn* ring-opening of the epoxide (Scheme 1-4).[15]



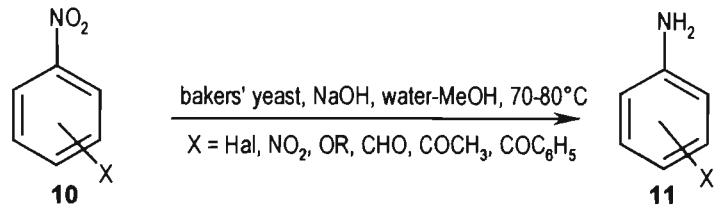
Scheme 1-4

In order to produce enantiomerically pure precursors of spiroketals, the double stereoselective reduction of 5-nitro-2,8-nonandione **8** was accomplished using yeast. A moderate yield of the diol **9** was attained (58%) but again with excellent stereoselectivity (95% ee) (Scheme 1-5).[16]



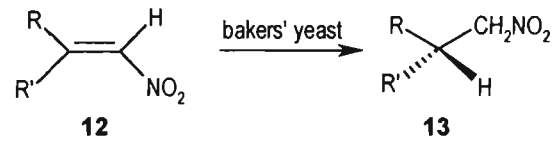
Scheme 1-5

Reduction of aromatic nitro compounds, for example the reduction of **10** to **11**, shows the selectivity of yeast-mediated reactions (Scheme 1-6).[17] With aromatic rings containing carbonyl groups, only the nitro group was reduced and when two nitro groups were present, only one was reduced. No dehalogenation was detected when halogens were present, in contrast to the result achieved with common reducing agents. In most cases high yields were obtained (>80%) in a short time (~5 h). Given the harsh conditions (pH>9, 70-80°C) it is surprising that the yeast is active.



Scheme 1-6

Organic nitro compounds are important synthetic intermediates because they can be easily converted into amines, carbonyl compounds or hydrocarbons. Asymmetric hydrogenation of nitroolefins **12** by bakers' yeast resulted in moderate yields of **13** with excellent stereoselectivity (Scheme 1-7).[18]



Scheme 1-7

These examples show the variety of substrates yeast can utilise and the range of reactions yeast is capable of mediating. Although the yeast-mediated reactions of certain substrates do result in excellent stereoselectivity, yeast reactions of other substrates do not.

There are two major factors associated with the lack of stereoselectivity found in some yeast reactions:

1. Only one enzyme is responsible for the reduction and the substrate does not exert its enantio- or diastereofacial difference in the enzyme active site, or

2. There are two or more enzymes operating simultaneously but some produce the (*S*)-enantiomer while others produce the (*R*)-enantiomer; both enzymes act with high stereospecificity.[19]

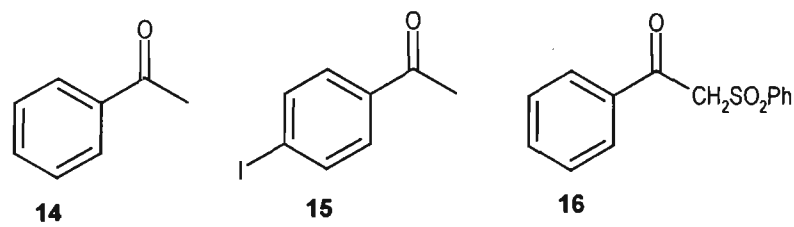
There has been a great deal of investigation of these factors and how to control them, in order that yeast can be successfully used as a catalyst for stereoselective reactions.

The main class of compounds of interest has been the β-keto esters, however many interesting and relevant studies have also investigated other keto esters, ketones and aldehydes. Some of the work has concentrated on the stereochemical control in yeast-mediated reductions of α-keto esters.[20] Unfortunately, α-keto esters are not a good model for yeast reactions as one enantiomer of the product is further broken down enzymatically in an aqueous environment and not until this reaction was performed in bulk organic solvent did this become apparent. Hence in this case, the stereochemistry of the reduction reaction is hard to quantify and the detection of the effects of modifications to the reaction system is difficult.

Methods reported to improve the stereoselectivity include substrate modification, addition of an inorganic salt, addition of a specific enzyme inhibitor, immobilisation, thermal treatment and use of organic solvents.

1.2.1 Substrate Modification

Reports concerning substrate modification as a way to control the stereoselectivity of yeast-mediated reductions first appeared in early 1980. Most researchers have exploited Prelog's rule that has been shown to apply to bakers' yeast reductions.[21] Nakamura[22] reported on the substrate modifications of acetophenone **14** by iodonation and sulfonylation (Figure 1-1).



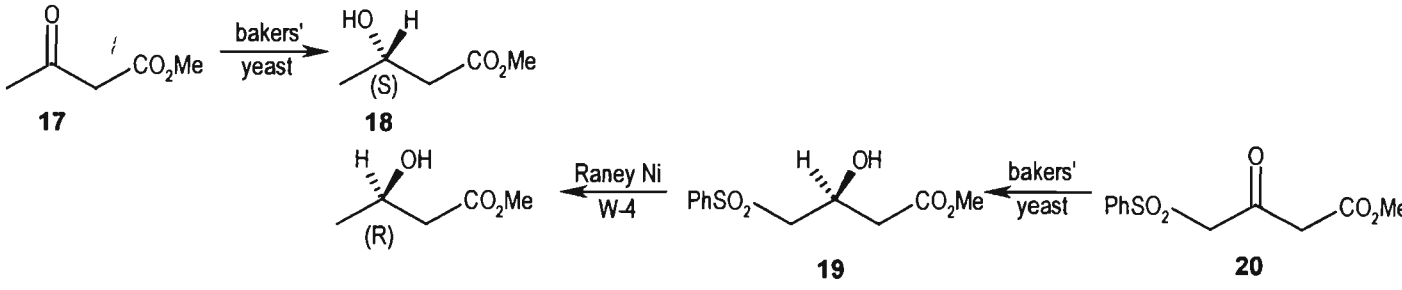
14 **15** **16**

Figure 1-1 Modifications of acetophenone 14 for yeast-mediated reductions.

Yeast-mediated reduction of **14** resulted in a product yield of 30%, with 60% ee, whilst reduction of the iodonated compound **15** improved the stereoselectivity of the reaction to give the product in 96% ee, without changing the yield. The effect of placing a large iodine

atom on the already large phenyl ring increased the difference between the small (methyl) and large (phenyl) groups attached to the carbonyl. The difference in enzyme binding to the *Re*- and *Si*-faces is greater and therefore better stereoselectivity results. The reduction of the sulfonylated ketone **16** with bakers' yeast afforded unsatisfactory stereochemistry (15% ee, 85% yield). This may have been due to the smaller group, α-phenyl, being too large to be comfortably accommodated within the enzyme's active site, resulting in non-specific binding. In this case, the product has the opposite stereochemistry since the relative size of the groups either side of the carbonyl had changed. Saké yeast (*Saccharomyces cerevisiae*) was also used for the reduction of **16** with greatly improved stereoselectivity (92% ee).

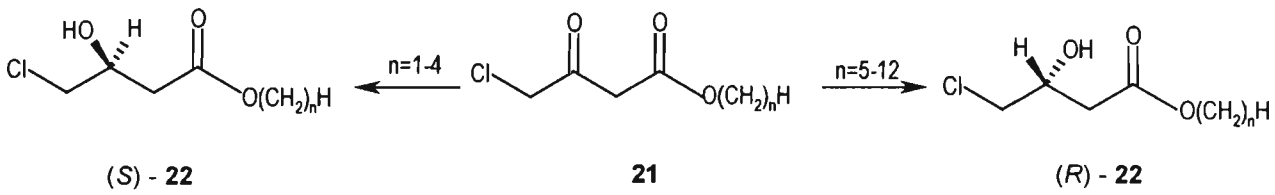
Sulfonylation was also applied to the reduction of β-keto esters (Scheme 1-8).[22] Methyl 3-oxobutanoate **17** is reduced to the corresponding (*S*)-alcohol **18** (23%yield, 87%ee) in an aqueous fermenting yeast medium. In order to obtain the opposite enantiomer with excellent stereoselectivity, methyl 4-phenylsulfonyl-3-oxobutanote **20** was reduced with bakers' yeast. Desulfonylation gave the (*R*) alcohol **18** (80% yield, 98%ee).



17 **18** **19** **20**

Scheme 1-8

According to Prelog's rule if the relative size of the groups either side of the carbonyl group being reduced is changed, then the opposite enantiomer is formed. Sih *et al.*[23] demonstrated this with the yeast-mediated reduction of an homologous series of γ-chloro-3-oxobutanoates **21** (Scheme 1-9). There was a dramatic shift in the stereochemistry of the product formed as the size of ester grouping changed from C_4 (27% $ee_{(S)}$) to C_5 (75% $ee_{(R)}$).



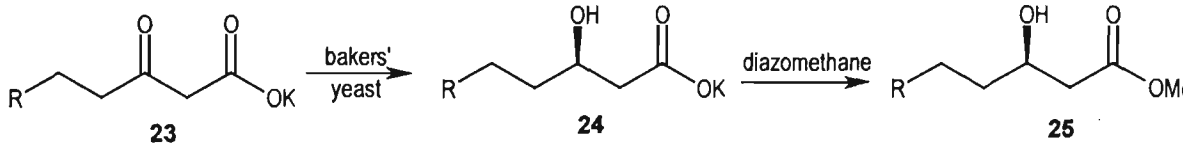
(*S*) - **22** **21** (*R*) - **22**

Scheme 1-9

This showed that the enzyme in yeast responsible for the reduction of **21** did in fact follow Prelog's rule. Sih *et al.*[23] also repeated the experiment with a different concentration of esters

and found that a different stereochemical profile was obtained, indicating that there is more than one enzyme present in the yeast catalysing this reaction.

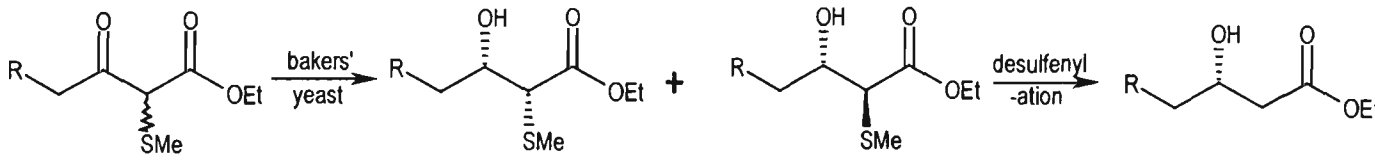
Hirama *et al.*[24] reduced the potassium salt **23**, rather than an ester, of a 3-oxoalkanoic acid with bakers' yeast (Scheme 1-10). Again the difference between the relative size of the groups either side of the carbonyl is increased, the carboxylate ion derived from the potassium salt being smaller than an alkoxy moiety. The product **24** was isolated and esterified with diazomethane to give **25** with excellent stereoselectivity (>99% ee). The enantiomeric ratios were determined by 360 MHz NMR using chiral shift reagents, which would appear to be at the limit for that methodology. The yields were moderate (38-59%).



23 **24** **25**

Scheme 1-10

Modifications were made to both the alkoxy moiety and at C_4 of the 3-oxoalkanoates in a different approach taken by Fujisawa *et al.*[25] A sulfenyl substituent was added to 3-oxoalkanoates at the C_2 position **26**. It was found that esters of 3-oxobutanoate with a 2-methylthio group are reduced by bakers' yeast in better yield than those containing a 2-phenylthio group. Addition of a 2-methylthio group to 3-oxopentanoates, gives the (*S*)-enantiomer **28** with high stereoselectivity (Scheme 1-11) while reduction of the unsubstituted keto ester gives the (*R*)-enantiomer in low optical purities (~40% ee).



26 **27** (threo) **27** (erythro) **28**

Scheme 1-11

The modified substrate resulted in the opposite enantiomer, as the smaller group was transformed into the larger group, and excellent stereoselectivity was obtained. Low yields were obtained for the desulfenylation step (R = CH_3, 23%; R = CH_2OCH_2Ph, 12%).

An excellent review of many of these modifications is presented by Sih and Van Middlesworth.[26] In general, substrate modification takes two forms; increasing the difference in the relative sizes of the groups either side of the carbonyl, essentially allowing for greater

discrimination between the *Re*- and *Si*-faces, and as a consequence improving the stereoselectivity; and swapping the relative size of the smaller and larger groups to obtain the opposite enantiomer. Modifying the substrate however will always involve two extra synthesis steps, adding the modifier before the yeast-mediated reduction, then removing it once the correct product is obtained. With some of the low yields quoted for yeast reductions, further synthetic steps could reduce the final product to impractical quantities.

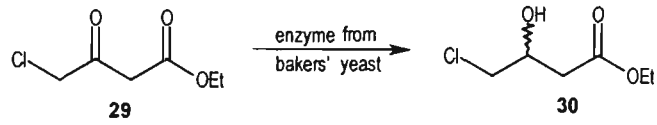
In order to determine why some reduction reactions are not as stereoselective as others, enzymes from the yeast have been isolated and characterised.

1.2.2 Isolated Enzymes and Diastereoselectivity

Bakers' yeast contains a complex mixture of enzymes and consequently is a good source for a variety of isolated enzymes. There are some very familiar enzymes like yeast alcohol dehydrogenase and fatty acid synthetase, but others have not been characterised or had their natural substrates determined. The two main research groups isolating and characterising enzymes from bakers' yeast, have been lead by Nakamura and Sih.

Nakamura *et al.* isolated eight enzymes from the cells of compressed bakers' yeast.[27,28] Three of these enzymes reduced α-keto esters, four reduced β-keto esters and one was yeast alcohol dehydrogenase. The molecular weight of each of the four β-keto oxidoreductases, and their activity towards the reduction of ethyl 4-chloro-3-oxobutanoate **29** were determined (Table 1-1).[28] This substrate was chosen for the investigation as the stereoselectivity of the yeast-mediated reduction of the compound was low (12%), implying that both the D- and L-enzymes displayed similar activity.

Table 1-1 Characteristics of the enzymes involved in the reduction of ethyl 4-chloro-3-oxobutanoate.[28]



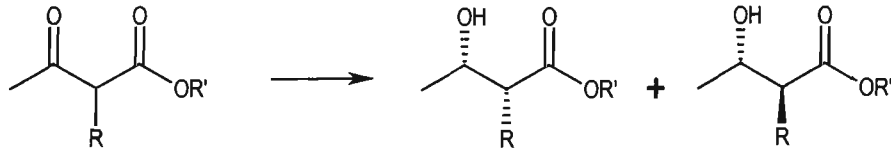
29 30

ENZYME	M_w (kDa)	TOTAL ACTIVITY*	CONFIG. OF PRODUCT	YIELD (%)	ee (%)
D-enzyme-1	25	26	D	45	>99
D-enzyme-2	1600	1442	D	84	>99
L-enzyme-1	32	246	L	62	>99
L-enzyme-2	32	3108	L	80	>99

*One unit of enzyme activity was defined as the amount of enzyme that catalysed the oxidisation of 1μmol of NADPH per minute at 30°C.

Of the four enzymes present, Nakamura *et al.* demonstrated that D-enzyme-1 contributed little (ca. 0.5%) to the reduction.[28]

Nakamura *et al.* did further work on the substrate specificity of the L-enzymes, 1[29, 30] and 2.[29] The two L-enzymes reduced alkyl 2-methyl (R=Me) and 2-allyl (R=allyl) 3-oxobutanoates **31** (Scheme 1-12).[29] The main difference in the stereoselectivity of the enzymes was at the 2-position with one producing *syn*-**32**, (2*R*, 3*S*) and the other *anti*-**32** (2*S*, 3*S*). L-Enzyme-1 produced the L-*syn*-hydroxy ester with exclusive stereoselectivity whereas the diastereoselectivity of L-enzyme-2 was dependant on both the structure of the alkoxy group and also the structure of the group at the C_2 position.



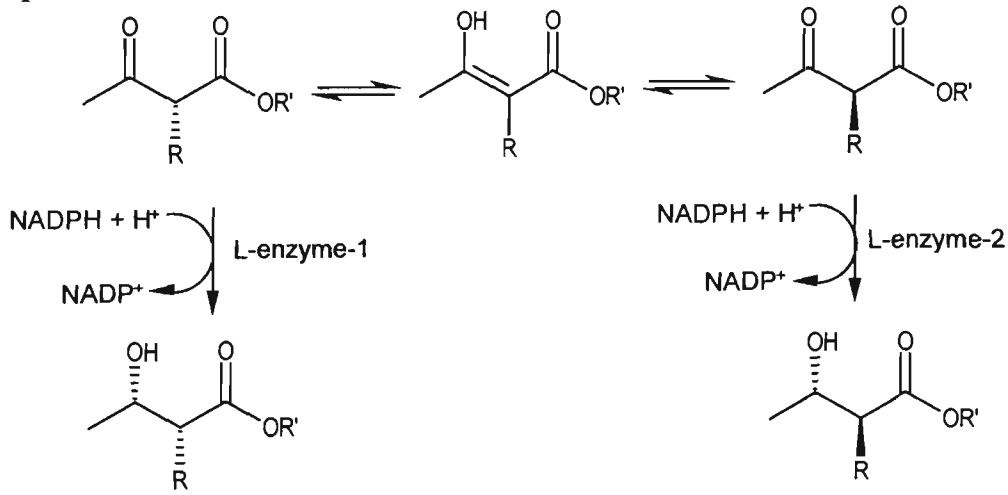
31 *syn*-**32** *anti*-**32**

Scheme 1-12

Sih *et al.* also isolated oxidoreductase enzymes from bakers' yeast[31] and performed a similar study which examined the substrate specificity of two L-enzymes by also reducing 2-alkyl-3-oxobutanoate esters **31**.[32] One enzyme, L-enzyme-1,[33] was the same as Nakamura's both in MW and substrate specificity however, the second enzyme had a different MW to Nakamura's L-enzyme-2, but was very similar in activity and substrate specificity. The MW of the second enzyme was approximately twice the MW of the L-enzyme-2 Nakamura isolated, suggesting the possibility of a dimeric form.

An earlier report by Nakamura *et al.* involving the yeast-mediated reduction of substrates similar to **31**, with variations in both the alkoxy group (R') and the group at C_2 (R) found that as the size of the substituent in the 2-position increased, the product changed from predominantly *syn* to *anti*.[34] Apart from esters containing a tertiary carbon attached to the alkoxyl oxygen, the 2-methyl butanoates (R=Me) gave the highest diastereomeric excess. The former compounds gave the *anti* product in very high diastereoselectivity. These results indicate that L-enzyme-1 predominates the reduction when there is a methyl group at the 2-position and as that group becomes larger, L-enzyme-2 becomes more active.

The production of optically pure 2-substituted 3-hydroxybutanoates **32** is very useful as these compounds contain 2 chiral centres as well as two functional groups that can easily be modified for the synthesis of various natural products.[30] With the purified enzyme, stereochemically pure compounds can be obtained. The starting material **31** stays as a racemic mixture due to the enolisation of the (2*R*)- and (2*S*)-enantiomers under the reaction conditions (Scheme 1-13). This allows for excellent resolution with the potential to convert all of the starting material into a single enantiomer of the desired product. This is in stark contrast to the alternative which involves resolution using a lipase where the reaction must be halted when conversion reaches 50%; the remainder is then racemised, if possible, and the procedure repeated.



Scheme 1-13

These studies have shown that the reason yeast can reduce such a wide range of substrates is because of the number of enzymes present with different substrate specificities. This also explains the variation in the stereoselectivity of yeast reactions, as different enzymes can act on the same substrate to give opposite stereochemistry. Although it might seem an insurmountable task to target one enzyme to obtain an enantiomerically pure product, each of

the enzymes have different properties which can be exploited in order to use yeast as a synthetically useful tool. The isolation of enzymes and elucidation of some of their properties has led to more targeted strategies being devised to achieve the desired product. Selective enzyme inhibition has been shown to work well on whole bakers' yeast cells and the inclusion of an additive has been an alternative way of controlling and improving the stereoselectivity of yeast-mediated reductions.

1.2.3 Incorporation of an Additive

The role of a additive falls into three main categories; an inhibitor that reduces the activity of the enzymes producing the unwanted enantiomer, an activator that increases the activity of the enzyme producing the desired enantiomer and a substance that alters the true or available concentration of the substrate.

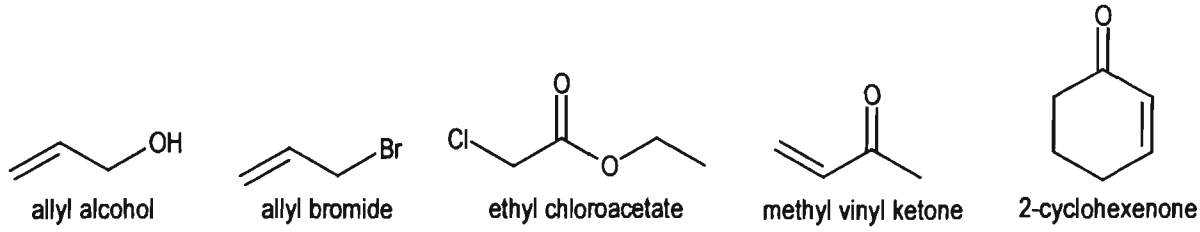
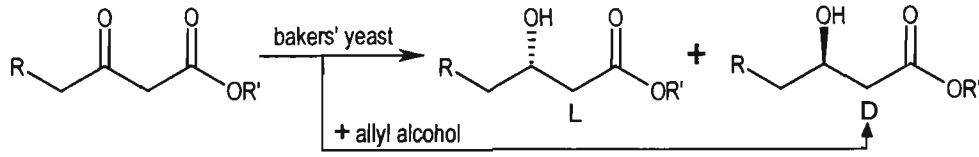


Figure 1-2 Some inhibitors of bakers' yeast-mediated reduction.

The addition of allyl alcohol (Figure 1-2) to the yeast-mediated reduction of 3-oxopentanoates greatly improved the relative production of the D-isomer, with some loss of yield (Scheme 1-14).[35]

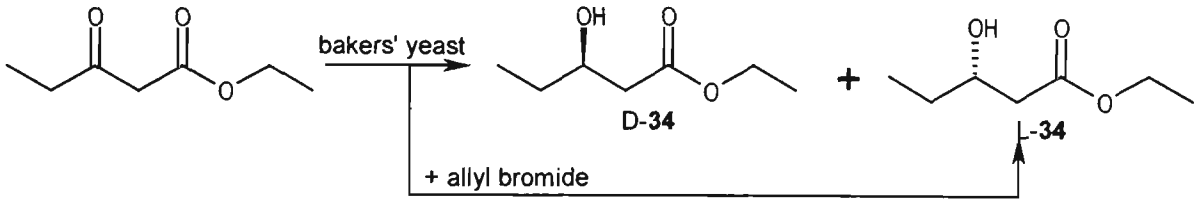


33 R=Me, R'=Me; **29** R=Cl, R'=Et; **17** R=H, R'=Me

Scheme 1-14

The stereoselectivity of the reduction of methyl 3-oxopentanoate **33** and ethyl 4-chloro-3-oxobutanoate **29** was improved, with only small quantities of the inhibitor (1g/L) required to approximately double the ee of the product. The stereoselectivity of the reduction of methyl 3-oxobutanoate **17**, which forms the L-isomer, was diminished. Although the most obvious assumption is that the L-enzyme is being inhibited, it is also possible that the D-enzyme is being activated.

Conversely, the addition of allyl bromide (Figure 1-2) to the yeast-mediated reduction system caused ethyl L-(*S*)-3-hydroxypentanoate **34** to be formed with ≥98% ee (Scheme 1-15).[36]



Scheme 1-15

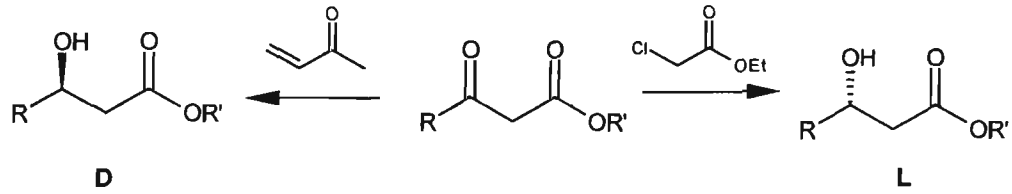
The allyl bromide was thought to covalently modify the enzyme, since increasing the preincubation time with the allyl bromide increased the stereoselectivity. Allyl bromide is quite reactive compared to allyl chloride which had no effect on stereoselectivity or yield.

A range of α,β-unsaturated carbonyl and hydroxy compounds were tested as inhibitors with methyl 3-oxopentanoate **33** as the substrate.[19] It was found that methyl vinyl ketone and 2-cyclohexenone (Figure 1-2) improved the stereoselectivity (60-70% ee) compared to reactions performed in the absence of an inhibitor (12% ee). Kinetic studies were also undertaken and it was found that the presence of the inhibitor decreased the production rate, indicating that in fact the methyl vinyl ketone was acting as an *inhibitor* towards the L-enzyme(s), not as an activator of the D-enzyme(s). Kinetic studies were also performed in the presence and absence of glucose with no inhibitor. Glucose increased the rate of reaction and improved the stereoselectivity of the reaction from 12% ee to 31% ee, indicating that glucose is an *activator* of the D-enzyme. The best carbonyl and hydroxy inhibitors were tested on five different β-keto esters and were found useful in preferentially obtaining the D-hydroxy esters. The reduction of ethyl 3-oxobutanoate in the presence of the same inhibitor however, did not result in the reversal of configuration since the stereoselectivity of the reduction of this substrate without additives is already strongly directed towards the L-enantiomer. The stereoselectivity of the reduction was reduced from 77% ee to 40% ee, effectively shifting the reaction about 40% towards the D-enantiomer, thus demonstrating the maximum effect of the additive.

The addition of α-haloacetates (Figure 1-2) has been shown to inhibit D-enzymes. Thus, the stereoselectivity of yeast-mediated reduction of β-keto esters was shifted towards the L-isomer.[37] The nature of the alkoxy group of the α-haloacetate is significant, with ethyl and butyl esters effective as inhibitors, but the allyl ester diminishing both the stereoselectivity and the yield of the reaction. This was most likely due to hydrolysis of the ester, releasing

allyl alcohol that, as discussed previously, inhibits L-enzymes. Both α-chloroacetates and α-bromoacetates gave excellent stereoselectivity, with the latter giving lower yields. Therefore, α-chloroacetates are the preferred inhibitors to shift the yeast-mediated reduction towards the L-enantiomer.

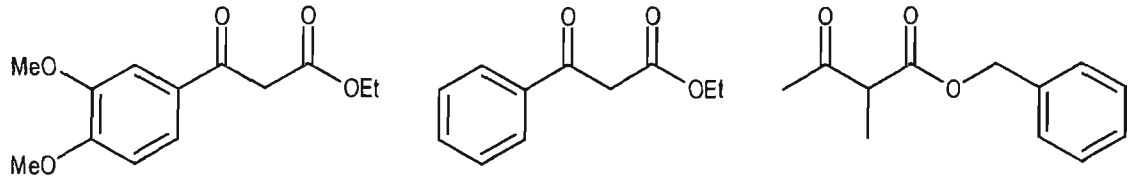
The activities of three of the four enzymes isolated by Nakamura *et al.* (see *Section 1.3.2*) were also examined in the presence of the inhibitors, methyl vinyl ketone and ethyl chloroacetate (Scheme 1-16).[38] D-Enzyme 1 was not included in this study because, as described earlier, it contributes very little to this reduction. Addition of methyl vinyl ketone resulted in the formation of the D-isomer, inhibiting both the L-enzymes. Alternatively, the addition of ethyl chloroacetate gave the L-isomer, inhibiting both D-enzyme-2 and L-enzyme-2. This confirmed the findings with whole yeast cells indicating that these are the main enzymes working in this reduction system.



D **L**

Scheme 1-16

The stereoselectivity of the reduction of ethyl 3-oxo-3-(3,4-dimethoxyphenyl)propionate **35** in a fermenting aqueous system was found to be 71% ee.[39] Four inhibitors were investigated using ethyl benzoylacetate **36** as a readily available model.

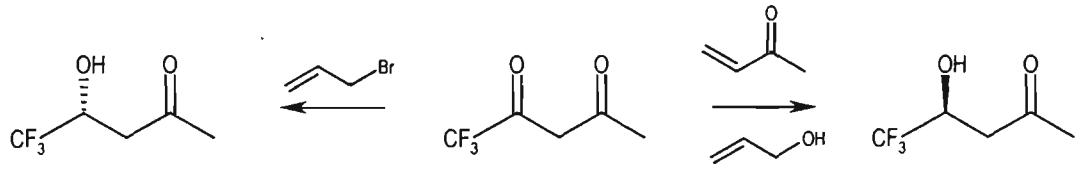


35 **36** **37**

Ethyl chloroacetate, methyl vinyl ketone, allyl alcohol and 2-cyclohexenone were applied to the model system and the best conditions were then applied to the substrate of interest. The inhibitor which worked best on the model compound **36**, completely halted the formation of any product from **35**, whilst the other inhibitors decreased the stereoselectivity rather than improving it. Furuichi *et al.*[40] isolated an enzyme from bakers' yeast that reduced a similar substrate to these, benzyl 2-methyl-3-oxobutanoate **37**.[40] The isolated enzyme appeared to have a very narrow substrate tolerance as no other compounds tested were reduced by the

enzyme, although neither compound **35** nor **36**, was tested. Although the two compounds, **35** and **36**, are structurally similar there may be two different enzymes responsible for their reduction. This could account for the lack of correlation between the inhibition of the reduction of **35** and **36**.

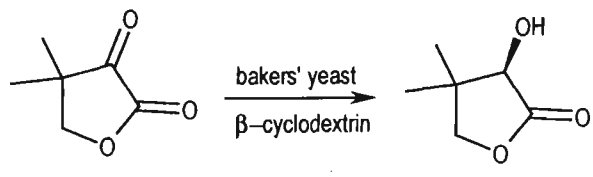
A successful use of inhibitors was achieved in the yeast reduction of fluorinated β-diketones **38** (Scheme 1-17).[41] Allyl alcohol and methyl vinyl ketone (Figure 1-2) increased the production of (*S*)-**39** (72% ee to 92% ee) whereas allyl bromide induced an inversion of configuration to (*R*)-**39** (81% ee). Other additives were also used such as acetic acid, fumaric acid and oleic acid but no significant effect was noticed.



(*R*)-**39** **38** (*S*)-**39**

Scheme 1-17

Addition of β-cyclodextrin to the yeast-mediated reaction of ketopantolactone **40** improved the stereoselectivity from 73% to 93% (Scheme 1-18).[42] Ketopantolactone fits into the cavity of β-cyclodextrin, therefore lowering the true concentration of the substrate in the bulk solution. This was confirmed when α-cyclodextrin, which does not bind ketopantolactone, was added and the enantioselectivity of the reaction was not affected.

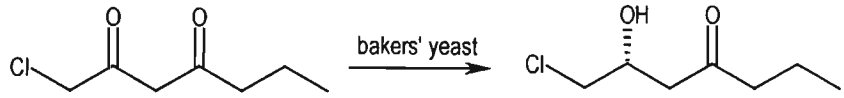


40 **41**

Scheme 1-18

Another additive that has been used to alter the concentration of the substrate is an organic solvent. Utaka *et al.*[43] used a combination of heat-treating the yeast, adding allyl alcohol as an inhibitor and adding a small amount (1·3%) of hexane or diethyl ether or both to enhance the stereoselectivity of the yeast-mediated reduction of 1-chloro-2,4-heptandione **42** (Scheme 1-19). This compound was chosen for the study as reduction in the absence of additives results in only 6% ee and therefore a shift to the (*R*)- or (*S*)-enantiomer would easily be seen. Trials were performed on combinations of the treatments and it was found that heat-treated

yeast, with allyl alcohol and a mixture of hexane and diethyl ether (total 1·3%) with dry bakers' yeast in a non-fermenting aqueous system gave (*S*)-**43** in 94%ee. This is an example of the use of a combination of methods to alter the stereochemistry of yeast-mediated reductions.



42 (*S*)-**43**

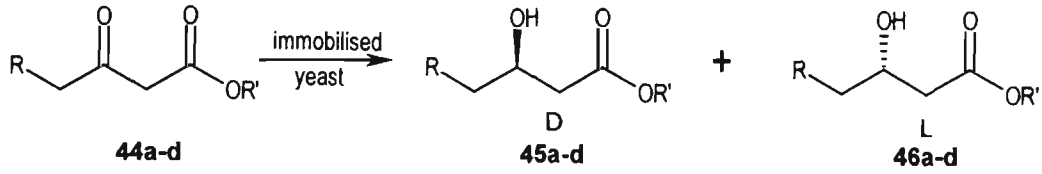
Scheme 1-19

D'Arrigo *et al.*[44] also employed organic solvents to improve the stereoselectivity of the yeast-mediated reduction of ethyl 3-oxobutanoate, although the amount of solvent was much greater than that used by Utaka *et al.* The best result obtained was with a 2:1 (v/v) ratio of hexane to water that gave the product in 95% ee. D'Arrigo's group also used another strategy to improve stereoselectivity; addition of resins that absorb organic compounds.[44, 45] A similar principle is behind the success of both of these methods, which is controlling the concentration of substrate available to the yeast in the aqueous phase.

1.2.4 Immobilisation

The ability to immobilise enzymes dramatically increased the feasibility of applying a much larger range of enzymes to organic synthesis. Immobilised enzymes have been used for a number of reasons but the principal advantages are that they are more stable and can be reused with minimal loss of activity. The added stabilisation is achieved by multiple attachment points preventing unfolding and denaturation of the enzyme. Immobilisation can increase stereoselectivity and prevent intermolecular inactivation processes, such as autolysis and aggregation.[46] The success of enzyme immobilisation encouraged the investigation of immobilisation of intact microorganisms.

Nakamura *et al.* used four different matrices to entrap bakers' yeast; calcium alginate, carrageenan, polyacrylamide and polyurethane.[47] Reduction of four β-keto esters at different substrate concentrations showed a significant effect was obtained with bakers' yeast entrapped in polyurethane (Scheme 1-20).



44a-d **45a-d** **46a-d**

a; R=Cl, R'=Et **b**; R=Cl, R'=Me **c**; R=H, R'=Et **d**; R=Me, R'=Me

Scheme 1-20

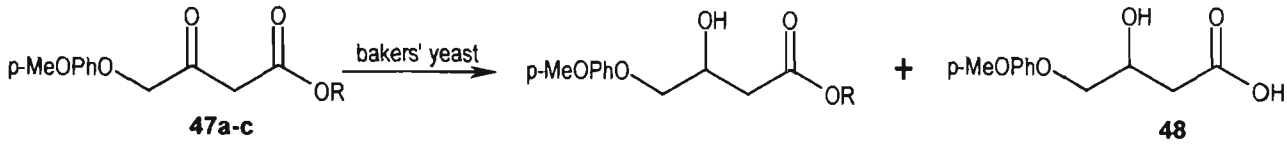
Free bakers' yeast reduction of **44a-d** resulted in poor stereoselectivity except for **44c** which resulted in 98% ee of the L-(+)-β-hydroxy ester **46c**. In the presence of immobilised yeast the stereoselectivity of the reduction was shifted towards the D-(-)-β-hydroxy ester **45a-d**, with the most dramatic shift occurring for yeast entrapped in the polyurethane matrix, for example **44b**; 12% ee_{46b} to 90% ee_{45b}. When **44c** was subjected to reduction with yeast entrapped in polyurethane the stereoselectivity decreased from 98% to 60% ee. Thus, polyurethane immobilisation can significantly increase the stereoselectivity of the reaction towards the D-(-)-β-hydroxy ester when the stereoselectivity is low, however, when the stereoselectivity is high, immobilisation of the yeast does not reverse the stereoselectivity of the reaction.

Another interesting property of immobilised yeast is the lack of dependence of stereoselectivity on substrate concentration. The stereoselectivity of the reduction of **44a** with free bakers' yeast at 10mM and at 50mM of substrate decreased from 42% ee to 15% ee, compared to 82% ee at both concentrations with yeast entrapped in polyurethane. This was reported to be due to the support controlling the amount of substrate in contact with the yeast, effectively keeping the substrate concentration at an optimum level.

By changing the immobilisation support to magnesium alginate and adding 4M $MgCl_2$ the yeast-mediated reduction of methyl 3-oxobutanoate **17** gave L-(+)-β-hydroxy ester in 89% ee and 44% yield.[48]

An excellent application of the use of immobilised fermenting bakers' yeast was demonstrated by Guanti *et al.* in the production of chiral building blocks in the synthesis of pharmacologically important β-lactam antibiotics (Table 1-2).[49] The yeast was immobilised onto calcium alginate beads and slow addition of the substrate increased the stereoselectivity. In the case of **47a**, the addition of ethyl chloroacetate suppressed the production of the hydroxy acid **48**. The potassium carboxylate **47c** was reduced to form (*S*)-**48** which was smoothly converted into the methyl ester, giving access to both enantiomers of the hydroxy ester with good yields and excellent stereoselectivity.

Table 1-2 Bakers' yeast reduction of three 4-(4-methoxyphenoxy)-3-oxobutanoates.

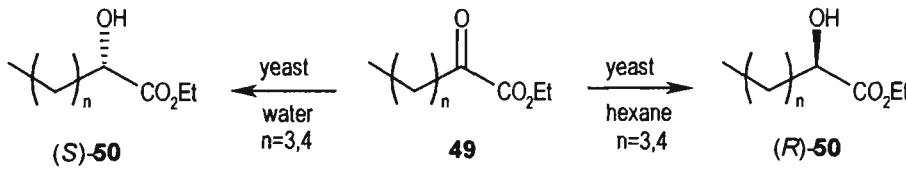


47a-c 48

	R	Hydroxy ester Yield %(ee%)	Hydroxy acid Yield %(ee%)	Configuration
a	Et	61 (89)	17 (n.d.)	*R*
b	Bn	69 (91)	n.d.	*R*
c	K	-	70 (90)	*S*

n.d. – not done

Nakamura *et al.* immobilised bakers' yeast on polyurethane prepolymer and compared the reduction properties with free bakers' yeast (Scheme 1-21).[50] An homologous series of α-keto esters **49** was reduced and isolated yields and enantiomeric ratios were calculated. Immobilisation of the yeast had little effect on the chemical yield (20-43%) or on the configuration of the product (*S*). The stereoselectivity was increased only for the larger esters (n= 3, 4), however the increase was not sufficient for the reaction to be useful for synthetic purposes.



(*S*)-**50** **49** (*R*)-**50**

Scheme 1-21

A comparison was also made with the immobilised yeast reduction in hexane.[50] In this case, the organic solvent was the bulk solvent rather than an additive or part of a biphasic system. The yields were still moderate (27-41%) and there was very little difference in the stereoselectivity between the three systems when n=0,1,2. The configuration was reversed to (*R*) however, when n=3,4 in the hexane system (Scheme 1-21). The change in configuration was due to the absence of stereoselective hydrolysis, which occurs under aqueous conditions. Unfortunately, the starting material was reported to be unstable with the immobilised yeast in hexane and since longer reaction times were required, the yields were still low.

Immobilisation had some effect on stereoselectivity, however the advantages of reuse and increased stability do not have the same importance with yeast as with enzymes. Reuse is not an issue when the cost of yeast is minimal and the enzymes in yeast are in effect already protected and supported by the cell.

1.2.5 Organic Solvents

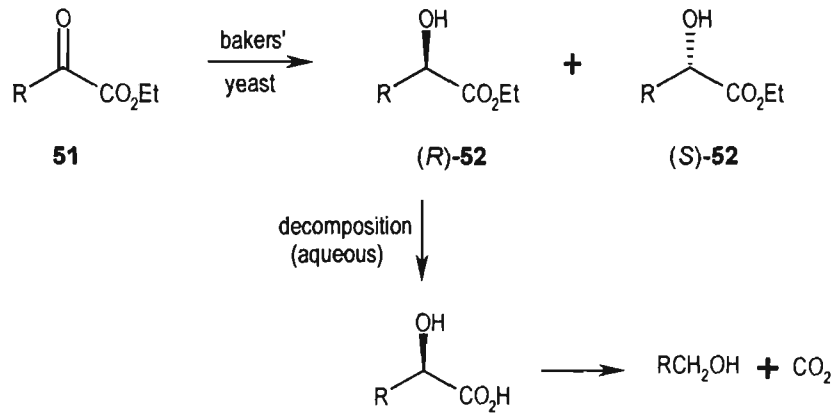
The use of bulk organic solvents with little or no water for biotransformations has been well reviewed for enzymes,[51,52,53,54] however little emphasis has been placed on whole cell systems. Nikolova and Ward reviewed whole cell biocatalysis in organic solvents and discussed choice of solvent, solvent toxicity, effect of water content, immobilization and briefly covered stereoselectivity.[55]

One of the main problems perceived with using whole cells in organic solvents is that the cell cannot survive in such an unnatural environment. Although this is true, there is no requirement for viable cells as long as the enzymes remain active. In the case of reduction reactions, the amount of NADPH recycling is limited by the amount of substrate (glucose) already present in the dried yeast. For this reason and because the yeast is not multiplying in the organic solvent the yeast to substrate ratio is very important.[56] This is in contrast to an aqueous system.

There is a requirement for the presence of a small amount of water for any reaction to occurin an organic solvent. The choice of solvent is therefore very important, as hydrophilic solvents have been shown to strip isolated enzymes of their water.[57] Hydrophobic solvents (such as hexane, toluene and petroleum ether) do not exert the same effect. When dried bakers' yeast is used, a roughly equivalent quantity of water is needed to hydrate the yeast. The yeast forms a viscous, sticky mass at the bottom of the reaction vessel, with the supernatant solvent containing both product and starting material. Consequently, isolation of the product is simply a matter of decanting the liquid and rinsing the vessel containing the yeast with a small quantity of fresh solvent. With an aqueous system, the yeast is finely dispersed in the medium along with the product, and it is necessary to employ extractions, which are usually time-consuming and messy, to isolate the product. Higher yields can therefore generally be obtained when an organic solvent is used.

Nakamura *et al.*[58] reduced α-keto esters **51a-c** (Scheme 1-22) using benzene as a solvent, instead of hexane which was the solvent used with immobilised yeast system, claiming that better stereoselectivity is achievable. The stereochemistry of the yeast-mediated reductions in benzene was shifted towards the (*R*)-product, therefore better stereoselectivity was achieved only for substrate **51b** ((*R*)-**52b**, 90% ee), which also gave the (*R*)-enantiomer in water ((*R*)-**52b**, 19% ee). Mechanistic studies of the reduction of α-keto esters in benzene and hexane by

bakers' yeast and reductase enzymes purified from bakers' yeast have been reported.[59, 27] The observed change in stereoselectivity is not only a change in the reduction reaction, but also a change in the stereoselective decomposition that occurs after reduction has taken place. The decomposition of (*R*)-**52** was demonstrated by placing the racemate in an aqueous dry bakers' yeast reaction and both the (*S*)-hydroxy ester and hexanol were recovered, hexanol being the decomposition product from (*R*)-**52c** (Scheme 1-22).

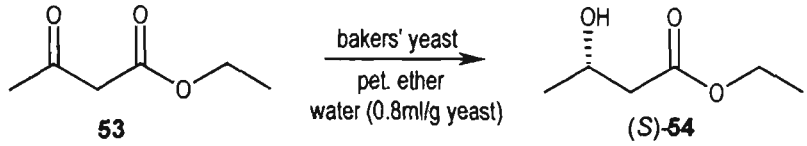


a: R=CH_3 **b**: R=$(CH_3)CH$ **c**: R=C_5H_{11}

Scheme 1-22

Reduction of **51c** in benzene was the only reaction that produced a marginally better yield than reductions performed in water (9% water, 26% benzene). However the yields reported were gas chromatographic conversions rather than isolated yields and one of the main advantages of an organic solvent system is the ease of isolation compared with the messy extractions required for aqueous systems.

Petroleum ether was the organic solvent of choice for the yeast-mediated reduction of a β-keto ester, ethyl 3-oxobutanoate, **53** (Scheme 1-23).[60] The stereoselectivity of the reaction was 94% ee, determined by optical rotation. A later report by the same group cited a higher stereoselectivity (98% ee) which was determined by chiral gas chromatographic analysis of the trifluoroacetyl derivative.[61] Complete consumption of starting material, as determined by gas chromatography, occurred with 1g yeast/ mmole substrate with moderate isolated yields (58%). The same reaction was also performed in toluene, carbon tetrachloride, and diethyl ether with more yeast required to effect complete conversion. The stereoselectivity of the reaction performed in toluene or carbon tetrachloride was similar to that found in petroleum ether; in diethyl ether the stereoselectivity was lower (96% ee). The isolated yields were also similar.



Scheme 1-23

North[62] followed the protocol set out by Smallridge and co-workers to reduce the β-keto esters **53, 17, 36, 56-58** (Figure 1-3). Some optimisation was performed by varying the quantity of yeast in the reactions. Excellent stereoselectivity (>98% ee) was achieved for all substrates, other than **57** and **36**. The (*R*)-alcohol was the predominant isomer (40% ee) resulting from the reduction of **57** in an aqueous system, however the (*S*)-alcohol was the major isomer formed in petroleum ether (45% ee). The ester **36** was not reduced at all in petroleum ether. Rotthaus *et al.*[63] also performed yeast-mediated reductions of **53, 56, 57** and **29** in a variety of organic solvents.

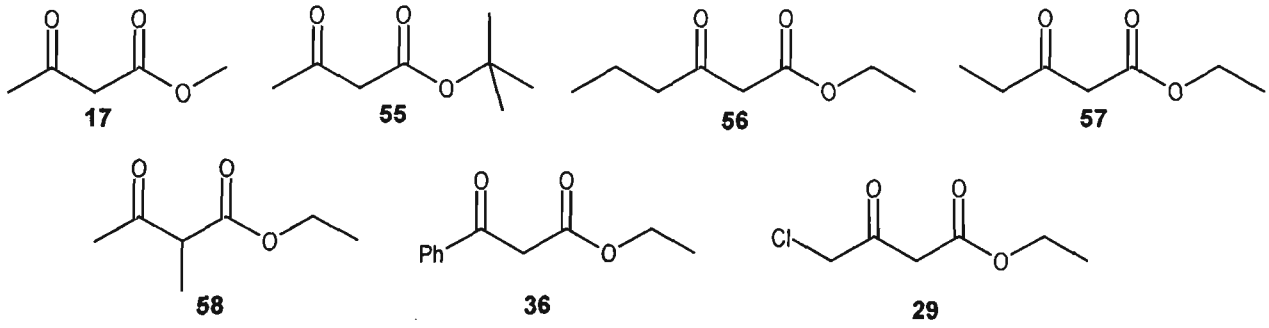


Figure 1-3 Esters used in the yeast-mediated reduction reactions performed by North[62] in petroleum ether.

The ratio of water, yeast, substrate and solvent are more critical under non-fermenting conditions since the yeast is not growing and the metabolites are not being recycled. Nakamura's group, North and Smallridge's group found that 0·8ml water /g yeast was required whereas Rotthaus *et al.* used only half this amount. Toluene and hexane were found to be better solvents than ethyl acetate and diethyl ether. This confirms the observation of Smallridge and co-workers, that non-polar solvents are more suited to yeast-mediated reductions than polar solvents.[61]

1.3 Aims

Much of the reported investigation of yeast in organic synthesis has been focussed on the control of the stereoselectivity in aqueous systems. Yeast reactions in an organic solvent is a relatively new development with a great deal of potential, however the yields vary markedly as does the quantity of yeast required for complete consumption of substrate.

The aim of the present project is to examine some of the mechanisms involved in yeast-mediated reactions. This will include an investigation of the effects of changing the reaction conditions such as temperature, yeast to substrate ratios and yeast pretreatment. The result of varying these parameters will be determined by changes in conversion, isolated yields and stereoselectivity. By following the kinetics of the reaction, information can be gained on optimum reaction conditions. Since conducting yeast-mediated reactions in organic media is a relatively new technique, the scope of possible reaction types has not been established. This study will attempt to broaden the scope of yeast reactions in an organic solvent by investigating esterification reactions.

Chapter 2 compares the yields and yeast requirements of the yeast-mediated reduction of six esters of 3-oxobutanoates. In Chapter 3 the yeast-mediated reduction of ethyl, isopropyl and benzyl 3-oxobutanoate are followed by NMR spectroscopy to examine their reaction kinetics. Chapter 4 investigates a reaction not previously reported using yeast in an organic solvent; esterification of carboxylic acids.

CHAPTER 2

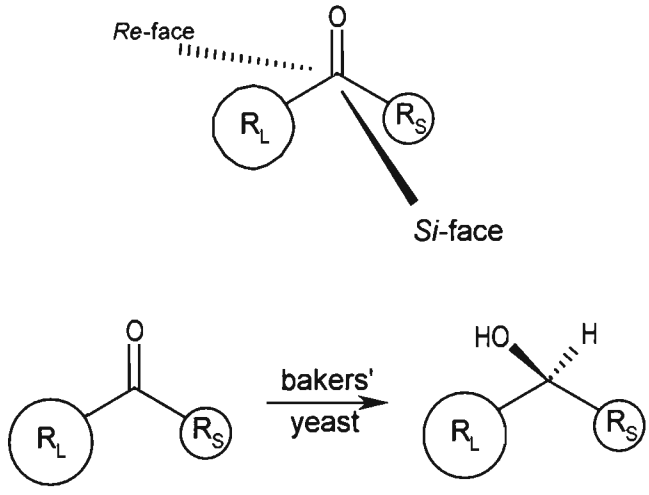
YEAST-MEDIATED REDUCTION OF 3-OXOBUTANOATES

2. Yeast-mediated Reduction of 3-Oxobutanoates

2.1 INTRODUCTION

The first report of the yeast reduction of an organic molecule was the transformation of furfural to furfuryl alcohol.[64,65] Since then the reductive properties of yeast have been applied to a wide variety of substrates, especially those that can be used as chiral precursors to pharmaceutical products.[66,10,67] Much of the interest in yeast-mediated reductions has been in the control of the stereochemistry of the final product.

This feature is well explained by Prelog's rule which states that an enzyme site has shape limitations which distinguishes between the *Re*- and *Si*-face of a molecule by the relative size of groups either side of the π-system (Scheme 2-1).[68] Changing the relative size of the groups either side of the carbonyl being reduced changes the face to which the hydrogen is transferred.



Scheme 2-1

Prelog's rule can not be exclusively applied to whole yeast cells as there are a number of active enzymes, some of which have similar catalytic action but result in opposite stereochemistry.[69] The reason that good selectivity still occurs is due to the difference in the optimum operating conditions of the competing enzymes (such as pH, substrate concentration (K_M), temperature).

The orientation of the substrate in the binding site and the effect it has on the stereochemistry of the reaction has been studied quite extensively.[23, 70] However, studies of size *vs* rate of reduction can show how well the substrate binds to the enzymes' active site/s. This does not involve changing the relative size of the groups either side of the carbonyl being reduced (Prelog's rule) but instead changing the size and shape of the larger group.

For esters of 3-oxobutanoate the ester moiety is the larger group and changing the ester can provide information regarding the shape of the active site of the enzyme/s involved in the reduction, without changing the stereochemistry of the reaction. This can further contribute to better substrate design that will result in more efficient reduction with the desired stereochemistry. It can also aid in the predictability of suitable substrates for yeast reduction reactions.

In order to investigate the relationship between ester size and reduction rate, a range of esters of 3-oxobutanoate were subjected to yeast-mediated reduction in petroleum ether (Figure 2-1). The esters chosen had different steric and electronic properties, ranging from methyl to butyl to benzyl.

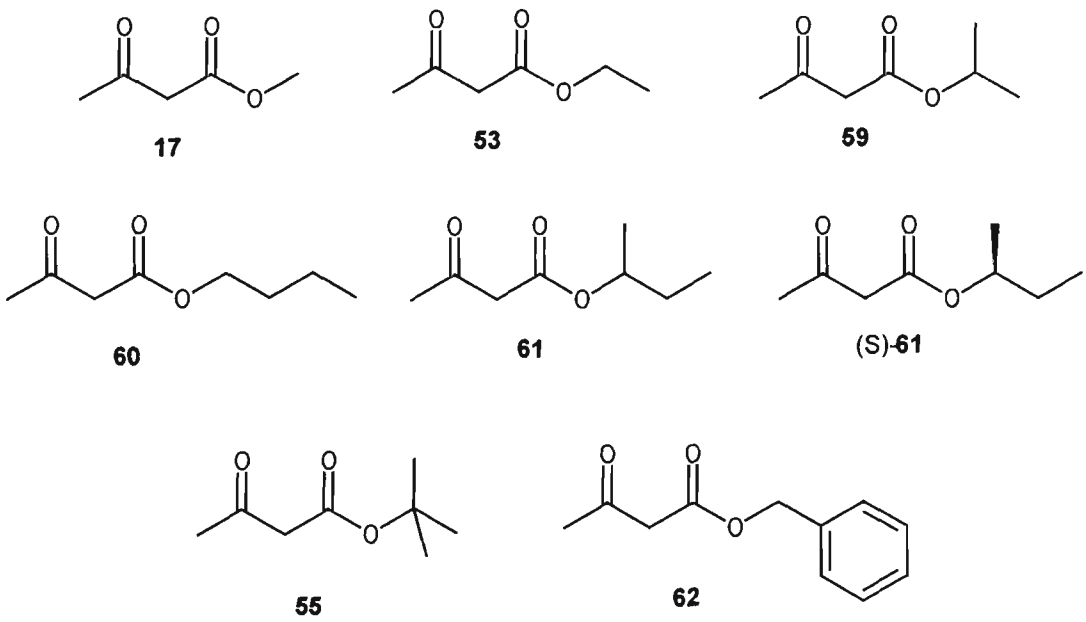


Figure 2-1 The esters of 3-oxobutanoate exposed to yeast in the study

2.2 PREPARATION OF ESTERS

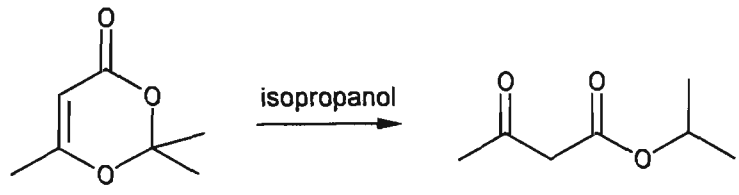
The methyl, ethyl, *tert*-butyl and benzyl esters are available commercially, hence only the isopropyl, butyl and *sec*-butyl esters were prepared. Transesterification is the most obvious

route to form the esters as ethyl 3-oxobutanoate is readily available, however there are problems with this approach;

i. keto-enol tautomerism stabilises the molecules making them less reactive than simple esters,
ii. decarboxylation of the ester may occur,
iii. the presence of two carbonyl groups gives the alcohol two points of attack resulting in the formation of ketals and enol ethers.

2.2.1 Isopropyl 3-oxobutanoate

Initial attempts to transesterify ethyl 3-oxobutanoate with isopropanol were unsuccessful. Use of hydrochloric acid and p-toluenesulphonic acid as catalysts returned only starting material. A general method to make esters of 3-oxobutanoates is by the reaction of an alcohol with diketene,[71] however since diketene is unavailable in Australia, an alternative approach was required. The acetone adduct of diketene, 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one **63**, is available and is used in the preparation of 3-oxobutanoate esters.[72] The isopropyl ester was prepared from **63** and isopropanol with a 50% conversion, where the remainder was the acetone adduct of diketene **63** (Scheme 2-2).



63 **59**

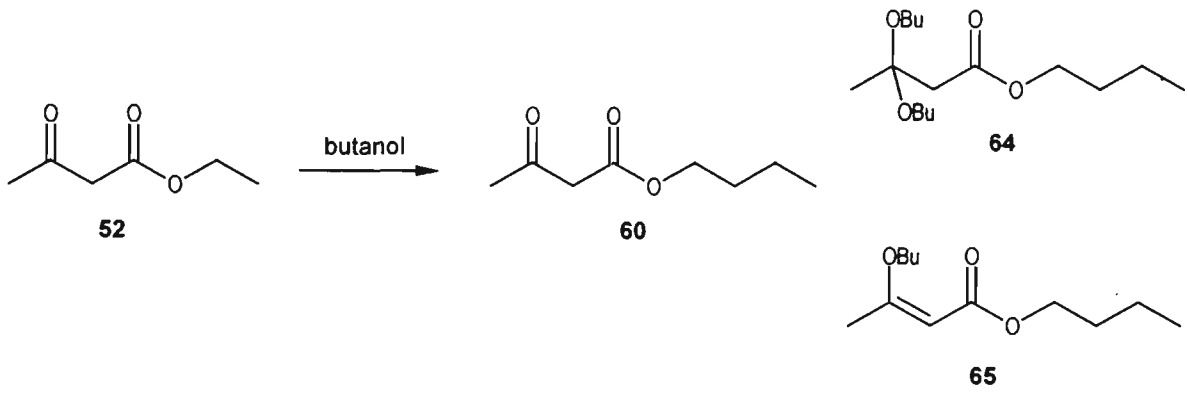
Scheme 2-2

Removal of the excess acetone adduct proved to be difficult by distillation or chromatography, therefore the desired ester was isolated as the copper chelate.[73] Subsequent treatment with sulphuric acid gave the pure compound in poor yield (11%).

In contrast, it was found that performing the reaction in a sealed vessel resulted in complete consumption of the starting material after 27 hours, presumably due to the increased pressure and better retention of the isopropanol. Formation of the copper chelate was therefore unnecessary, resulting in a good yield of the isopropyl ester (64%).

2.2.2 Butyl 3-oxobutanoate

Attempts to transesterify ethyl 3-oxobutanoate with butanol using p-toluenesulphonic acid as a catalyst gave complete consumption of starting material, however a mixture of products was isolated (Scheme 2-3). Attack at both carbonyl groups had occurred, giving the ketal **64** and enol ether **65** along with the desired butyl ester **60**.

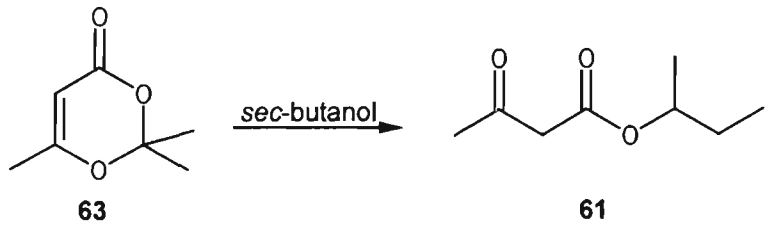


Scheme 2-3

Attempts to separate these compounds were unsuccessful. To overcome this problem, the acetone adduct of diketene **63** was again employed. Although this strategy prevented the production of the enol ether **65**, there was still about 5% of the ketal **64** present in the final product. Attempts to remove the ketal **64** using the copper chelate gave an oil rather than crystals and resulted in poor yield (3%), which still contained 5% ketal. Hydrolysis of the ketal with acetic acid in THF was also unsuccessful.[74] The mixture containing 5% ketal was therefore used for the yeast reactions.

2.2.3 sec-Butyl 3-oxobutanoate

The *sec*-butyl ester **61** was also prepared from the acetone adduct. In this case, complete conversion occurred and, after distillation, pure product was obtained in a 72% yield (Scheme 2-4). The (*S*)-*sec*-butyl ester was prepared in the same manner to give a pure product in 47% yield.



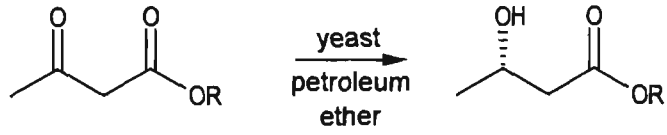
Scheme 2-4

2.3 YEAST REDUCTIONS

The 3-oxobutanoate esters were subjected to yeast-mediated reduction using the conditions previously reported to be optimum for the reduction of ethyl 3-oxobutanoate **53**: 1mmol substrate, 1g yeast, 0·8ml water, 50ml petroleum ether at room temperature for 24h.[61]

For the majority of the esters, 1g of yeast was insufficient to effect complete reduction. Longer reaction times did not lead to increased conversion due to a significant decrease in yeast reductase activity after exposure to the reaction system for 24 hours. This was demonstrated by stirring yeast in petroleum ether with 0·8ml water for 24 hours prior to the addition of substrate (ethyl 3-oxobutanoate **53**) and then allowing the reaction to proceed for a further 24 hours. Very little reduction (13%[¶]) was observed in this case compared to complete consumption of substrate if no pretreatment was performed. Reactions utilising increasing amounts of yeast were therefore conducted to determine the minimum amount of yeast required for the complete consumption of starting material. Removal of the yeast by filtration, followed by washing with ethyl acetate and distillation gave high to near quantitative yields of the *(S)*-3-hydroxy esters (Table 2-1).

Table 2-1 - Yields and stereoselectivity of the yeast-mediated reduction of a series of 3-oxobutanoate esters.



66-72

COMPOUND (SUBSTRATE/PRODUCT)	R GROUP	YEAST (g/mmol)	ISOLATED YIELD (%)	ee[b] (%)
17/66	methyl	1	57	98*(S)*
53/67	ethyl	1	69	99*(S)*
59/68	isopropyl	2	96	97*(S)*
60/69	butyl	3	89	>99*(S)*
61/70	*sec*-butyl	4	89	97*(S)*[a]
55/71	*tert*-butyl	11	68	98*(S)*
62/72	benzyl	5	72	94*(S)*

[a] ee at the hydroxy centre, no enantiomeric excess was observed at the ester centre.
[b] determined by chiral gas chromatography of the trifluoroacetates.

[¶] Ratio of product to starting material as determined by gas chromatography.

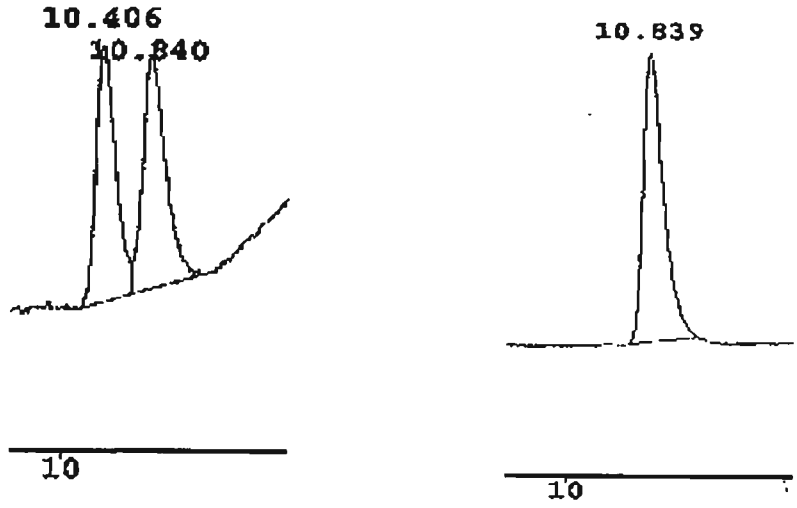


Figure 2-2 Chiral gas chromatogram of racemic and isolated butyl 3-hydroxybutanoate.

2.3.1 Stereoselectivity

In all cases chiral gas chromatography indicated that the reduction proceeded with a high degree of stereoselectivity (94-99% ee, Table 2-1) to give the *(S)*-β-hydroxy esters. The preferential formation of the *(S)*-enantiomer, as determined by specific rotation, is consistent with reported reductions of these esters utilising both aqueous and organic reaction systems although the enantioselectivity achieved is generally higher than that reported using the other systems.

Rotthaus *et al.*[63] reported the yeast-mediated reduction of ethyl 3-oxobutanoate in a range of solvents (Table 2-2). In hexane, they obtained an ee of 93%, which is substantially lower than the 99% obtained here in petroleum ether. The significantly lower cost and reduced occupational exposure hazard associated with petroleum ether led to its routine use in this laboratory, since no difference was detected in the yield or ee of the product when the reaction was performed in petroleum ether, compared with hexane. The reason for the lower enantioselectivity observed by Rotthaus *et al.* is therefore probably related to the yeast preparation used in their study since they also reported a lower ee in diethyl ether than was previously reported using our yeast preparation.[61]

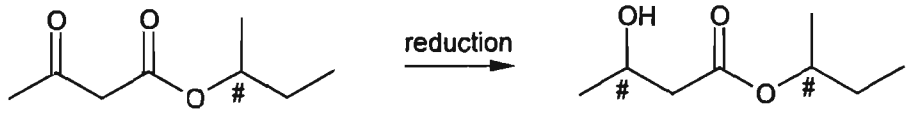
Table 2-2 Comparison of various solvents for the yeast-mediated reduction of ethyl 3-oxobutanoate using the ee of ethyl 3-hydroxybutanoate.

SOLVENT	ee[a]	ee[b]
Hexane	93%	99%[c]
Toluene	100%	98%
Diethyl ether	77%	96%
Ethyl acetate	57%	[d]
Water	96%	

[a]reported by Rotthaus *et al.*[63]; [b]reported by Smallridge and co-workers[61]; [c]reaction in pet ether 40-60; [d]no reaction.

North[62] incompletely reduced methyl, ethyl and *tert*-butyl 3-oxobutanoate with yeast in petroleum ether and recovered a mixture of starting material and product. He measured the specific rotation of each isolate and corrected the result for the amount of starting material present; comparison of the corrected value with reported specific rotations for each of the esters led him to conclude that the reduced products had ee values >98%. Although North's approach was somewhat indirect, his results were in close agreement to the ee values obtained by direct measurement (using chiral gas chromatography) in the present study. This suggests that North's yeast had similar behaviour to that used in this work.

sec-Butyl 3-oxobutanoate **61** already has one chiral centre and reduction to the corresponding alcohol creates a second chiral centre producing diastereomers (Scheme 2-5); the product has the potential to exhibit four stereoisomers.



61 # - chiral centre **72**

Scheme 2-5

Yeast reduction of the *sec*-butyl ester **61** gave high enantioselectivity at the 3-hydroxy centre, but showed no evidence of diastereoselectivity. Incomplete reduction (62% conversion by gas chromatography) of *sec*-butyl 3-oxobutanoate was achieved using 1g of yeast/mmol and examination of the product using chiral gas chromatography showed no evidence of diastereoselectivity.

To confirm whether yeast-mediated reduction had a preference for a single enantiomer, (*S*)-*sec*-butyl 3-oxobutanoate was subjected to yeast reduction conditions. It was found that the

(*S*)-*sec*-butyl ester did not react with yeast any differently than the racemic material and required the same amount of yeast for complete reduction. This indicates that both enantiomers of the β-keto ester were reduced by the yeast at a similar rate that is consistent with reported results for this compound using an aqueous reaction system.[75]

2.3.2 Yields

In all cases, the reaction went to completion as evidenced by the absence of starting material in the gas chromatographic analysis of the reaction mixture. The product was isolated from the reaction mixture, and as seen in Table 2-1, the yield of isolated material varied considerably with the nature of the ester group. The methyl, ethyl, *tert*-butyl and benzyl esters gave good yields, 57-72%, whilst virtually quantitative yields were obtained for the isopropyl, butyl and *sec*-butyl esters.

The yields obtained in this study are generally better than those reported for reduction with fermenting yeast in an aqueous system. (*S*)-Ethyl 3-hydroxybutanoate has been prepared by Seebach *et al.*[76] from an aqueous fermenting yeast suspension in a 59%-76% yield which is comparable to the yield achieved here (69%). Spiliotis *et al.*[77] also reduced the ethyl ester but with the yeast enclosed in a dialysis tube to facilitate product isolation, however a lower yield was obtained (48%). The butyl ester also was reduced by Spiliotis *et al.*[77] in the same manner with a yield of 50%, which although similar to Seebach and Züger's[78] result of 58%, was considerably lower than that obtained in the present study (89%). The *tert*-butyl ester has also been reduced in fermenting systems by Seebach and Züger[78] in good yield (61%) and by Hirama *et al.*[24] in moderate yield (45%), both lower than that obtained in petroleum ether (68%). Hirama *et al.*[24] reduced the methyl ester with a yield of 23% which is about half of what was achieved here (57%). Hudlicky, Tsunoda and Gadamasetti[75] reduced the *sec*-butyl ester in an aqueous system and obtained a 69% yield compared to the 89% obtained in petroleum ether in the present study. The aqueous yeast reduction of the isopropyl or benzyl esters has not been reported. These examples are typical of the results reported for aqueous yeast reduction and show that not only is the tedious isolation procedure avoided by conducting the reaction in organic solvents but the yields are comparable in the case of the ethyl and *tert*-butyl esters and considerably better with the remainder of the esters.

The yields obtained in this study are also generally better than those reported for reduction with yeast in an organic solvent system. There have been relatively few reports of the non-

immobilized yeast reductions of keto esters in organic solvents, with the most common solvents being petroleum ether,[60, 62] diethyl ether[79] and benzene.[58] A somewhat unusual organic solvent used for yeast reduction was isopropyl palmitate with asolectine (soybean phospholipids) as surfactant.[80] *tert*-Butyl 3-oxobutanoate was reduced in this system with a good isolated yield (72%), marginally better than that obtained in the present study (68%). The yeast was removed by centrifugation and the product was distilled from the supernatant and isolated after a second distillation step. Whilst the yield obtained in the present study using petroleum ether is slightly lower, the extraction method employed, is simpler than that described with isopropyl palmitate and asolectine.

North[62] reduced the ethyl, methyl and *tert*-butyl esters in petroleum ether using dried bakers' yeast with some success. He found that 1.5g yeast/mmol of substrate was required to attain complete conversion for the ethyl and methyl ester whereas only 1g/mmole was necessary in the present study; the difference in yeast requirements is probably due to different yeast preparations. The isolated yields obtained were 38% for the ethyl ester and 15% for the methyl ester, both considerably lower than those obtained here, 69% and 57% respectively. With the *tert*-butyl ester complete consumption of the starting material was not achieved as the maximum amount of yeast included in the reaction system was 4.7g/mmol whereas we have found that the *tert*-butyl ester requires 11g/mmol for complete conversion.

Rotthaus *et al.*[63] reduced ethyl 3-oxobutanoate in hexane, toluene, diethyl ether and water and although gas chromatographic conversions were reported, isolated yields were not. In the present study the reaction conditions were considered to be optimised when gas chromatographic analysis showed complete consumption of starting material (100% conversion in the terms of Rotthaus *et al.*). However as Table 2-1 shows, complete consumption of starting material can result in yields that vary from 57%-97%; the gas chromatographic conversions give no indication of the amount of useful product that can be isolated from the system.

Whilst the yields achieved using our solvent system are better than those previously reported, there are large differences between the yields obtained for different esters. The reason for the marked variation in the yields is not clear, the differences could originate from a number of causes: irreversible binding to the yeast, decomposition of the starting material or the product, ease of extraction of the product from the yeast biomass into the solvent.

The variation in isolated yields does not appear to be due to adsorption of substrate or product onto the yeast since similar yields are obtained using 1g yeast/mmol (**53**, 69%), and 11g yeast/mmol (**55**, 68%). If material was being adsorbed onto the yeast it would be expected that the greater the amount of yeast employed the more material would be adsorbed and the lower would be the isolated yield. Consistent with this idea, North[62] found that doubling the amount of yeast used for the reduction of the methyl ester, actually did decrease the yield by 83%. However in the case of the methyl ester the reduced product is highly volatile and it is likely that the isolation procedure described by North may have led to unforeseen losses. He commented that in the case of the tert-butyl ester more material was retained when large amounts of yeast (up to 60g) were used. In our experience considerably more ethyl acetate is required to completely extract material from the yeast than was used by North. This could account for the lower recovery of material with larger amounts of yeast.

Decomposition by yeast catalysed ester hydrolysis is also unlikely to be the cause of the difference in isolated yields as no trace of liberated alcohol could be detected in the reaction mixture using gas chromatography and ^{13}C NMR (*See Chapter 3*).

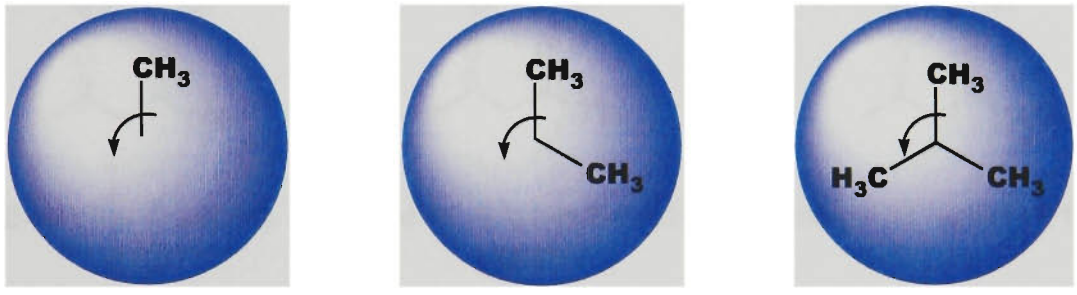
The trend in the yield variation indicates there is an optimum size for the ester group in order to obtain a quantitative yield, since the larger and smaller esters give the lower yields. The ethyl and methyl, having yields of 69% and 57% respectively, are the smaller ester groups, possibly small enough to fit into other enzyme sites, resulting in them being irretrievably bound. Whereas the *tert*-butyl and benzyl esters are much larger, possibly being trapped in the active site, unable to return to the solvent to be isolated. This effect may be contributing to the lower yields of 68% and 72% respectively. The isopropyl, butyl and *sec*-butyl may be just the right size to fit the enzyme pocket and also be mobile enough to move back into the solvent. Therefore it appears that to obtain excellent yields of 89-96%, even though a little more yeast is required, the ester group should be an alkyl group containing 3 or 4 carbon atoms.

2.4 SUBSTRATE REACTIVITY

The amount of yeast required to effect complete consumption of starting material (as measured by gas chromatography) increased with the size of the ester group. The smaller methyl and ethyl esters are reduced with just 1g of yeast whilst the more sterically

demanding *tert*-butyl group requires 11g. The requirement for increased amounts of yeast strongly suggests that the compounds with the larger ester groups react at a slower rate. One possible reason is slower substrate trans-membrane transport. The only way to determine whether trans-membrane kinetics are a factor would be to perform comparative studies with isolated enzymes. Another possibility is increased steric interactions between the larger ester group and the enzyme binding site.

It is anticipated that the keto and carboxyl groups will be held in a conformation dictated by the enzyme active site, irrespective of the alkyl moiety. The alkyl moiety has free rotation about the carbon-oxygen bond and is flexible enough to adopt a conformation to suit the available space. The ethyl, isopropyl and *tert*-butyl groups have the same swept volume when free rotation is considered (Figure 2-3).



ethyl isopropyl *tert*-butyl

Figure 2-3 The swept volume of an ethyl, isopropyl and *tert*-butyl group.

Yet the yeast requirement for the *tert*-butyl ester (11g/mmol) is considerably higher than the ethyl (1g/mmol) and isopropyl (2g/mmol) esters, and so there must be some constraint. Volume is defined by length, width and height and these alkyl groups do not differ in length, but they do differ in width (or height as these are interchangeable with rotation). Assuming a width constraint within the enzyme active site, the molecules were drawn with the ester occupying the narrowest width using Chem3D© (Figures 2-4 & 2-5).

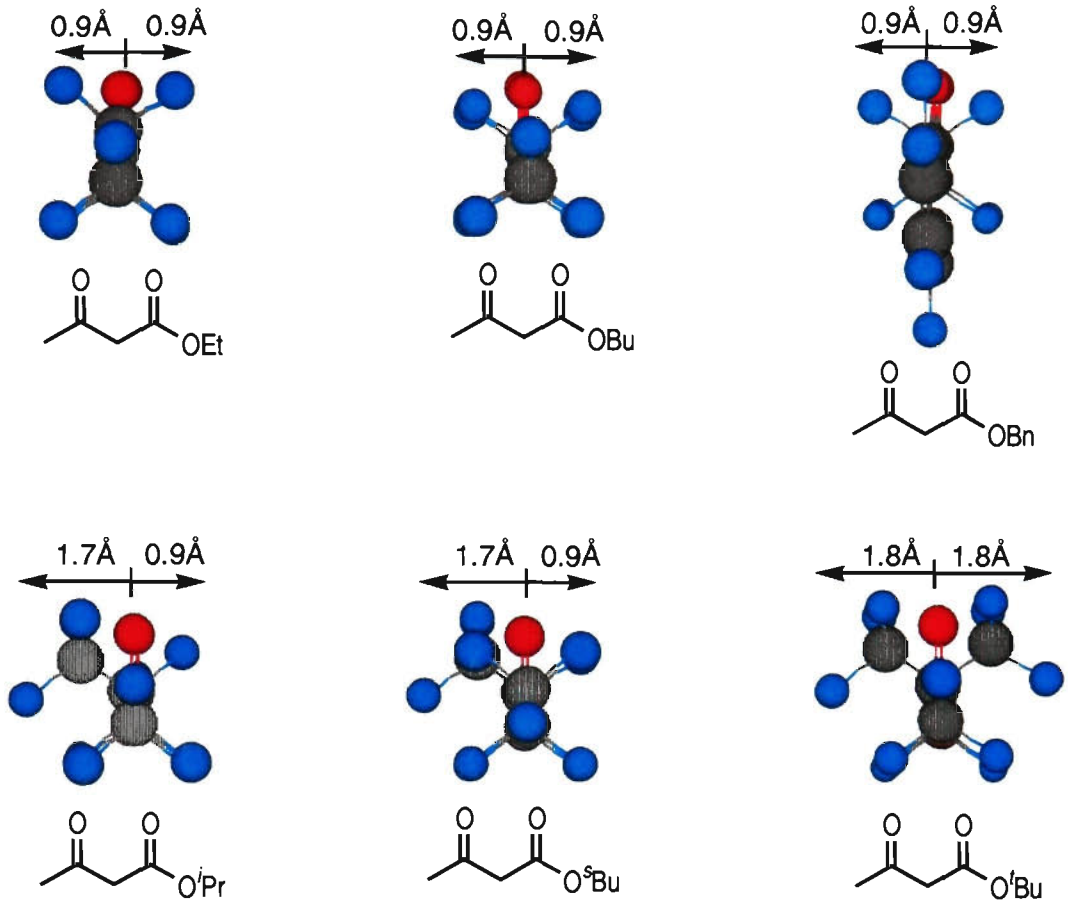


Figure 2-4 View from the alkyl end of 3-oxobutanoates indicating the width of the alkyl moiety.

Figure 2-4 shows each molecule viewed from the alkyl end along the length of the chain with the width of the ester marked. The ethyl ester, which requires only 1g of yeast/mmol, is narrow, only having H atoms extending either side. The isopropyl and *sec*-butyl esters each have a methyl group protruding to one side leaving the other side unobstructed. These esters need 2g and 4g of yeast/mmol respectively. However the *tert*-butyl ester which requires considerably more yeast, 11g/mmol, has methyl groups protruding on both sides with no clear face, possibly making access to the active site more difficult. Although both the butyl and benzyl esters are as narrow as the ethyl ester, they require more yeast for complete reduction (3g and 5g respectively) indicating that width is not the only restricting factor in enzyme binding.

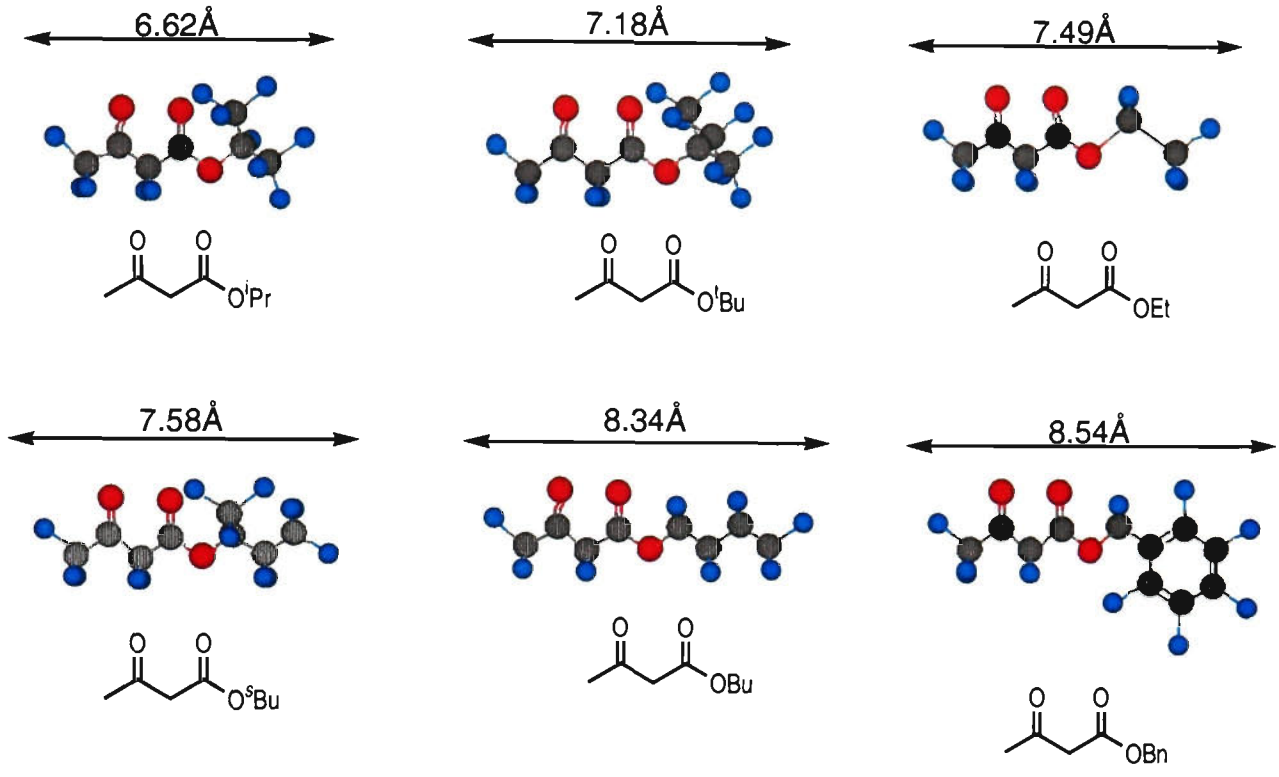


Figure 2-5 View along the length of 3-oxobutanoates.

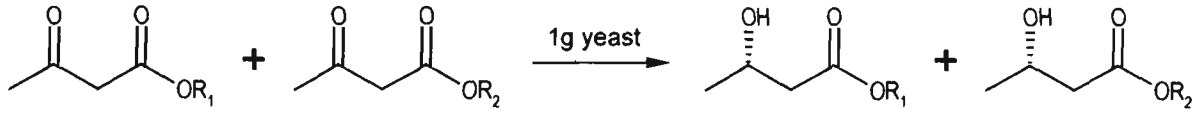
Figure 2-5 shows the different lengths of the 3-oxobutanoates. The butyl and benzyl esters are somewhat longer than the other esters indicating that possibly they are too long to be comfortably accommodated within the active site of the enzyme. This results in a slower reaction and consequently more yeast is required for complete reduction, given that the yeast has a limited reaction period in the petroleum ether solvent system. The length of these two esters are comparable but there is still a 2g difference, which may be due to greater flexibility of the butyl group which could adopt a configuration similar to the isopropyl group in Figure 2-3. The extra bulk in the benzene ring and repulsion forces arising from the π clouds in close proximity to charged side chains around the active site may account for the requirement for more yeast.

This suggests that width/height and length of the alkyl portion of the 3-oxobutanoate ester are important in binding to the active site and that the optimum substrate would have an alkyl moiety with one clear face (free from protruding groups) and less than four carbon atoms in length.

2.5 COMPETITION REACTIONS

The previous section determined how the 3-oxobutanoate esters react individually and models were drawn in an attempt to compare their reactivity and to make some comment on the ideal substrate. In order to compare the esters directly and to investigate how well they compete for the enzyme active site, a series of reactions involving equal amounts of two different esters, was conducted. Table 2-3 shows the results of reactions carried out with 1g of yeast and 1mmol each of two different esters of 3-oxobutanoate. The amount of reduction was determined by the gas chromatographic ratio of starting material to product.

Table 2-3 Conversions from the yeast-mediated reduction reactions containing two esters of 3-oxobutanoates in petroleum ether.



1 mmol 1 mmol

EXPERIMENT NO.	R_1 R_2	REDUCTION (%)[b]	YEAST REQUIRED (g/mmol)[a]	REDUCTION WITHOUT COMPETITION (1g YEAST)[b]
1	Me	40	1	100
	Et	36	1	100
2	iPr	19	2	77
	Et	74	1	100
3	Bn	17	5	49
	Et	95	1	100
4	iPr	63	2	77
	Bn	16	5	49

[a] for complete reduction

[b] gas chromatographic ratio of product to starting material.

No ester was found to be reduced to the same extent as in the absence of a competitor, although the ethyl ester was close to this when combined with the benzyl ester (Figure 2-6). This indicates that there is a finite amount of reduction that can take place within the 24h interval, presumably due to the effect of the solvent system on the yeast and enzymes as already briefly discussed. The effect of the solvent system on the enzyme system in yeast is the subject of further investigation carried out in Chapter 3.

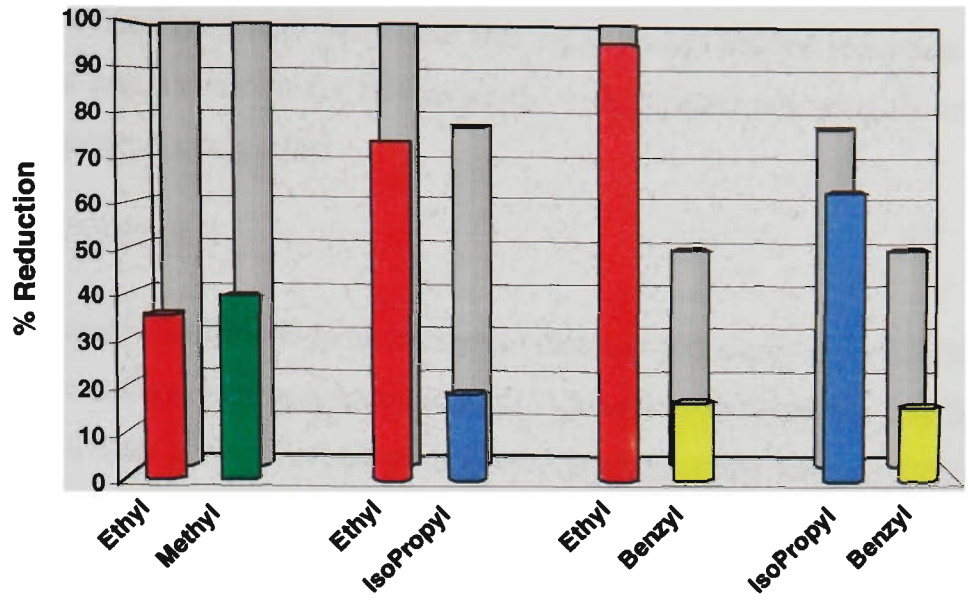


Figure 2-6 The amount of reduction obtained for the yeast-mediated reduction reactions containing one ester (grey) and two esters (coloured) with 1g of yeast in petroleum ether.

The esters that require the same amount of yeast, ethyl and methyl, compete equally for reduction (Experiment 1) resulting in approximately the same amount of reduction, 36% and 40%. The fact that they only reached half the amount of reduction they would achieve alone suggests that there is a limited total amount of reduction that occurs in 24 hours. This is most likely to be due to the enzyme system slowly becoming deactivated over this period and in that time it is working at the maximum rate. Another contributing factor could be the amount of NADPH recycling is limited by the amount of substrate(glucose) already present in the dried yeast.

Experiments 1, 2 and 3 show the ability of the methyl, isopropyl and benzyl esters to compete with the ethyl ester for reduction. As mentioned previously the methyl ester competed well only allowing the ethyl ester to reach 36% conversion. The isopropyl ester was not as effective as the methyl ester, as the ethyl ester yielded 75% conversion. The benzyl ester was the poorest competitor of the three, with the ethyl ester being reduced almost as much as it would be in the absence of a competitor. The order of competition is therefore, methyl>isopropyl>benzyl. To confirm this, the isopropyl and benzyl esters were compared and it was clear that the isopropyl ester was a better competitor for the active site than was the benzyl ester. This order correlates with the yeast requirement for esters; methyl 1g, isopropyl 2g and benzyl 5g yeast.

These results are consistent with the notion that the yeast has limited reduction capacity and that the different esters compete for this capacity, with the ester requiring the most amount of yeast being the poorest competitor.

2.6 CONCLUSIONS

The yeast-mediated reduction of 3-oxobutanoate esters in petroleum ether is a simple procedure for the stereoselective preparation of (*S*)-3-hydroxybutanoates. The best substrate was the isopropyl ester giving the best yield (96%), high enantioselectivity (97% ee) using only a small amount of yeast (2g). It was also significantly easier to prepare than the butyl ester which also resulted in a good yield (89%) and excellent stereoselectivity (>99% ee). The yields and stereoselectivity obtained using this method are superior to those reported for reductions using fermenting yeast in an aqueous environment.

CHAPTER 3

^{13}C NMR KINETIC STUDIES

3. ^{13}C NMR Kinetic Studies

3.1 *Introduction*

A number of different classes of yeast-mediated reactions in organic solvents have been reported with varying amounts of success; from low yields of a racemic product[56] to 96% isolated yield and >99% ee (*Chapter 2*). The reasons for this variability are difficult to determine since the whole cell system is a complex matrix of enzymes and other cell metabolites.

Yeast reduction of alkyl 3-oxobutanoates in petroleum ether is a high yielding, stereoselective process. Chapter 2 reported studies involving substrates with varying ester sizes aimed at achieving complete conversion to a single enantiomer in each case. Interestingly, the amount of yeast required for complete reduction varied from 1g/mmol to 11g/mmol of substrate, depending upon the ester. It is proposed that this difference is due to the ease with which the ester fits into the binding site of the enzyme, however it is not clear what effect an ill-fitting substrate has upon the reaction, other than the requirement for more yeast.

Two possible explanations for the greater yeast requirement of some substrates are that,

i. the reaction proceeds at a slower rate, or
ii. the reaction occurs at the same initial rate, but slows to a stop due to the substrate directly or indirectly deactivating the reductase enzymes in the yeast.

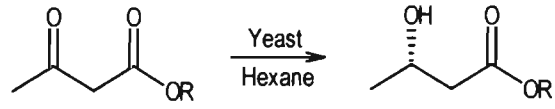
By monitoring the consumption of substrate and product formation over the course of the reactions, the enzyme activity can be profiled. This information can be used to determine the effect of different substrates, different temperatures, different solvents and the role of water.

In vivo NMR has been used to follow the consumption of substrates in a range of organisms. ^{13}C labelled substrates were first used in an *in vivo* study to examine yeast metabolism in 1972 using labelled glucose.[81] Since then, the technique has been used extensively to study the metabolism and assimilation of various substrates in yeast, including acetate[82],

methylamine[83] and methanol.[84] All of the studies have been carried out with yeast cells suspended in water, with some aeration of the system.

Using NMR spectroscopy to follow the course of a reaction in an organic solvent system is less complicated than in the corresponding aqueous system. Yeast rests at the bottom of the tube, below the receiver coil, and consequently shimming is simpler, the spectra are cleaner and there is no confusion between intra- and extracellular material.

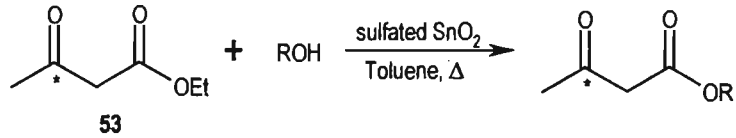
The reaction chosen was the reduction of alkyl 3-oxobutanoates (Scheme 3-1). The ratios of water, yeast and 3-oxobutanoate were the optimum values reported in Chapter 2.



Scheme 3-1

3.2 PREPARATION OF ESTERS

Benzyl and isopropyl 3-oxobutanoate-3-^{13}C were prepared *via* transesterification of ethyl 3-oxobutanoate-3-^{13}C in good yields (88% & 47% respectively) using solid superacid as outlined by Chavan and co-workers[85] (Scheme 3-2). In order to obtain a good yield of the isopropyl ester, it was necessary to prevent the loss of isopropanol at the high temperature required for the reaction to proceed (140°C). This was achieved by conducting the reaction in a stainless steel pressure vessel rather than using a reflux setup.



53

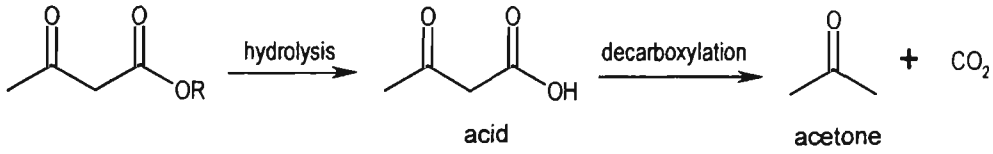
R = $CH(CH_3)$, CH_2Ph

Scheme 3-2

3.3 FORMATION OF SIDE PRODUCTS

The investigation of yeast-mediated reduction of esters of 3-oxobutanoates has uncovered some interesting differences in the isolated yield and in the amount of yeast required for complete reduction (see *Chapter 2*). Initial NMR studies were performed to investigate the possibility of the formation of side products as an explanation of lower isolated yields; for example, the benzyl ester is reduced in 74% isolated yield whereas the isopropyl ester gives

an isolated yield of 96%. Potential side products are 3-oxobutanoic acid and acetone, formed from yeast hydrolysis of the ester and decarboxylation respectively (Scheme 3-3).



Scheme 3-3

^{13}C NMR spectra were run on the reaction mixture from the yeast-mediated reduction of both single labelled ethyl 3-oxobutanoate-3-^{13}C and double labelled ethyl 3-oxobutanoate-1,3-^{13}C. No peaks due to ^{13}C labelling apart from substrate and product could be detected, indicating that no extracellular side products were being formed during the reaction. Gas chromatographic analysis of the supernatant obtained from a laboratory-scaled version of the same reaction, following washing the yeast with ethyl acetate, also showed no extraneous peaks. This was consistent with the NMR results and suggests that the lower yield was not a function of decomposition of the substrate or product.

Another possible cause of lower isolated yields was the retention of substrate and/or product by the yeast. Washing the yeast with ethyl acetate removed some product, however the yield did not rise above 75%. There may be more substrate and/or product bound to the yeast which is not easily removed. Solid-state NMR could be used to examine the yeast following a reaction with labelled substrate, and after washing the yeast with ethyl acetate, however this was outside the scope of the present work.

It became apparent that a great deal of information about the mechanism of yeast reduction could be gleaned from following reactions using ^{13}C NMR and therefore a series of time lapse ^{13}C NMR experiments was planned.

3.4 TECHNIQUES

3.4.1 Time lapse ^{13}C NMR

Petroleum ether was used as the solvent for the yeast reactions performed on a laboratory scale, however hexane was used for the NMR experiments because it gave a simpler spectrum; there was no difference in yeast reactivity between the two solvents.

The results obtained for the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane in an NMR tube is shown in Figure 3-1 as a typical time-lapse sequence of ^{13}C NMR spectra. Each spectrum was the result of 128 scans collected over about 7 min and the time between each spectrum was 1 h. The first spectrum was taken around 15-30 min after yeast reduction was initiated by the addition of water. The peaks at 198 ppm and 175 ppm are due to C3 of the keto and enol forms of the unreacted starting material and the peak appearing at 63 ppm is due to C3 of the reduced product.

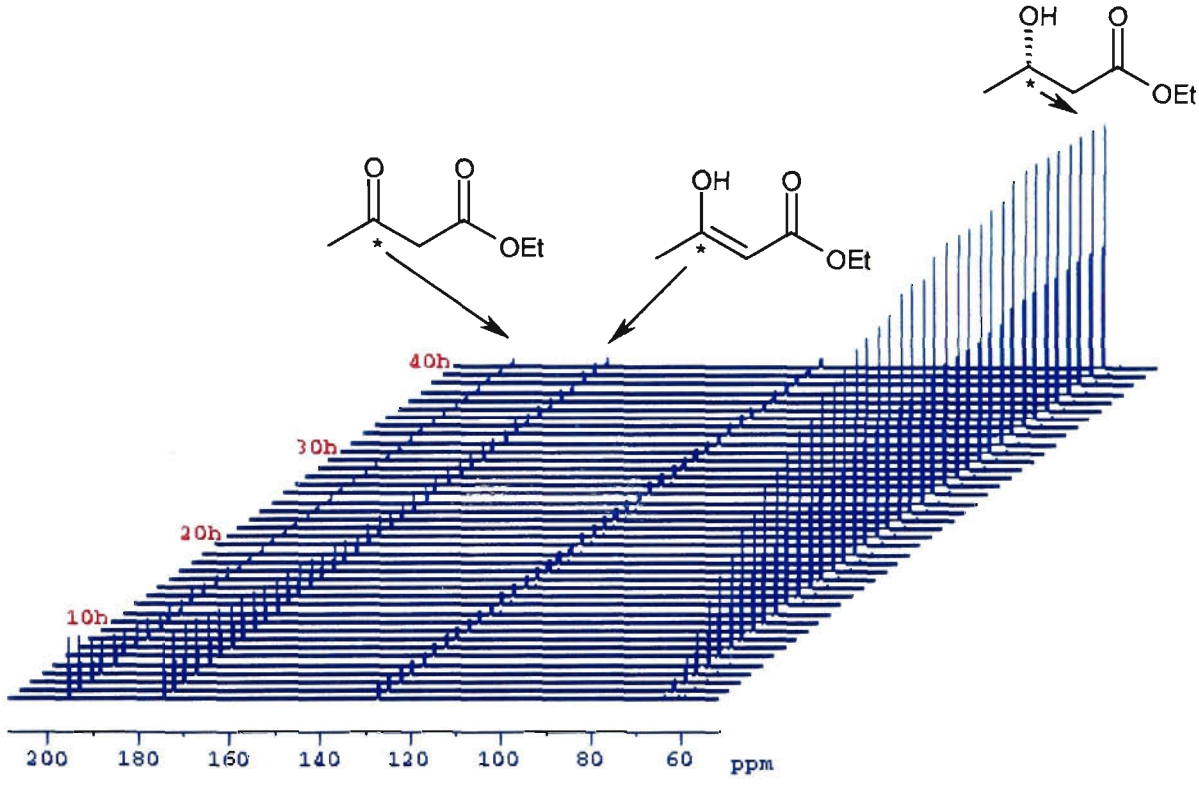


Figure 3-1 A time lapse sequence of NMR spectra obtained from the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane; 0·1g yeast, 0·1mmol substrate, 0·08ml water, 2·5ml hexane, 125µl benzene-d_6.

Benzene-d_6 provided both a lock substance and a reference for intensity measurements. The volume of benzene-d_6 was accurately added to allow comparison between experiments. The ratio of the keto and enol forms of the starting material varied from experiment to experiment and was not useful for determination of extent of conversion. The intensity of the product peak (63 ppm) was measured relative to the benzene-d_6 peaks and plotted as a function of time (Figure 3-2). The supernatant of the reaction mixture in the NMR tube was analysed by gas chromatography.

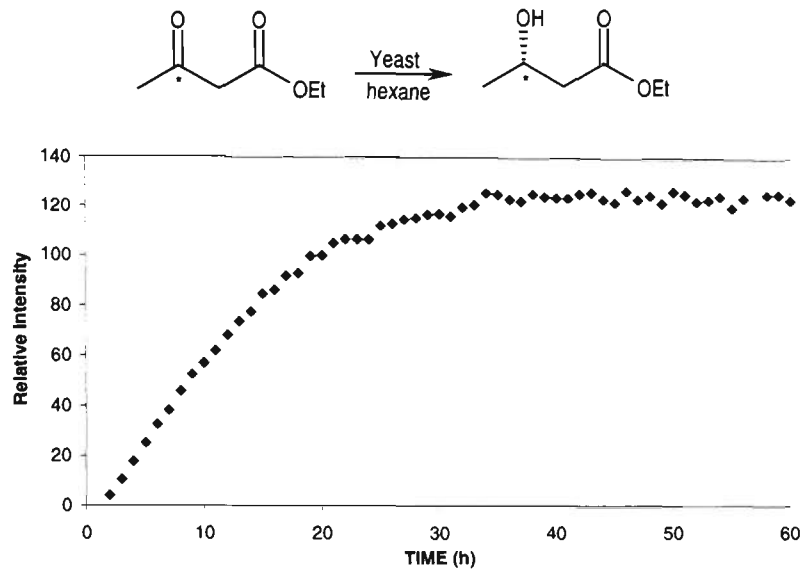


Figure 3-2 Reaction profile of the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane performed in an NMR spectrometer; relative intensity of the C3 peak of ethyl 3-hydroxybutanoate-3-^{13}C compared to d_6-benzene over 60 hours.

One problem with these reactions was that on some occasions the yeast swelled unevenly in the NMR tube and moved into the receiver coil region altering peak shape and generally decreasing peak heights. A broad peak adjacent to the product peak would also appear, making integration of the product peak difficult, hence the peak intensity was used as a measure of product concentration. The cause of the unpredictable yeast swelling was not clear, however the distribution of the water in the yeast appeared to play a part.

The initial rate of the enzyme reaction is the gradient of the relative intensity *vs* time graph at time zero and was calculated by taking the first eleven points and fitting them to Equation 3-1. This equation describes the shape of the first part of the profile better than a straight line, as it allows for a degree of curvature in the latter part of the profile which is present for some of the reactions.[86]

$$\text{relative intensity} = R_I \frac{t - D_I}{1 + m(t - D_I)} \quad \text{.....} \qquad \textbf{Equation 3-1}$$

where

D_I = initial delay (time taken for substrate to reach the active site, react, and for the product to return to the solvent)

R_I = initial rate

m = degree of curvature

Fitting the data presented in Figure 3-2 to Equation 3-1 yields an initial rate of 8·14 and indicates that the initial delay is about 1·5h.

3.4.2 Spinning vs Stationary

Spinning a sample in an NMR tube can be approximated to stirring a reaction. Stirring yeast cells in the presence of some organic solvents has been shown to increase the speed at which cell death occurs.[87] Salter and Kell showed that by increasing the agitation of the sample, the time of cell viability decreased. Although cell viability and enzyme activity are not necessarily related, it was of interest to examine the effect of agitation upon the yeast reduction of ethyl 3-oxobutanoate. Jayasinghe *et al.* have reported that stirring has little effect on yield in the laboratory scale reduction.[88]

NMR spectra were obtained for both a spinning and a stationary system and the results are shown in Figure 3-3.

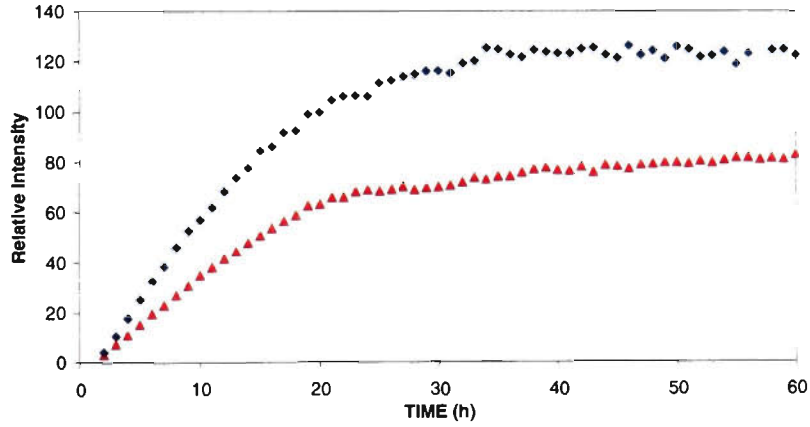


Figure 3-3 Reaction profiles of the yeast-mediated reduction of ethyl 3-oxobutanoate in hexane performed in an NMR spectrometer with the tube stationary (▲) and spinning (●); relative intensity of the C3 peak of ethyl 3-hydroxybutanoate-3-^{13}C compared to d_6-benzene over 60 hours.

Table 3-1 Yeast-mediated reduction of ethyl 3-oxobutanoate in hexane performed in an NMR spectrometer with the tube stationary and spinning; calculated initial reaction rates and gas chromatography conversions.

CONDITIONS	INITIAL RATE	CONVERSION (%)
Spinning	8·14	82
Stationary	4·06	68

In contrast to the laboratory scale experiments, the stationary reaction gave a lower initial rate and gas chromatographic conversion (Table 3-1). The difference between the laboratory scale and NMR experiments is probably due to the smaller surface area of the yeast in an NMR tube hindering mass transfer and slowing the reaction. The shape of the two curves is similar in that both tend to plateau after about 30h. This indicates that the enzyme activity is

retained for a similar period in both experiments; the reaction is slower when the tube is not spun. The sample was therefore spun for the remainder of the experiments.

3.4.3 Conclusion

The reaction in the NMR tube (0·1g yeast/0·1mmol ethyl 3-oxobutanoate-3-^{13}C) gave 82% conversion after 60h, whilst in the laboratory complete conversion was achieved; the difference is probably due to the enhanced mass transfer in the laboratory scale reaction. Notwithstanding this difference the data suggests the reaction in an NMR tube is a reasonable representation of the large scale reaction.

3.5 EXPERIMENTAL REPRODUCIBILTY

The spinning reaction described above was performed in triplicate to assess the reproducibility of the method. In each case, the extent of conversion was determined by gas chromatography. The profiles are presented graphically in Figure 3-4 and results are tabulated in Table 3-2.

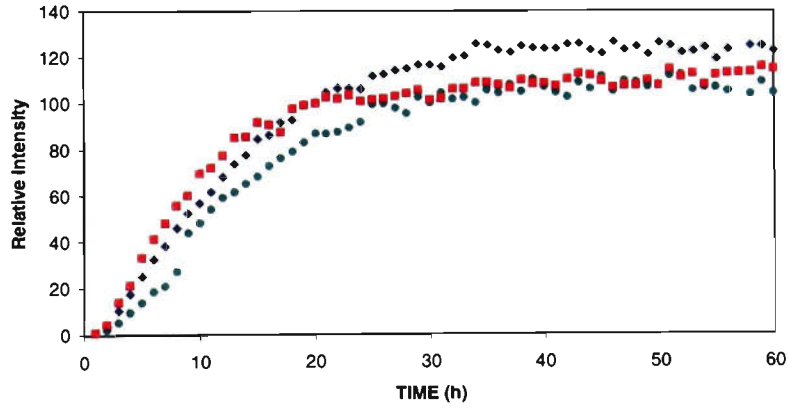


Figure 3-4 Reaction profiles of the yeast-mediated reduction of ethyl 3-oxobutanoate in hexane performed in an NMR spectrometer in triplicate; 1 (◆), 2 (■) and 3 (●); relative intensity of the C3 peak of ethyl 3-hydroxybutanoate-3-^{13}C compared to d_6-benzene over 60 hours.

Table 3-2 Yeast-mediated reduction of ethyl 3-oxobutanoate in hexane performed in an NMR spectrometer in triplicate.

EXPERIMENT	FINAL RELATIVE INTENSITY	CONVERSION (%)
1	125	82
2	112	96
3	108	78

It is apparent from Table 3-2 that the final relative intensity of the product peak did not reflect the conversion of substrate to product as measured by gas chromatography. The reason for this lack of correlation is not immediately evident, however it is important that it be rationalised so that meaningful comparisons can be made between results obtained from different NMR experiments. The approach taken was to calculate a ratio described as the maximum relative intensity (MRI) according to Equation 3-2.

$$MRI = \frac{RI_f}{C_{GC}} \quad \text{.....} \quad \textbf{Equation 3-2}$$

where

RI_f = final relative intensity

C_{GC} = gas chromatographic conversion

This MRI corresponds to the relative intensity of the product peak, had complete conversion occurred. Since the reaction has 1:1 stoichiometry, the MRI value also provides a measure of the initial substrate intensity (the initial relative peak intensity of the substrate cannot be measured directly as the substrate appears as two peaks, the keto and enol forms).

The MRI value can consequently be used to modify the initial rate derived using Equation 3-1 to calculate a corrected initial rate that is independent of the amount of substrate added to the reaction (Equation 3-3). The corrected initial rate can be used to compare the results of experiments on a common basis.

The initial rate, MRI and corrected rate for the three experiments are given in Table 3-3.

$$\text{Corrected rate} = \frac{\text{initial rate}}{MRI} \quad \text{.....} \quad \textbf{Equation 3-3}$$

Table 3-3 A comparison of the initial rate, MRI and the corrected rate for the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane performed over 60h in an NMR spectrometer in triplicate.

EXPERIMENT	INITIAL RATE	MRI	CORRECTED RATE
1	8·14±0·31	152	$5{\cdot}35 \times 10^{-2}$
2	8·54±0·86	119	$7{\cdot}17 \times 10^{-2}$
3	5·73±0·92	141	$4{\cdot}06 \times 10^{-2}$

There is a difference in initial rates, however a larger difference is obtained when the corrected rates are calculated. The reason for the difference is unclear since there was insufficient time to optimise the procedure fully due to the amount of NMR time required for each experiment. The mean of the corrected rates is $5{\cdot}53 \times 10^{-2}$ with a standard error of $0{\cdot}9 \times 10^{-2}$. In later experiments a full set of 60 data points is required for comparison purposes and so Experiment 1 was used as the standard because the corrected rate was closest to the mean.

3.6 DETERMINATION OF THE REACTION KINETICS

In order to analyse the data obtained in this study, it was necessary to determine the parameters which influence the reaction kinetics of the yeast-mediated reduction of 3-oxobutanoates. A mathematical model of the enzyme system was adapted from Coller *et al.*[89] based upon the Michaelis-Menten equation, the standard rate equation for a one-substrate enzyme catalysed reaction (Equation 3-4).

$$R_I = \frac{k[S][E]}{[S] + K_M} \quad \text{Equation 3-4}$$

where R_I = initial rate

k = rate constant

K_M = Michaelis-Menten constant

[S] = initial substrate concentration

[E] = total enzyme concentration

In order to construct the model it was necessary to determine the way in which enzyme activity changed over time and to determine the constants, k and K_M.

3.6.1 Enzyme Activity

Preliminary experiments on a laboratory scale indicated that after 24 hours exposure to the solvent system (including the small amount of water) the enzyme system in yeast responsible for reduction was substantially deactivated. For example, addition of the substrate to yeast after 24 hours exposure to the solvent system resulted in only about ~10% reduction compared to 100% when the substrate was added immediately. A series of experiments was designed in which the yeast was pretreated with the solvent system for successively longer

periods, prior to the addition of the substrate. All of the reaction components except the substrate, were added to the NMR tube and placed inside the NMR spectrometer for the indicated time. The sample was spinning during this time and the temperature was held constant at 22°C. Four pretreatment times of 3, 6, 12 and 24 h were chosen and the plot of relative intensity *vs* time for each reaction is presented in Figure 3-5 along with that of the standard reaction with no pretreatment time.

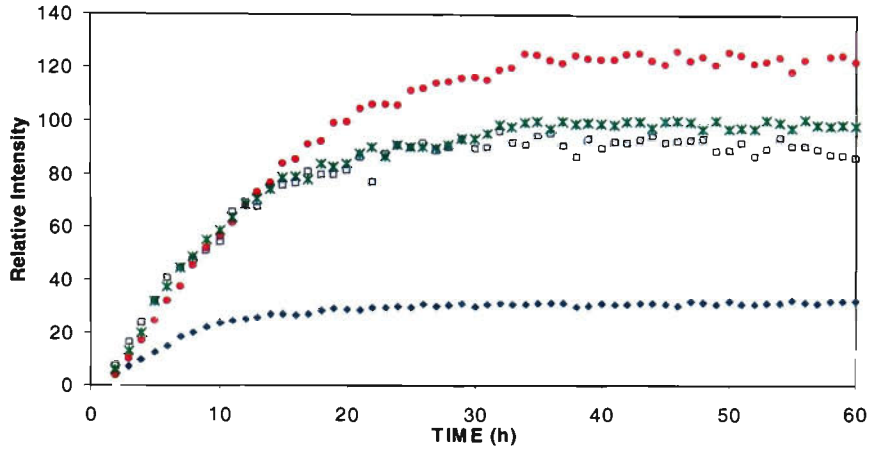


Figure 3-5 Reaction profiles of the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C under standard conditions(●), and after 3h(✱), 6h( ), 12h(◆) and 24h( ) pretreatment; relative intensity of the C3 peak of ethyl 3-hydroxybutanoate-3-^{13}C compared to d_6-benzene over 60 hours.

The 3h pretreatment reaction shows a similar profile to the 6h pretreatment reaction, whilst the 12 and 24h pretreatment profiles indicate that the reductase enzyme activity in the yeast has diminished considerably after the extended exposure to the solvent system. The initial rate, MRI and corrected rate were calculated for each reaction (Table 3-4).

Table 3-4 A comparison of the initial rate, MRI and the corrected rate for the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane performed over 60h in an NMR spectrometer with 0, 3h, 6h, 12h and 24h pretreatment time.

Pretreatment Time (h)	Initial Rate	MRI	Corrected Rate
0	8·14	152	$5{\cdot}36\pm0{\cdot}2 \times 10^{-2}$
3	10·77	167	$6{\cdot}99\pm0{\cdot}3 \times 10^{-2}$
6	9·93	143	$6{\cdot}45\pm0{\cdot}9 \times 10^{-2}$
12	5·03	82	$6{\cdot}13\pm0{\cdot}6 \times 10^{-2}$
24	1·41	203	$0{\cdot}69\pm0{\cdot}01 \times 10^{-2}$

A plot of corrected rate *vs* pretreatment time (Figure 3-6) yields a profile of enzyme activity as a function of time. This suggests that after a short activation period, followed by about 10h of fairly constant activity, the enzyme system undergoes progressive deactivation until after 24h very little activity remains.

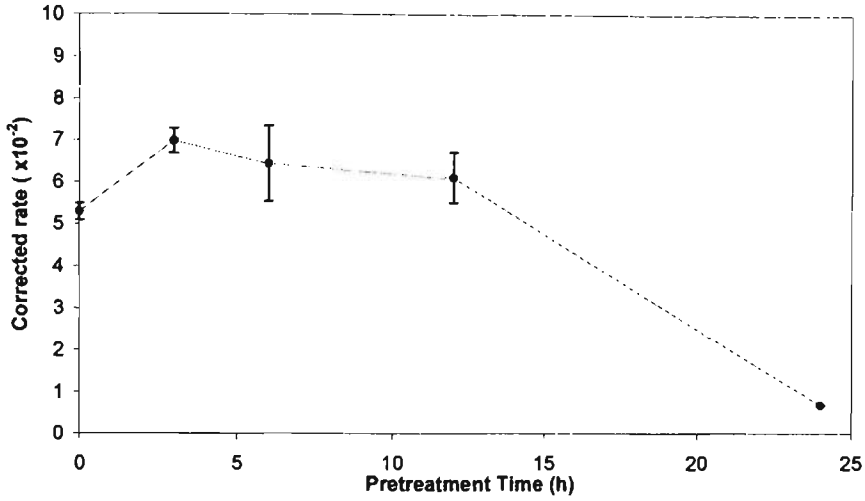


Figure 3-6 Corrected rate of the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C as a function of time of pretreatment of the yeast with the solvent system.

The initial activation is most likely caused by the slow rehydration of the dried yeast following the addition of the small amount of water required for enzyme hydration. The deactivation observed is presumed to be due to the exposure of the yeast to the organic solvent system, however it appears that the activity is maintained for a period of about 12h before the deactivation process affects the reaction. Although the number of data points is limited, two equations were used to describe the rates of activation and deactivation of the yeast enzyme system. Equation 3-5 describes the shape of a curve similar to that seen in the activation period.

$$y = 1 - e^{-t/C} \quad \text{.....} \quad \textbf{Equation 3-5}$$

Equation 3-5 was fitted to the experimental data by the addition of two constants. The activation factor, actF, controls the height of the curve and describes the extent to which enzyme system is activated. The activation constant, A, describes how quickly the maximum activity is reached. The initial delay, D_I, which was calculated in Equation 3-1, is included in the time term. The final equation is presented in Equation 3-6.

$$\text{activation} = \text{actF}(1 - e^{-(t-D_I)/A}) \quad \text{.....} \quad \textbf{Equation 3-6}$$

The general equation to describe the deactivation of the enzyme system is presented in Equation 3-7.

$$y = e^{-t/C} \quad \text{.....} \quad \textbf{Equation 3-7}$$

The activation factor, actF, employed in Equation 3-6 is also required in the deactivation equation. The deactivation time, t_D, is the time at which the activity of the enzyme system begins to decrease and is included in the time term. The rate at which the decay occurs is accounted for by the decay constant, D, which is the time it takes for the activity to decrease by 1/e. With all these parameters included the final deactivation equation is presented in Equation 3-8.

$$\text{deactivation} = \text{actF}(e^{-(t-t_D)/D}) \quad \text{..... Equation 3-8}$$

Fitting the experimental data to Equations 3-6 and 3-8 gave an activation factor of 1·2, an activation constant of 1·5, a decay constant of 7 and a deactivation time of 11 (Figure 3-7).

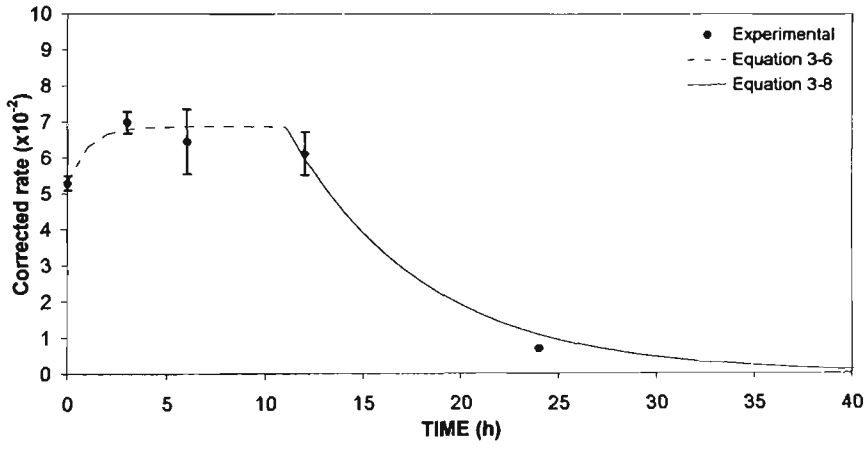


Figure 3-7 Profile of enzyme activity as a function of time, calculated using Equations 3-6 and Equations 3-8 compared with the corrected rate of the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane in an NMR tube as a function of pretreatment time.

3.6.2 Relative Rate

In order to avoid the difficulty of determining k directly (Equation 3-4), the data set obtained from Experiment 1, Table 3-3, which was performed under optimum conditions was taken as the standard and other data sets were assessed relative to this standard. The initial rate, R_I, of any reaction was therefore expressed as a relative initial rate R_{rel} according to Equation 3-9.

$$R_{rel} = \frac{R_I}{R_{std}} \quad \text{..... Equation 3-9}$$

where R_{std} is the initial rate of the standard reaction.

Since,

$$R_I = \frac{k[S][E]}{[S]+K_M} \quad \text{and} \quad R_{std} = \frac{k[S]_{std}[E]}{[S]_{std}+K_M}$$

It follows from Equation 3 - 9 that,

$$R_{rel} = \frac{k[S][E]}{[S]+K_M} \times \frac{[S]_{std}+K_M}{k[S]_{std}[E]}$$

This can be simplified to:

$$R_{rel} = \frac{[S]}{[S]_{std}} \times \frac{[S]_{std}+K_M}{[S]+K_M} \quad \text{..... Equation 3-10}$$

Equation 3-10 relates the relative initial rate to the initial substrate concentration and the K_M value and has the double advantage of not involving either k, the rate constant or [E], the total enzyme concentration.

In order to solve for K_M, a series of experiments was performed with three different concentrations of substrate. The reaction profiles from these experiments are shown in Figure 3-8.

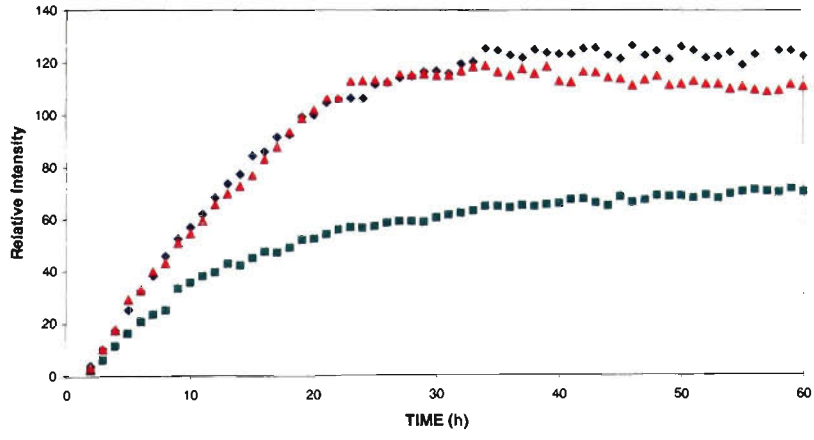


Figure 3-8 Reaction profiles of the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane with 0·2mmol (▲), 0·05mmol (■) and the standard 0·1mmol (◆) of substrate; relative intensity of the C3 peak of ethyl 3-hydroxybutanoate-3-^{13}C compared to d_6-benzene over 60 hours.

The initial rate of reduction of the yeast-mediated reaction with twice the amount of substrate is only slightly greater than that of the standard reaction. This indicates that with 0·1mmol of substrate, the reaction is working at near maximal rate, indicating that enzyme saturation has been reached. This is consistent with Michaelis-Menten kinetics. The MRI values, initial rates and relative rates were calculated and the relative rate was plotted against MRI (Table

3-5 and Figure 3-9). The data was fitted to Equation 3-11, with an extra term included, m, so the curve was not constrained to pass through one point in particular, to give K_M = 100±34 and m = 0·94±0·034.

$$R_{rel} = m \times \frac{[S]}{[S]_{std}} \times \frac{[S]_{std} + K_M}{[S] + K_M} \quad \text{..... Equation 3-11}$$

Table 3-5 A comparison of the initial rate, MRI and the relative rate for the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane performed over 60h in an NMR spectrometer with 0·05, 0·1 and 0·2mmol substrate.

SUBSTRATE (mmol)	MRI	INITIAL RATE	RELATIVE RATE (R_{REL})
0·05	82	5·36±0·57	0·66±0·07
0·1	152	8·14±0·31	1·00±0·02
0·2	266	8·93±0·64	1·10±0·08

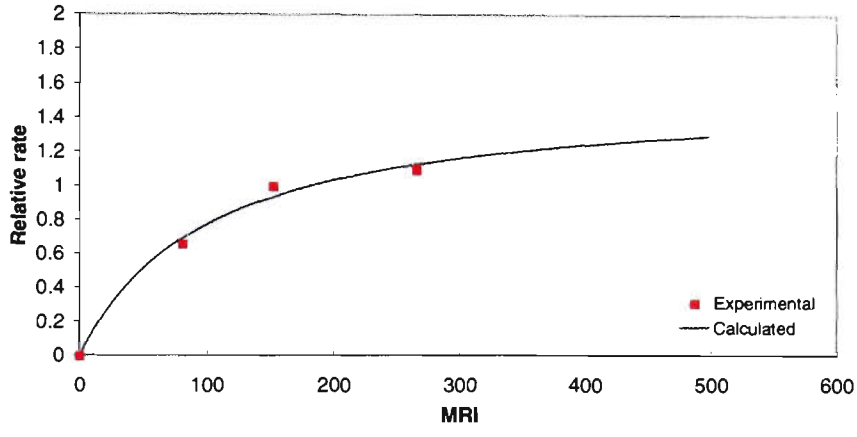


Figure 3-9 A plot of the calculated and experimental relative rate vs MRI for the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane performed over 60h in an NMR spectrometer with 0·05, 0·1 and 0·2mmol substrate.

3.6.3 Product Inhibition

Product inhibition is a common means of regulating the concentration of metabolites in biological systems. However, since ethyl 3-oxobutanoate-3-^{13}C is not a natural substrate for yeast, it is difficult to predict whether the product does inhibit the reaction. An experiment was performed involving the addition of (*S*)-ethyl-3-hydroxybutanoate (not ^{13}C labelled) into the standard reaction (0·1mmol ethyl 3-oxobutanoate-3-^{13}C, 0·1g yeast, 80µl water, 2·5ml hexane, 125µl d_6-benzene). The maximum amount of product that could be formed in the standard reaction (0·1mmol) was added to produce the maximum inhibiting effect; the results are presented in Figure 3-10 and are compared with the results obtained from the standard reaction (in the absence of added product).

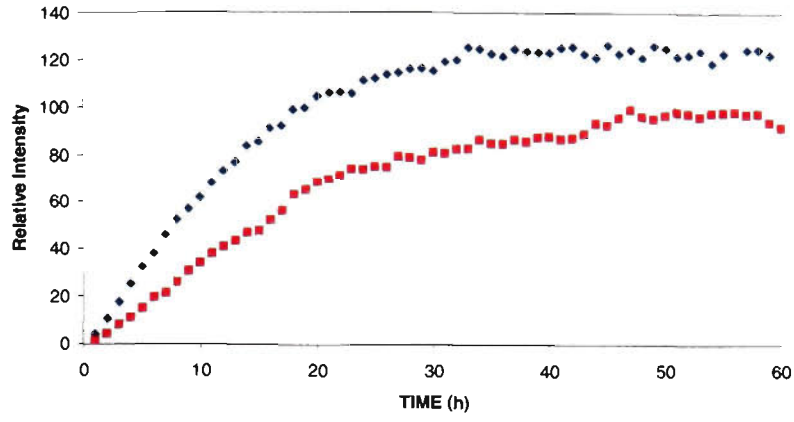


Figure 3-10 Reaction profiles of the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane under standard conditions(◆) and with the addition of (S)-ethyl 3-hydroxybutanoate(■); relative intensity of the C3 peak of ethyl 3-hydroxybutanoate-3-^{13}C compared to d_6-benzene over 60 hours.

The initial rate (3·67±0·22) is affected by the addition of product but since the two curves show a similar profile, the enzyme system remains active for approximately the same length of time. Therefore the presence of the product does not contribute to the deactivation but as expected, does inhibit the reaction rate. An accurate MRI could not be calculated for this reaction since gas chromatographic analysis cannot distinguish between labelled and unlabelled product. The value for the standard reaction of 152 was used for both thesubstrate and product concentration.

In order to account for the accumulation of product in the reaction mixture over the course of the reaction extra terms, K_P and product concentration [P], were included in the relative rate equation (Equation 3-11) to give Equation 3-12.

$$R_{rel} = 0{\cdot}935 \times \frac{[S]}{[S]_{std}} \times \frac{[S]_{std} + K_M}{[S] + K_M + (K_P \times [P])} \quad \text{.....} \quad \textbf{Equation 3-12}$$

Using the relative initial rate of the reaction described above (Figure 3-10), K_P was calculated to be 1·78 using Equation 3-12.

3.6.4 The Model

Three equations (3-6, 3-8, and 3-12) have been developed to describe the yeast-mediated reduction of ethyl-3-oxobutanoate. Equation 3-12, based upon Michaelis-Menten kinetics as a function of substrate concentration describes the relative rate and includes product inhibition terms, while Equations 3-6 and 3-8 describe the change in enzyme activity over

time. From these equations, it was possible to create a model to enable detailed information about yeast-mediated reductions in organic solvents to be elucidated.

To create a model of the system the equations were put into a form in which the enzyme activity could be followed over the course of the reaction and the product formation profiles could be compared to the experimental data.

The standard rate equation (Equation 3-4) makes two assumptions that can not be made in this situation:

a) The activity of the enzyme does not change over time. However, in this situation it has been shown that the activity increases initially due to the addition of water and decreases exponentially due to the organic solvent system. The change in enzyme activity is calculated using Equations 3-6 and 3-8.

b) The rate is an initial rate and assumes no product is present, therefore the reverse reaction (product back to enzyme-substrate complex) and product inhibition effects are not accounted for. The relative rate equation (Equation 3-12) includes product terms and therefore, calculation of rate at any time over the course of the reaction is possible.

Rearranging Equation 3-9,

$$R_I = R_{rel} \times R_{std} \quad \text{..... Equation 3-13}$$

where R_{rel} is calculated using Equation 3-12 and R_{std} is the initial rate for the standard reaction. Since the relative rate, R_{rel}, includes the product terms, Equation 3-13 is also valid when there is product present in the reaction mixture.

If Equation 3-13 has the enzyme activity included then the rate at any time during the course of the yeast-mediated reduction reaction can be calculated (Equation 3-14).

$$\text{rate} = R_{rel} \times R_{std} \times \text{enzyme activity} \quad \text{..... Equation 3-14}$$

These equations were incorporated into a Visual Basic program based on a design by Coller for a chemical catalysed reaction written in QuickBasic.[90] The program allows the input of

various values such as substrate concentration, pretreatment time, decay constant and deactivation time (Table 3-6).

Table 3-6 Variables used in the Visual Basic program to model the yeast-mediated reduction reactions.

Variable name	Variable meaning	How determined	Equation reference
cSstd	MRI, [S]	experiment	3-2
Rtstd	Initial rate	experiment	3-1
K_M	Michaelis-Menten	experiment	3-10
K_P	Product constant	experiment	3-11
D_I	Initial delay	experiment	3-1
A	Activation by water	unknown	3-6
actF	Activation factor	experiment	3-6
t_D	Deactivation time	unknown	3-8
D	Decay constant	unknown	3-8

Figure 3-11 shows the relationship of these variables to the enzyme activity and the product profiles.

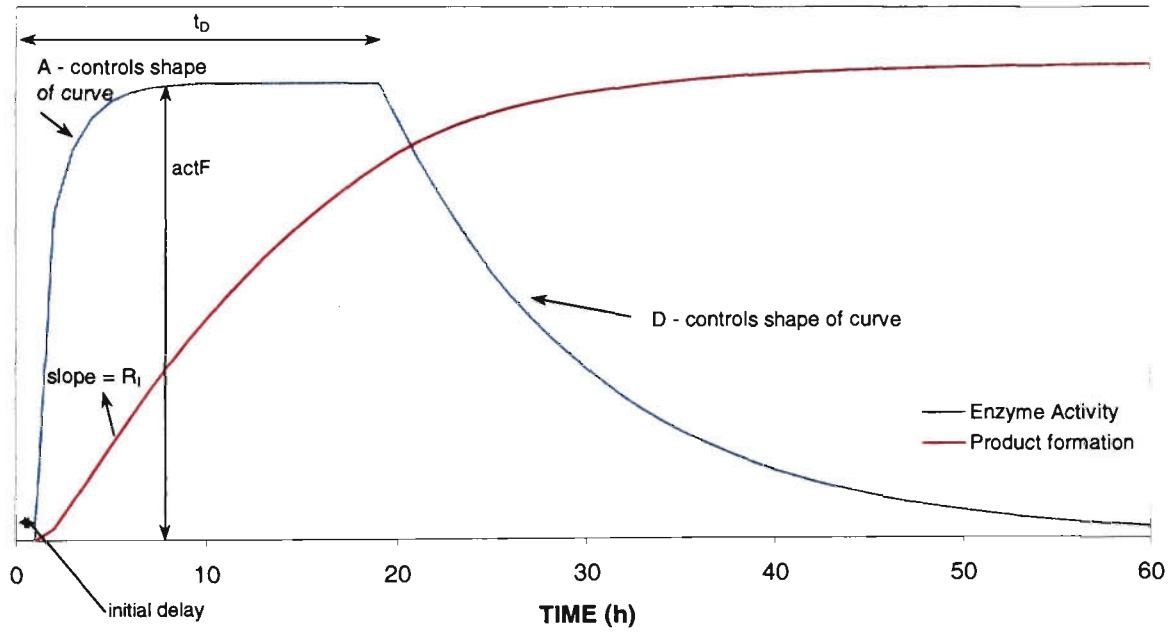


Figure 3-11 Diagram indicating the parameters used in the model with respect to the profiles obtained.

The experimentally determined parameters, initial delay, MRI and initial rate, for a particular reaction were entered into the model. The other variables except deactivation time and decay constant, were set as calculated in previous sections.

The computer program begins by calculating the initial enzyme activity at time zero using Equations 3-6 and 3-8. The relative rate is calculated at time zero (Equation 3-12) for a given amount of substrate (MRI). The enzyme activity is multiplied by the relative rate and by the standard rate to give the rate of the reaction at time zero. The amount of product formed in the first 0·1h is calculated by multiplying the rate by 0·1. The amount of substrate remaining is determined by subtracting the amount of product formed from the initial amount of substrate.

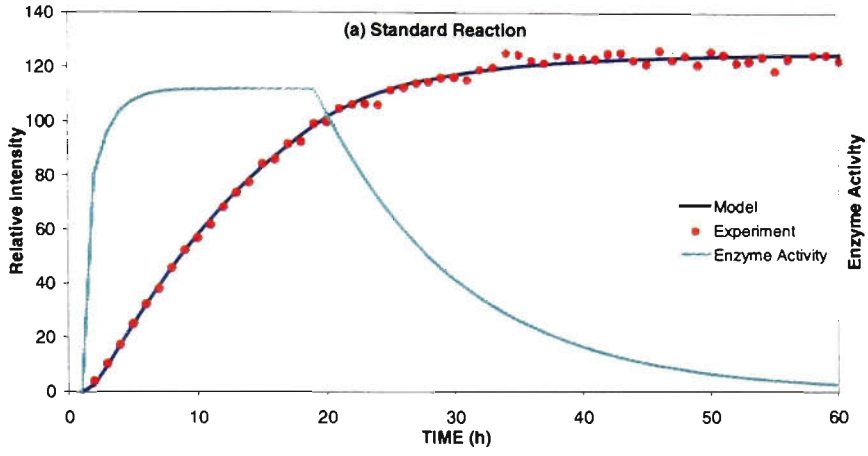
The enzyme activity is recalculated and the relative rate is also recalculated using the remaining amount of substrate and the newly formed product. This leads to a new rate and the amount of product formed in the next 0·1h is calculated. This process is repeated 10 times for a one hour period and then the enzyme activity and the amount of product are inserted in a Microsoft Excel® sheet. After 60h, profiles of the enzyme activity and product formation (Figure 3-11) are drawn in a Microsoft Excel® chart.

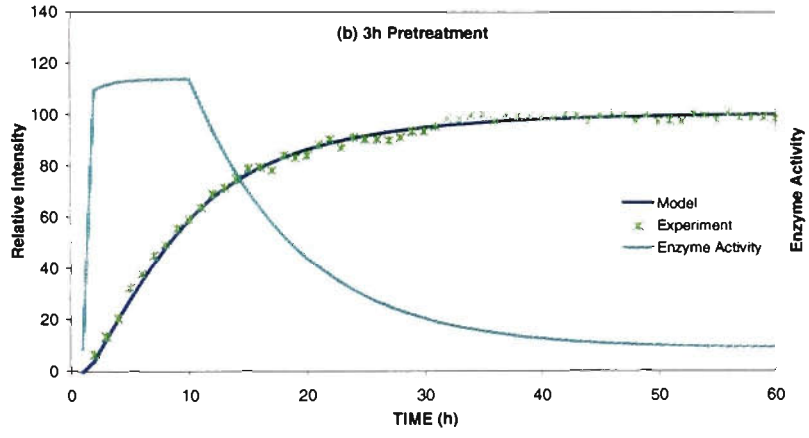
The goodness of fit of the model profile to the experimental results is assessed using the sum of the squares of the residuals when each of the 60 pairs of experimentally determined and calculated values are compared. The deactivation time and decay constant were varied systematically until the best fit was obtained. In some cases an improved fit could be obtained by varying the activation factor and the product constant. The activation factor enabled a greater amount of product to be formed and changing the product constant had the greatest impact between 15 and 30h, smoothing the curve of the product formation during this period. While modelling the reactions the effect of changing the product constant appeared to indicate that this constant incorporates more than just product inhibition, encompassing a multitude of effects.

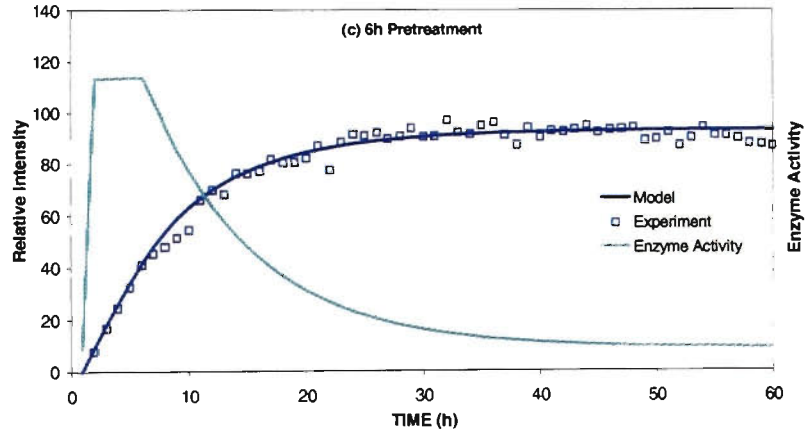
3.7 Comparisons of the Experimental and Computer Data

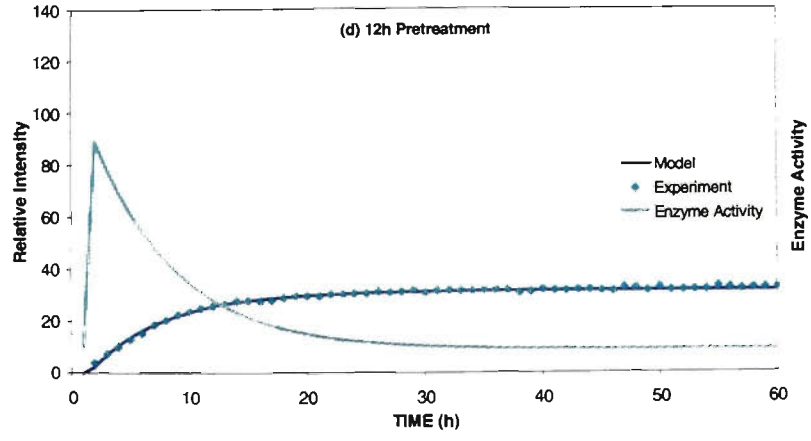
In order to derive information about the deactivation of the enzyme system, the experimental results from the pretreatment reactions were fitted to the model using the calculated initial rates and MRI values. The deactivation time and the rate of decay were systematically altered to obtain the best fit. It was found that an improved fit could be obtained with a value for K_P lower than that obtained by calculation in *Section 3.6.3*. The lower value is presumed to be a reflection of lower product concentrations than the maximum level used for the calculation.

The results are shown in Figure 3-12 and Table 3-7 gives the values obtained. The deactivation time is measured from the beginning of the pretreatment period whereas the experimental data are all measured from the addition of the substrate.









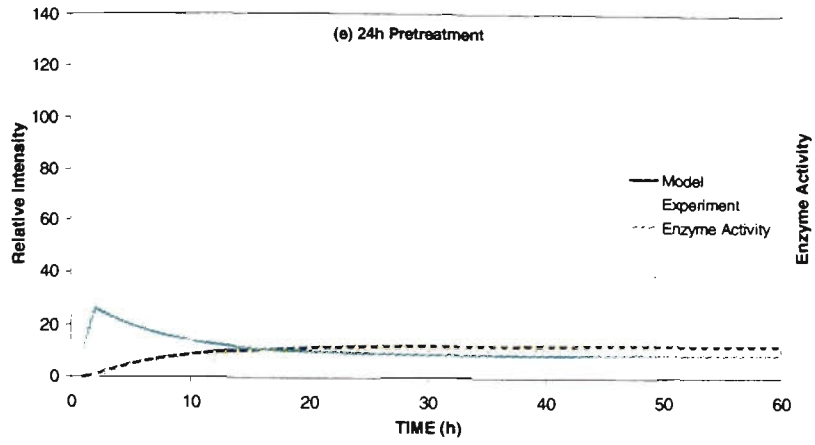


Figure 3-12 Comparison of the computer simulation with the experimental results for the yeast-mediated reduction of ethyl-3-oxobutanoate-3-^{13}C in hexane in a NMR tube. Computer simulation(—), enzyme activity(—), (a) standard reaction(●), (b) 3h( ), (c) 6h( ), (d) 12h(◆) and (e) 24h( ) pretreatment.

Table 3-7 Values used in the model giving the best fit to the experimental results for the yeast-mediated reduction of ethyl-3-oxobutanoate-3-^{13}C in hexane in an NMR tube for the standard reaction and for the reactions involving pretreatment of the yeast for 3-24h.

PRETREATMENT TIME (h)	DEACTIVATION TIME (h)	DECAY CONSTANT
0	19	11
3	12	11
6	12	9
12	12	7
24	13·5	7

These results indicate that there is consistency in the deactivation time determined for each of the pretreatment reactions although, the standard reaction appeared to have a longer deactivation time. The decay constant of the enzyme system was determined to be about 9h. This suggests that the enzyme system involved in the reduction of ethyl 3-oxobutanoate is unaffected by the solvent system for at least 12h at 20°C and then decays exponentially.

3.7.1 Temperature

Recent studies have shown that some yeast reactions work better at lower temperatures.[91] This effect could be due to repression of competing reactions, or to different temperature optima of the enzymes involved in the reaction. The effect of temperature on the initial rate and deactivation time of the yeast reductions in hexane was investigated by performing the reaction at 10°C, 20°C and 30°C.

3.7.1.1 NMR Study of the Reduction of Ethyl 3-oxobutanoate.

Ethyl 3-oxobutanoate-3-^{13}C was reduced with yeast in hexane in an NMR tube at 10°C, 20°C and 30°C for 60 hours, using 0·1g yeast/0·1mmol substrate as described previously. The results are shown in Figure 3-13.

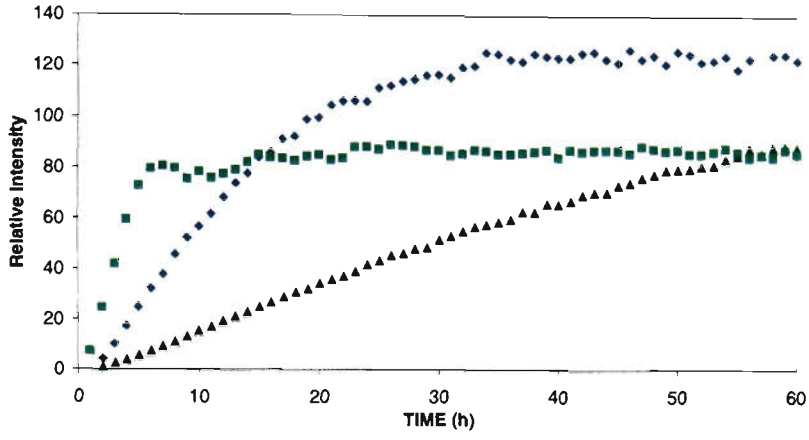


Figure 3-13 Reaction profiles of the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C at 10 (▲), 20 (◆) and 30°C (■); relative intensity of the C3 peak of ethyl 3-hydroxybutanoate-3-^{13}C compared to d_6-benzene over 60 hours.

The MRI, initial rate and the initial delay for each temperature was calculated as previously described and are shown in Table 3-8.

Table 3-8 Calculated values and measured conversions for the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane at different temperatures in an NMR tube.

TEMP (°C)	MRI	INITIAL DELAY (h)	INITIAL RATE	CONVERSION (%)
10	109	2·5	2·02±0·04	82
20	152	1·5	8·1±0·3	82
30	143	0·5	16·5±0·7	63

Each temperature yielded a different profile. The reaction conducted at 10°C had a much slower initial rate than those performed at the higher temperatures. The slope of the curve is nearly constant indicating that the yeast has retained most of its activity over the 60h period. The initial delay was greater indicating that movement of the substrate and product into and out of the yeast was retarded. Gas chromatographic analysis of the product of the 10°C reaction showed a similar conversion (82%) to that exhibited by the reaction at 20°C.

Although the reaction performed at 30°C had an initial rate of 16·5, double that of the experiment performed at 20°C, the higher level of activity was not sustained for as long and the reaction appeared to stop completely after about 6h. As a consequence, the maximum

intensity reached was lower than that reached when the experiment was performed at 20°C; the gas chromatographic analysis of the reaction mixture showed 63% conversion, confirming what was indicated by the reaction profile. The initial delay was also shorter (0·5h compared with 1·5h) than those at lower temperatures.

Each of these reactions was modelled by incorporating the calculated values for initial rate and initial delay; the deactivation time and decay constant were systematically altered until the best fit was achieved (Figure 3-14 and Table 3-9).

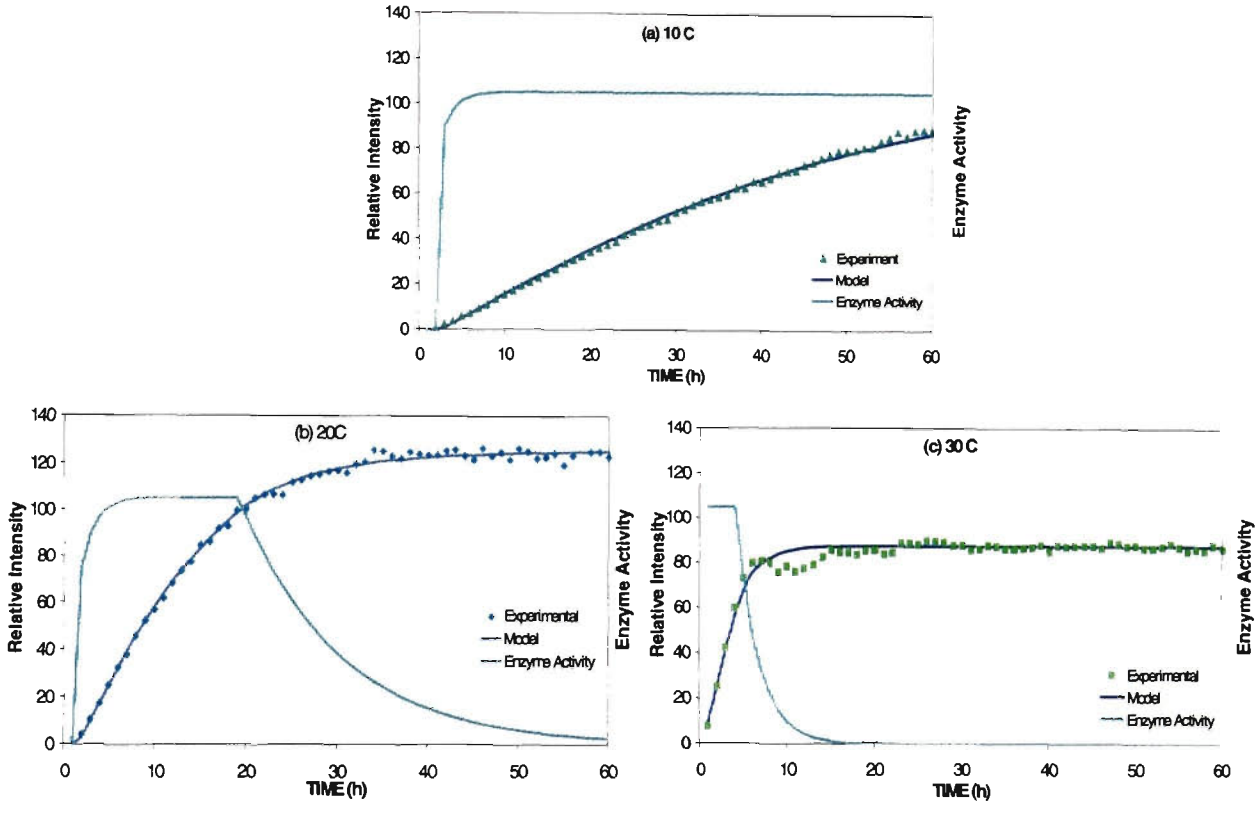


Figure 3-14 Comparison of the results of the model with the experimental results for the yeast-mediated reduction of ethyl-3-oxobutanoate-3-^{13}C in hexane in a NMR tube at different temperatures. Model(—), enzyme activity(—), (a) 10°C (▲), (b) 20°C (◆) and (c) 30°C (■)

Table 3-9 Parameters used in the model of the yeast-mediated reduction of ethyl-3-oxobutanoate-3-^{13}C in hexane in an NMR tube at three different temperatures.

TEMP (°C)	DEACTIVATION TIME (h)	DECAY CONSTANT	ACTIVATION FACTOR	PRODUCT CONSTANT
10	>60	-	1·2	0·00178
20	19	11	1·2	0·89
30	4	2·5	0·12	0·178

As is evident in Table 3-9, at 10°C the model yields a deactivation time of greater than 60h and consequently no indication of decay within the 60h period. This indicates the lower

temperature preserves the enzyme activity despite exposure to the organic solvent system. Retention of enzyme activity over the full reaction time suggests that further amounts of substrate could be reduced using reaction periods in excess of 60h at this temperature.

The 30°C reaction was found to have a much shorter deactivation time and an increased decay constant compared with both reactions at lower temperatures. To obtain this fit the activation factor was reduced by a factor of 10. This indicates that the enzyme system is both activated and deactivated at a faster rate at the higher temperature, resulting in an increased initial rate but with a lower extent of reduction.

It was found that at both 10°C and 30°C the value for the product constant, K_P required to give the best fit was lower than that associated with the 20°C data. This suggests that the product constant is temperature dependent.

3.7.1.2 NMR Study of the Reduction of Isopropyl 3-oxobutanoate.

Isopropyl 3-oxobutanoate-3-^{13}C was reduced with yeast in hexane in an NMR tube at 10°C, 20°C and 30°C for 60h (Figure 3-15). Only 0·1g yeast/0·1mmol substrate was used although as described earlier, the isopropyl ester required 0·2g of yeast for complete reduction; the purpose of the study was to investigate the reason for the difference in amounts of yeast required, rather than to achieve complete reduction.

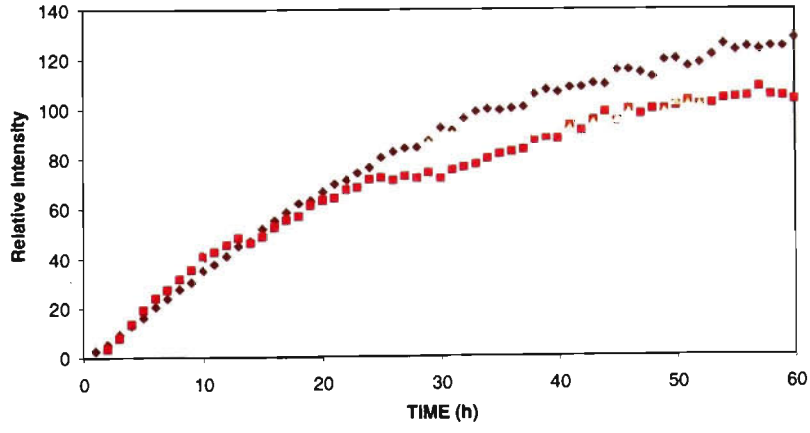


Figure 3-15 Reaction profiles of the yeast-mediated reduction of isopropyl 3-oxobutanoate-3-^{13}C in hexane in an NMR tube at 10 (◆), 20 (■) and 30°C ( ); relative intensity of the C3 peak of isopropyl 3-hydroxybutanoate-3-^{13}C compared to d_6-benzene over 60 hours.

The MRI, initial rates and initial delays of the yeast-mediated reduction of isopropyl 3-oxobutanoate-3-^{13}C at the three temperatures were calculated as previously described and are

shown in Table 3-10. The initial rates of the reactions at the three temperatures follow a similar pattern to those of ethyl 3-oxobutanoate, with the reaction at 30°C having the fastest rate (12·8) and the reaction at 10°C having a slightly slower rate (3·3) than the reaction performed at 20°C (5·5).

Table 3-10 Calculated values and measured conversions for the yeast-mediated reduction of isopropyl 3-oxobutanoate-3-^{13}C at different temperatures in an NMR tube.

TEMP (°C)	MRI	INITIAL DELAY (h)	INITIAL RATE	CONVERSION (%)
10	138	1·2	3·3±0·17	93
20	138	1·0	5·5±.0·44	76
30	145	0·7	12·8±1·8	69

Each of these reactions was modelled using the calculated values for initial rate and initial delay. The parameters which gave the best fit of the modelled reaction profile to the experimental data for each temperature, are listed Table 3-11 and the modelled profiles are compared with the experimental data in Figure 3-16.

Table 3-11 Parameters used for the modelling of the yeast-mediated reduction of isopropyl 3-oxobutanoate-3-^{13}C in hexane in an NMR tube at three temperatures.

TEMP (°C)	DEACTIVATION TIME (h)	DECAY CONSTANT	ACTIVATION FACTOR	PRODUCT CONSTANT
10	>60	-	1·38	0·267
20	9	50	1·2	1·78
30	4	10	0·84	0·267

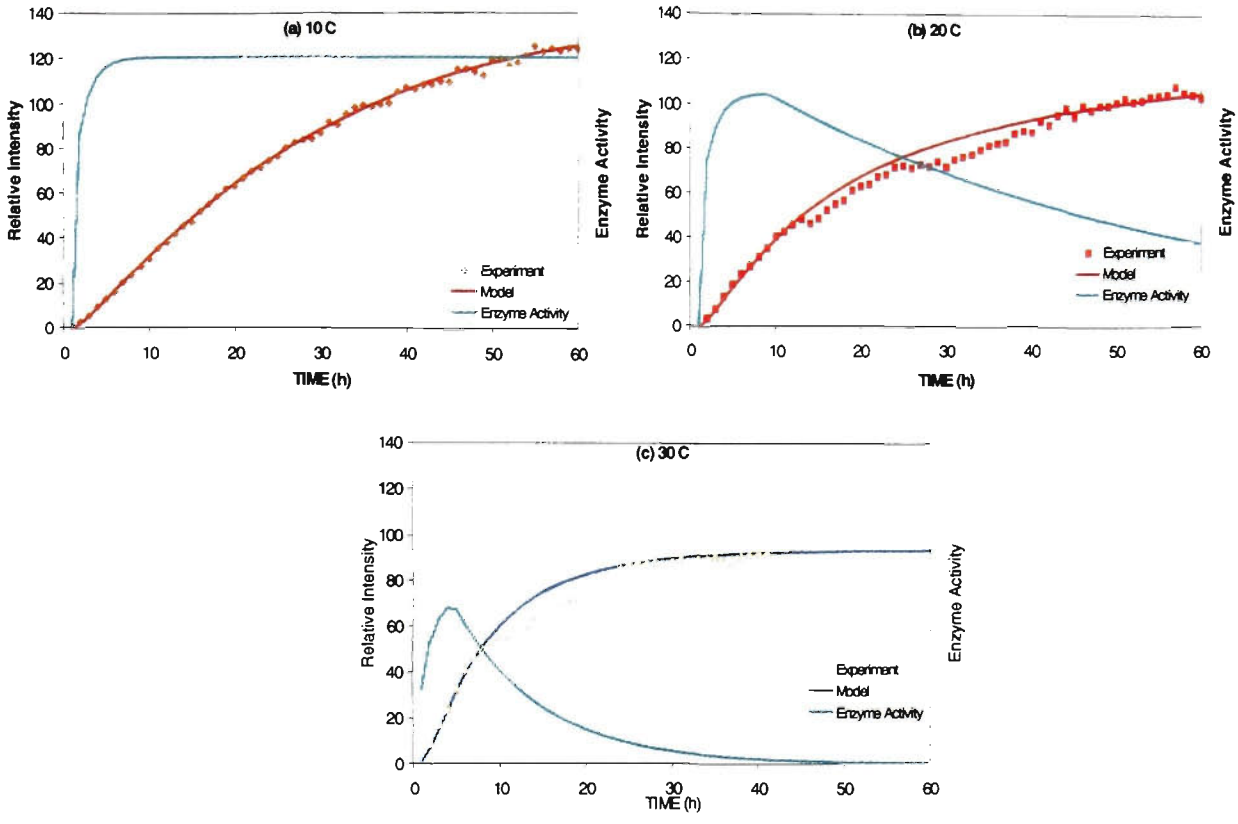


Figure 3-16 Comparison of the results from the model with the experimental results for the yeast-mediated reduction of isopropyl 3-oxobutanoate-3-^{13}C in hexane in a NMR tube at different temperatures. Model(—), enzyme activity(—), (a) 10°C (◆), (b) 20°C (■) and (c) 30°C ( ).

Fitting the reactions performed at 10°C, 20°C and 30°C yielded deactivation times which were generally consistent with those obtained for the ethyl ester. The decay constant however was generally greater for the isopropyl ester, indicating that the decay is slower than that for the ethyl ester. This suggests that although the onset of deactivation occurs at about the same time for both substrates, during the deactivation period the enzyme system retains an ability to bind the isopropyl ester more effectively than the ethyl ester. This is consistent with the earlier finding in Chapter 2 that isopropyl 3-oxobutanoate was the best substrate.

When fitting the data obtained from these three reactions to the model the activity of the enzyme system appeared to be different from that evident for ethyl 3-oxobutanoate. In the simulation, the activation factor is responsible for setting the maximum activity the enzyme system reaches during the activation period. At 10°C the activation factor for isopropyl 3-oxobutanoate was increased by 15% over that for the ethyl ester whilst at 30°C it was decreased by 30%; at 20°C the activation factor was similar for both substrates.

As with the ethyl ester the value of the product constant, K_P, which facilitated the best fit of the model to the 10°C and 30°C data was again lower than that associated with the 20°C data Again this is consistent with the product constant being temperature dependent.

The profiles of the reactions at 20 and 30°C (Figure 3-16) are not of the same shape as the corresponding ethyl 3-oxobutanoate reactions (Figure 3-14); there appears to be a tendency at both temperatures for the curves to flatten out after the initial rapid reaction but this is followed by an increase in reaction rate. A possible explanation for this behaviour is that two enzymes are involved in the reduction of isopropyl 3-oxobutanoate and therefore the model based upon the assumption of one working enzyme is inadequate. The model was modified to include a second enzyme that deactivated after a longer time; the activity of the two enzymes was weighted at 0·8 and 0·2, with the first enzyme having a deactivation time of 4h and a decay constant of 10 and the second enzyme having a deactivation time of 23h and a decay constant of 18 (Figure 3-17). This set of numbers yielded a better fit to the experimental data, as judged by the sum of the squares of the residuals, than was possible with a single enzyme model and therefore provided at least *prima facie* support for the two-enzyme concept.

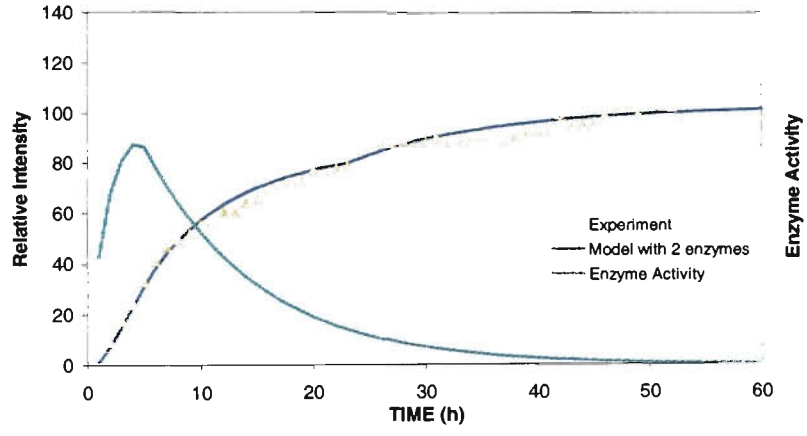


Figure 3-17 Comparison of the results from the 2-enzyme model with the experimental results for the yeast-mediated reduction of isopropyl 3-oxobutanoate-3-^{13}C in hexane in a NMR tube at 30°C ( ). Model(—) and enzyme activity(—).

Despite the slower initial rate of the reaction at 10°C, the yeast enzyme was active for a considerably longer period resulting in higher ultimate conversion. Indeed, the highest conversion of isopropyl 3-oxobutanoate-3-^{13}C over 60 hours was achieved at 10°C (93%).

3.7.1.3 NMR Study of the Reduction of Benzyl 3-oxobutanoate.

Benzyl 3-oxobutanoate-3-^{13}C was reduced with yeast in an NMR tube at 10°C, 20°C and 30°C for 60h (Figure 3-18). Only 0·1g yeast/0·1mmol substrate was used although the benzyl ester was shown earlier to require 0·5g of yeast for complete reduction at 20°C. The purpose of the study was to investigate the reason for the difference in amounts of yeast required, rather than to achieve complete reduction.

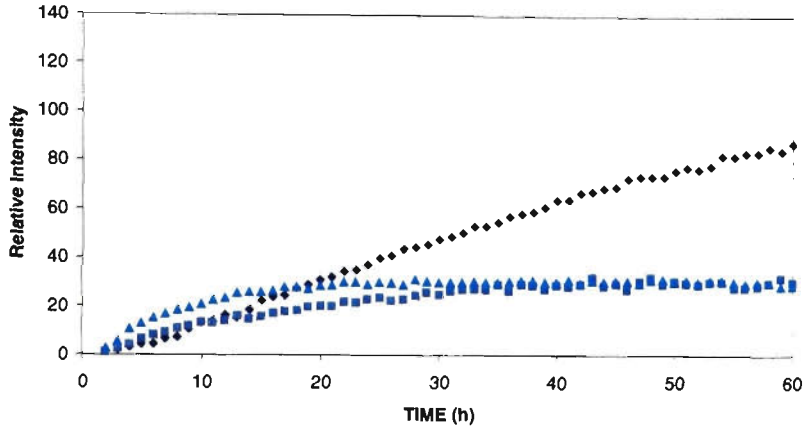


Figure 3-18 Reaction profiles of the yeast-mediated reduction of benzyl 3-oxobutanoate-3-^{13}C at 10 (◆), 20 (■) and 30°C (▲); relative intensity of the C3 peak of benzyl 3-hydroxybutanoate-3-^{13}C compared to d_6-benzene over 60 hours.

The MRI, initial rate and initial delay of the yeast-mediated reduction of benzyl 3-oxobutanoate-3-^{13}C at each temperature was calculated as previously described and are shown in Table 3-12.

Table 3-12 Calculated values and measured conversions for the yeast-mediated reduction of benzyl 3-oxobutanoate-3-^{13}C in hexane at different temperatures in an NMR tube.

Temp. (°C)	MRI	Initial Delay (h)	Initial Rate	Conversion (%)
10	158	2·0	1·7±0·08	55
20	52	1·5	2·1±0·23	58
30	200	1·6	5·8±0·69	15

The reaction profile at 10°C is very similar to the profile of the ethyl ester at 10°C (Figure 3-14), with the initial rate being maintained virtually throughout the 60h of the experiment. The reaction profiles at 20°C and 30°C are similar to each other, although the initial rate of the reaction at 30°C is higher than that at 20°C.

The experimental data derived from these reactions were fitted to the model using the calculated initial rates and initial delays and the other parameters were systematically altered until the best fit was achieved (Figure 3-19 and Table 3-13).

Table 3-13 Parameters used for the model of the yeast-mediated reduction of benzyl 3-oxobutanoate-3-^{13}C in hexane in an NMR tube at different temperatures.

TEMP. (°C)	DEACTIVATION TIME (h)	DECAY CONSTANT	ACTIVATION FACTOR	PRODUCT CONSTANT
10	>60	-	1·2	0·142
20	0·5	28.5	1·08	1·78
30	0·5	7·5	0·96	1·78

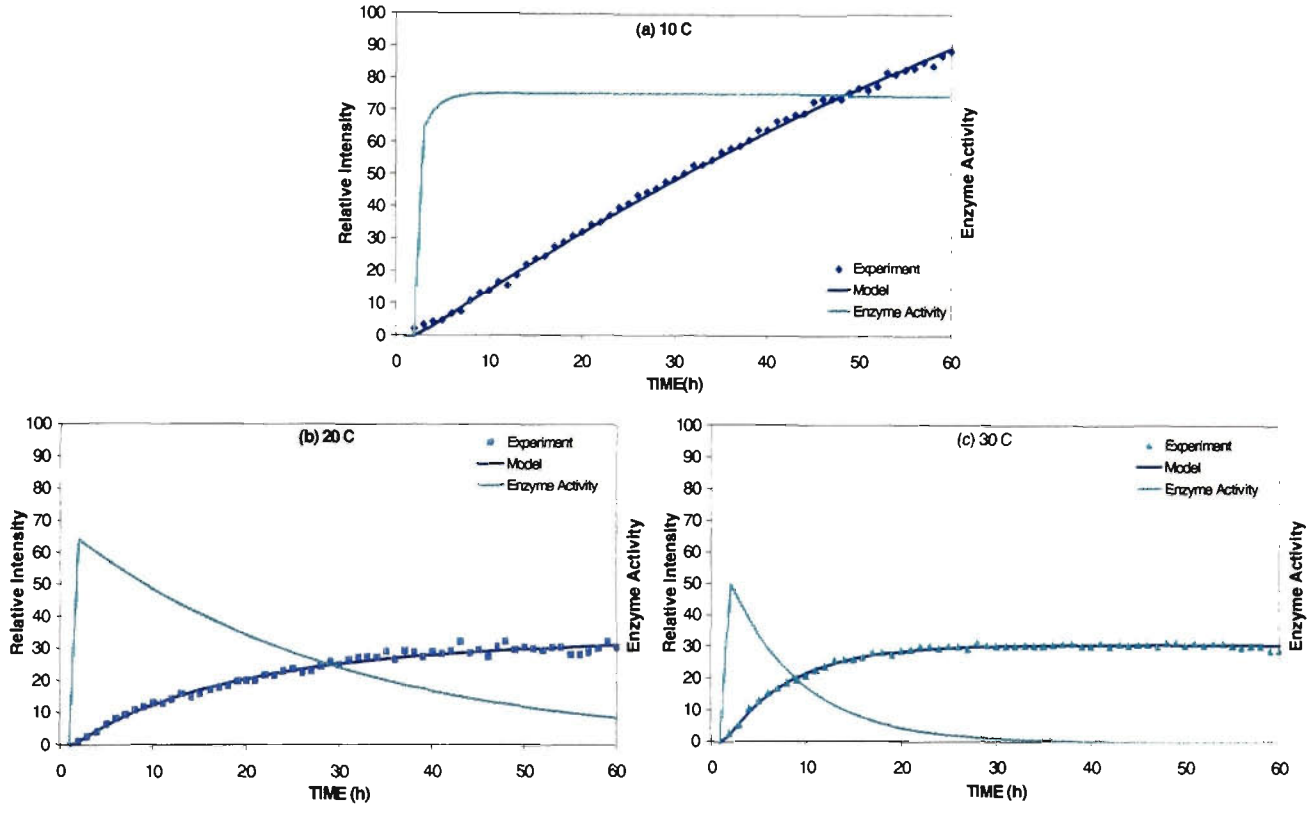


Figure 3-19 Comparison of the results from the model with the experimental results for the yeast-mediated reduction of benzyl 3-oxobutanoate-3-^{13}C in hexane in a NMR tube at different temperatures. Model(—), enzyme activity(—), (a) 10°C (◆), (b) 20°C (■) and (c) 30°C (▲).

Fitting the data obtained from the yeast-mediated reduction of benzyl 3-oxobutanoate at 10°C showed no evidence of enzyme deactivation over the whole of the 60h exposure to the organic solvent, consistent with the reactions of the isopropyl and ethyl esters at the same temperature. The deactivation time for the reactions at 20°C and 30°C was found to be 0·5h which is considerably shorter than that found for the two previous esters. It was concluded in Chapter 2 that the benzyl ester fits relatively poorly into the active site of the enzyme. This is consistent with the present results which suggest that alteration to the enzyme caused by

exposure to the solvent system has an immediate impact upon the reaction rate. Again, the decay constant decreased with increasing reaction temperature; for each of the three esters the increase in temperature from 20°C to 30°C resulted in the decay constant being reduced by approximately one quarter.

When fitting the reactions at 20°C and 30°C, the activity of the enzyme system appeared to be considerably lower than that for ethyl 3-oxobutanoate. The activation factor was decreased by 10% for the reaction at 20°C and a further 10% for the reaction at 30°C.
As for each of the other esters it was found that the value of the product constant, K_P, required to give the best fit to the benzyl ester data at 10°C was considerably lower than those for 20°C and 30°C.

3.7.2 Overall

The deactivation times and decay constants for the yeast-mediated reduction reactions involving ethyl, isopropyl and benzyl 3-oxobutanoate-3-^{13}C at 10°C, 20°C and 30°C are presented in Table 3-14.

Table 3-14 Deactivation times and decay constants for three esters of 3-oxobutanoate-3-^{13}C at different temperatures.

Ester	Ethyl		Isopropyl		Benzyl	
Temp (°C)	Deactivation time (h)	Decay constant	Deactivation time (h)	Decay constant	Deactivation time (h)	Decay constant
10	>60	-	>60	-	>60	-
20	19	11	9	50	0·5	28·5
30	4	2·5	4	10	0·5	7·5

The reaction rate of each of the esters at 10°C remained reasonably constant over the 60h period. The deactivation time of the enzyme system at this temperature was greater than 60h although, the rate of reaction was slower than at the other temperatures.

At 20°C the deactivation times appear to be substrate dependent, with the bulkiest ester, benzyl, having the shortest time. The data suggests that one interpretation of the results is that the enzyme system is deactivating at the same rate in each case but that poorly fitting substrates are more sensitive to deactivation. This relationship between substrate size and deactivation time suggests that the deactivation of the enzyme system involves disruption of the active site. It appears that the organic solvent system progressively alters the spatial

characteristics of the active site and the deactivation time is the point at which substrate binding becomes affected. It is also conceivable that other enzyme systems are being affected by the organic solvent, in particular the cofactor recycling mechanism. This may also have the effect of changing the deactivation time of the reduction reaction at different temperatures. However, the variation in deactivation times of the different substrates cannot be explained by deactivation of the cofactor recycling system.

At 30°C the deactivation time was reduced to 4h for the ethyl and isopropyl esters; the deactivation time for the benzyl ester was only 0·5h at 20°C and remained about the same value at 30°C.

3.7.3 Activation Energy

The Activation Energy, E_A, is a measure of the energy barrier which must be overcome for a reaction to occur and is related to the reaction rate by the Arrhenius equation (Equation 3-15). It can be calculated by plotting ln initial rate (ln k) vs inverse temperature (1/T) (Equation 3-16) and measuring the gradient.

$$k = Ae^{-E_A/RT} \quad \text{.....} \quad \textbf{Equation 3-15}$$

$$\ln k = \ln A - \frac{E_A}{RT} \quad \text{.....} \quad \textbf{Equation 3-16}$$

The activation energy was calculated for all three esters (Table 3-15).

Table 3-15 Activation Energy for each of the three esters of 3-oxobutanoate.

ETHYL kJmol^{-1}	BENZYL kJ mol^{-1}	ISOPROPYL kJ mol^{-1}
65 ± 5	43 ± 17*	46 ± 7

*Corrected rate could not be used due to the abnormally low MRI for 20°C

It was anticipated that the activation energy for each of the substrates would reflect their different affinities for the active site. No significant differences could be identified however, perhaps because of the excessive experimental uncertainty in the values

3.8 THE ROLE OF WATER

Yeast requires a small amount of water for reduction to occur in hexane and the optimum amount is 80µl water/0.1g yeast for a 24h reaction time.[61] The addition of water contributed significantly to the deactivation of the reductase enzyme system, as was shown by two

experiments involving pretreatment of yeast, in the presence and absence of water, for 24h before addition of the substrate, ethyl 3-oxobutanoate-3-^{13}C (Figure 3-20 and Table 3-16).

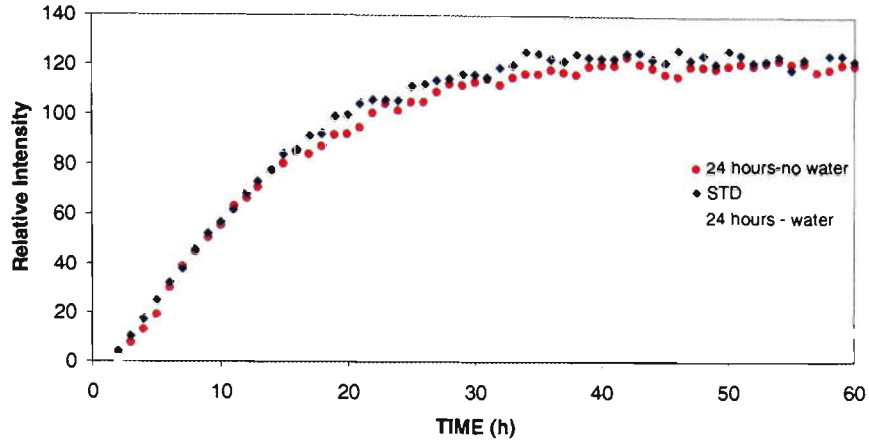


Figure 3-20 Profiles of yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C reactions in hexane in an NMR tube after pretreatment for 24 hours with ( ) and without water(◆) compared to the standard reaction (◆); relative intensity of the C3 peak of ethyl 3-hydroxybutanoate-3-^{13}C compared to d_6-benzene over 60 hours.

Table 3-16 Extent of conversion of the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane in an NMR tube; reactions pretreated for 24 hours with and without water compared to the standard reaction

PRETREATMENT CONDITIONS	CONVERSION (%)
24 hours, water	6
24 hours, no water	90
Standard	82

The difference in reduction between the two pretreated reactions is likely to be due to the water hydrating the yeast and exposing vital components to the solvent resulting in deactivation of the reductase enzyme.

It was thought that decreasing the amount of water would possibly lead to continuing activity over a longer period. Figure 3-21 shows the results of two reactions using 40µl water/0·1g yeast, compared to the standard 80µlwater/0·1g yeast reaction. While performing this reaction with half the amount of water it was noticed that it was possible to completely wet half the yeast (uneven distribution) or alternatively, to half wet all of the yeast (even distribution). A reaction employing 0·05g of yeast is also shown. The initial and corrected rates for these reactions are shown in Table 3-17.

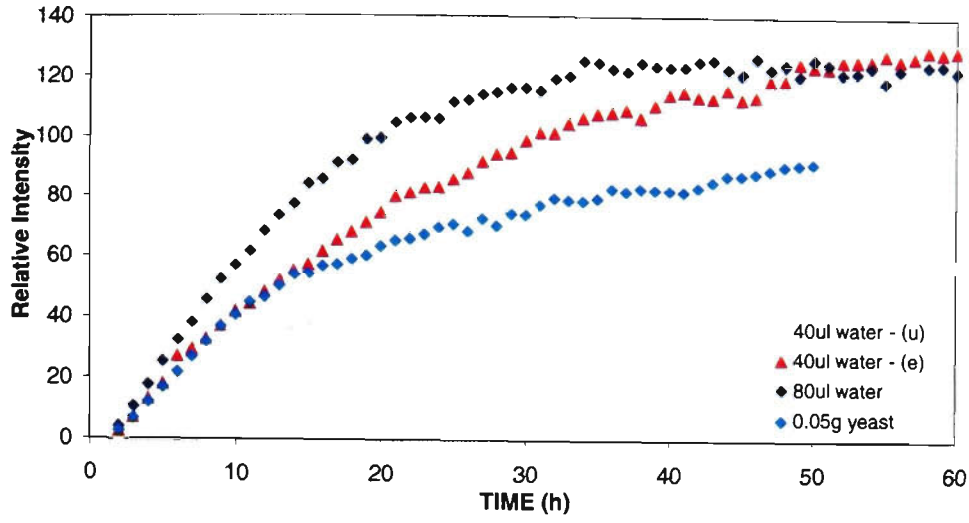


Figure 3-21 Reaction profiles of the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane with 40µl water (u) uneven water distribution( ), (e) even water distribution(▲), with 0·05g yeast(◆) and with 80µl water(◆); relative intensity of the C3 peak of ethyl 3-hydroxybutanoate-3-^{13}C compared to d_6-benzene over 60 hours.

Table 3-17 Values for the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane under standard conditions, with 40µl water (u) uneven water distribution, (e) even water distribution and with 0·05g yeast.

EXPERIMENT	CONVERSION	INITIAL RATE	MRI	CORRECTED RATE
Standard, 80µl water, 0·1g yeast	82%	8·14±0·31	152	$5·36±0·20×10^{-2}$
40µl water – (u)	62%	4·76±0·411	92	$5·17±0·45×10^{-2}$
40µl water – (e)	95%	6·66±0·73	137	$4·86±0·53×10^{-2}$
0·05g yeast	44%	5·11±0·24	211	$2·42±0·11×10^{-2}$

There is a clear difference in the profiles of the reactions containing less water, the difference being more pronounced after 10h. The reaction in which half the yeast was fully hydrated (u), shows the typical shape indicating its similarity to the standard reaction, in that after a period of activity the enzyme system is deactivated and the reaction stops. However when all the yeast was half wet (even distribution) a different profile was obtained. The reaction appeared to continue for a longer time, only slowing after 50 hours. This indicates the enzyme system is deactivating more slowly than the standard reaction resulting in a slower decay constant. The corrected initial rates for reactions containing 40µl of water are similar to that calculated for the standard reaction (Table 3-17). This indicates that addition of less than the optimum amount of water has little effect on the initial rate.

The conversion obtained for the reaction with uneven water distribution is considerably less than that obtained for the standard reaction. It was expected that this reaction would be very similar to the reaction containing half the amount of yeast (Figure 3-21). The corrected initial

rate for the reaction containing 0·05g yeast is approximately half that of the reaction with uneven water distribution indicating that even though only half the yeast was hydrated the activity is still greater than a reaction with less yeast.

These results indicate that the amount of water and the distribution of water within the yeast mass have a significant effect on the deactivation of the yeast reductase enzymes but not on initial enzyme activity.

It is also apparent that water optimisation studies need to be conducted for longer periods than 24h since smaller amounts of water can retard enzyme deactivation leading to higher conversions after extended reaction times.

3.9 *Diethyl Ether as a Solvent*

The effect of the organic solvent on the yeast reductase system was investigated to see if a different solvent resulted in a different deactivation time and decay constant. A variety of different organic solvents for the yeast-mediated reduction of ethyl 3-oxobutanoate were trialled by Jayasinghe *et al.*[61] It was found that diethyl ether gave 50% conversion in 24h. Ethyl 3-oxobutanoate-3-^{13}C was reduced with yeast in diethyl ether in an NMR tube at 20°C for 60h. The results are presented in Figure 3-22 and compared with the reaction in hexane. The initial and corrected rates are presented in Table 3-18.

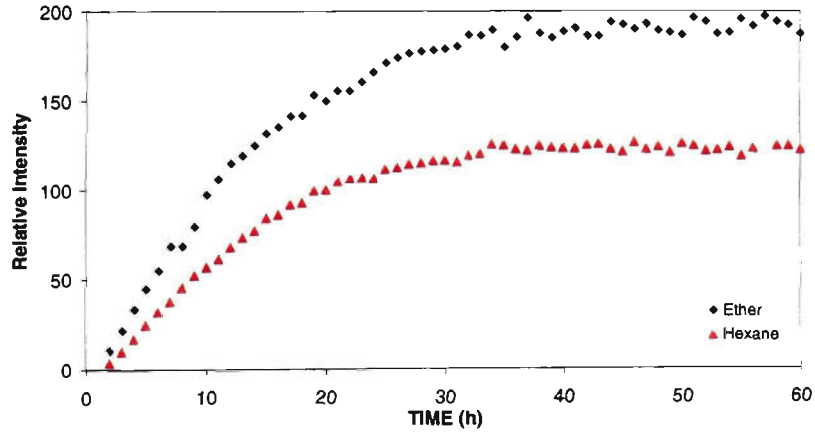


Figure 3-22 Comparison of the reaction profiles of the yeast-mediated reduction of ethyl 3-oxobutanoate in hexane and diethyl ether in an NMR tube; relative intensity of the C3 peak of ethyl 3-hydroxybutanoate-3-^{13}C compared to d_6-benzene over 60 hours.

Table 3-18 Calculated values and measured conversions of the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane and diethyl ether.

SOLVENT	INITIAL RATE	MRI	CORRECTED RATE (x10^{-2})	CONVERSION (%)
HEXANE	8·14	152	5·36	82
DIETHYL ETHER	15·0	260	5·77	74

The profiles of the two reactions are similar, as are the corrected rates although the conversion of substrate to product in diethyl ether is slightly lower. The results obtained from the reaction in diethyl ether were fitted using the computer program described earlier (Figure 3-23) and the parameters obtained are shown in Table 3-19.

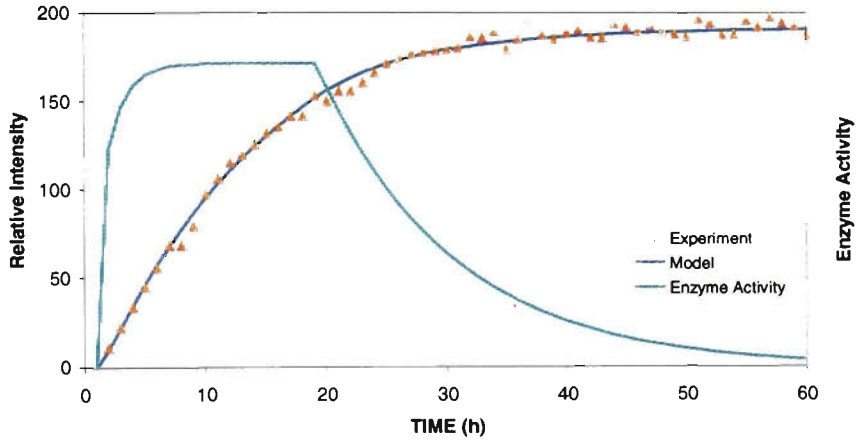


Figure 3-23 Comparison of the results from the model with the experimental data from the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in diethyl ether in a NMR tube. Model (—), enzyme activity(—), diethyl ether(▲).

Table 3-19 Comparison of the values used in the model of the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane and diethyl ether in an NMR tube.

SOLVENT	DEACTIVATION TIME (h)	DECAY CONSTANT	PRODUCT CONSTANT
HEXANE	19	11	0·89
DIETHYL ETHER	19	11	2·136

The best fit was achieved using the same deactivation time and decay constant found for the reaction in hexane. This suggests that changing the solvent has little impact on the activity of the enzyme system. A higher product constant factor, K_P, was required, indicating that it is both temperature and solvent dependent.

3.10 Competition with Other Esters

To further examine the competition experiments discussed in Chapter 2, two reactions were performed in an NMR tube in which labelled ethyl 3-oxobutanoate-3-^{13}C was combined with a different (unlabelled) ester. Methyl 3-oxobutanoate requires the same amount of yeast as the ethyl ester for complete reduction to the 3-hydroxy ester, and therefore the two esters compete equally well for the enzyme system. The *tert*-butyl ester requires 11 times as much yeast as the ethyl ester and would not be expected to compete as well. The production of ethyl 3-hydroxybutanoate-3-^{13}C under these competitive conditions is shown in Figure 3-24.

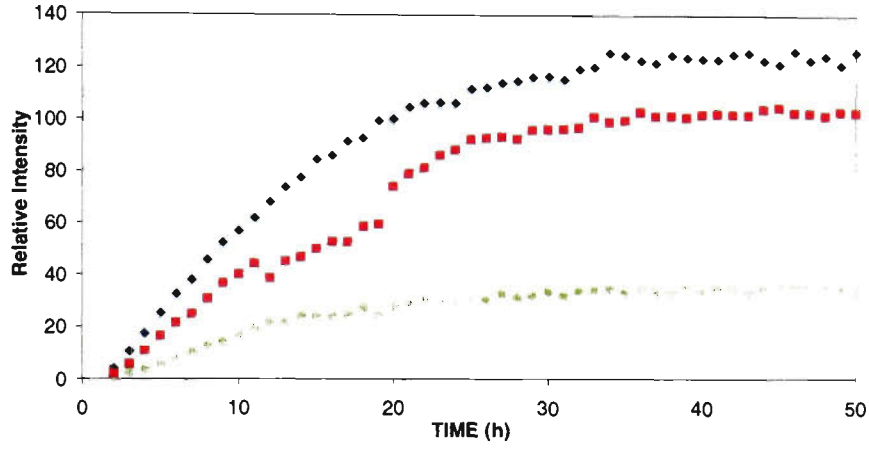


Figure 3-24 The yeast-mediated reduction of ethyl 3-oxobutanoate(◆) compared with the same reaction with the addition of methyl 3-oxobutanoate(●) or the addition of *tert*-butyl 3-oxobutanoate(■); relative intensity of the C3 peak of ethyl 3-hydroxybutanoate-3-^{13}C compared to d_6-benzene over 60 hours.

Table 3-20 Calculated values and measured conversions for the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane in the absence and presence of different esters in an NMR tube.

Experiment	Initial Rate	Conversion (%)	MRI	Corrected Rate
Standard	8·14±0·31	82	152	$5{\cdot}36\pm0{\cdot}20\times10^{-2}$
with methyl ester	1·58±0·13	24	140	$1{\cdot}13\pm0{\cdot}09\times10^{-2}$
with *tert*-butyl ester	5·06±0·34	69	149	$3{\cdot}40\pm0{\cdot}23\times10^{-2}$

The profiles of the three reactions are very similar indicating that the addition of a competitor does not alter the enzyme deactivation. The addition of a competitor does however reduce the initial rate of the yeast-mediated reduction of ethyl 3-oxobutanoate; the better competitor has the greater effect. This is consistent with the results reported in *Section 2.5*.

3.11 CONCLUSIONS

Studying the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C using NMR spectroscopy has revealed the nature of the effect of the organic solvent system on the reductase enzyme system in yeast. Exposure to the solvent system deactivates the enzyme system and slowly decreases the reaction rate until no further reduction occurs. The deactivation appears to be consistent with a gradual disruption the spacial arrangement of the enzyme active site making the binding of substrates less effective. The substrates which fit less well are more susceptible to these changes and therefore require more yeast for complete reduction to occur. At 10°C enzyme deactivation does not occur within the 60h period.

The small amount of water in the organic solvent system enables reduction to occur. The distribution of the water in the yeast biomass is critical to the extent of conversion when good mixing is not possible. The deactivation of the enzyme system occurs faster with increasing water content.

There appears to be little difference on the effect of polar (diethyl ether) and non-polar (hexane) organic solvents on enzyme deactivation, initial rate and decay constant however the product constant was much higher.

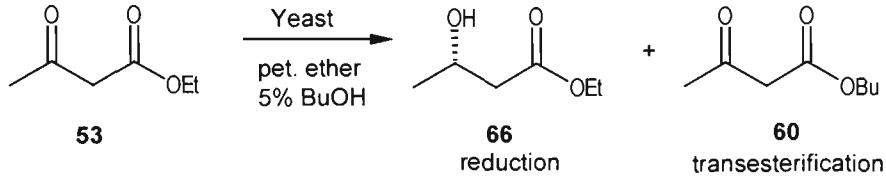
CHAPTER 4

YEAST-MEDIATED ESTERIFICATION OF CARBOXYLIC ACIDS

4. Yeast-mediated Esterification of Carboxylic Acids

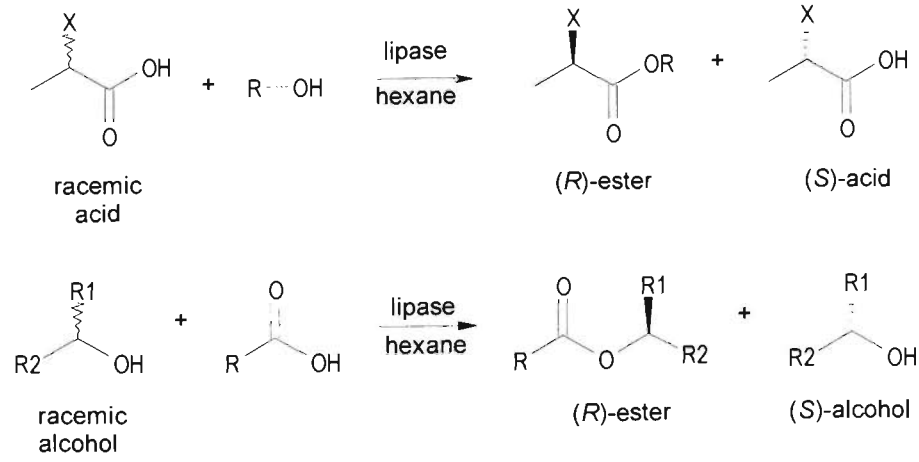
4.1 INTRODUCTION

Yeast has been extensively investigated by Smallridge *et al.*[60, 56, 92, 61] as a catalyst for reduction reactions in organic solvents. One part of this investigation focussed on the extent of reductase activity in a range of solvents and one of the more interesting results was obtained when the reduction was carried out in petroleum ether containing 5% butanol.[93] Not only had reduction taken place, but also some transesterification was observed (Scheme 4-1) suggesting that whole yeast cells might be used for transesterification and perhaps also esterification reactions.



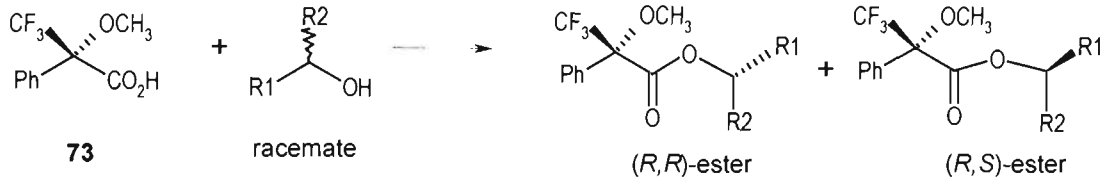
Scheme 4-1

Isolated yeast enzymes have been used extensively for esterification reactions in organic solvents with remarkable success. The yeast lipase most frequently used has been that from *Candida cylindracea* (*Candida rugosa*) but lipases from *Candida antarctica* have also been exploited. Because of their stereoselective action, these enzymes have often been used for the resolution of racemic acids[7, 94] and alcohols;[95] one enantiomer of a racemic mixture is esterified while the other enantiomer remains unaffected (Scheme 4-2).



Scheme 4-2

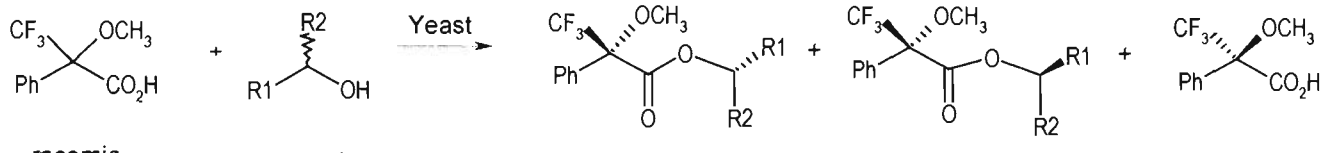
An interesting application for the esterification reaction would be to use yeast and *racemic* Mosher's acid **73** for the differentiation of alcohols. Currently a single enantiomer of Mosher's acid is esterified with a chiral alcohol to produce two diastereomers, differing in configuration at the alcohol centre (Scheme 4-3). Quantification of the two diastereomeric esters is then possible by NMR, which permits evaluation of the ee of the chiral alcohol.



73 racemate

Scheme 4-3

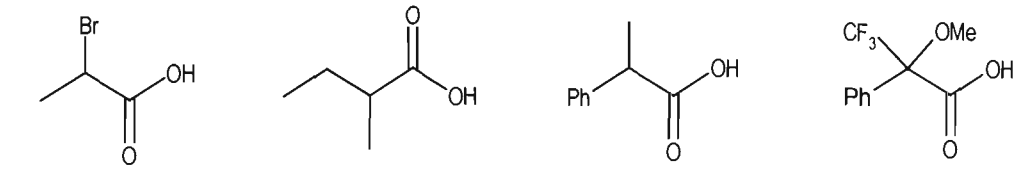
Mosher's acid must be present as a single enantiomer to ensure that only two of the possible four stereoisomers are produced, since the esterification is not stereoselective. This makes differentiation of alcohols an expensive procedure as the cost of enantiomerically pure Mosher's acid is high ($360/g). Since yeast catalysed reactions are generally stereoselective, yeast has the potential to selectively esterify one only enantiomer of the acid with both enantiomers of the racemic alcohol (Scheme 4-4). This would enable racemic Mosher's acid to be used, a considerably cheaper alternative ($43/g) to the enantiomerically pure material.



racemic Mosher's acid **73** racemate *R,R* *R,S* (*S*)-**73**

Scheme 4-4

In order to determine whether yeast is capable of stereoselectively catalysing esterification reactions, a variety of acids exhibiting different steric and electronic properties, were investigated (Figure 4-1).

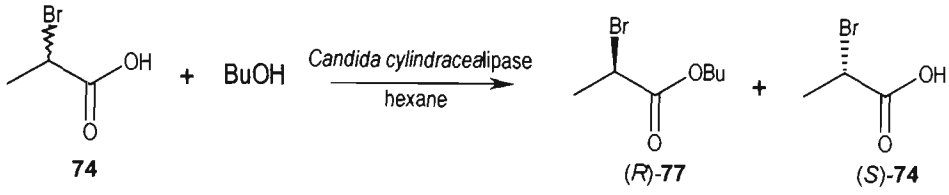


2-bromopropanoic acid **74** 2-methylbutanoic acid **75** 2-phenylpropanoic acid **76** Mosher's acid **73**

Figure 4-1 The four acids used in the present study.

4.2 ESTERIFICATION OF 2-BROMOPROPANOIC ACID

A range of 'non-natural' substrates has been used to show the ability of isolated yeast enzymes to function in anhydrous organic solvents.[96] *Candida cylindracea* lipase was employed to selectively esterify racemic 2-bromopropanoic acid **74** in the presence of butanol to yield butyl (*R*)-(+)-2-bromopropanoate **77** (Scheme 4-5). The (*S*)-enantiomer of the acid remained unchanged.



Scheme 4-5

It was shown that either enantiomer of the acid could be obtained with high optical purity by choosing when to halt the reaction. *Candida cylindracea* lipase preferentially esterifies the (*R*)-acid to the (*R*)-ester **77** before esterifying the (*S*)-acid. To obtain the (*R*)-acid the reaction was stopped at 45% conversion (6h) ensuring none of the (*S*)-ester was formed. The (*R*)-ester **77** was then hydrolysed to give the (*R*)-acid. Alternatively the reaction was allowed to proceed to 78% conversion (14.5h) and the (*S*)-acid could be isolated directly from the reaction mixture. In either situation, the unwanted product could be epimerised and hydrolysed, if necessary, with HBr to give racemic **74** again, and the resolution procedure repeated. Enzyme catalysis is much slower for esterification of the (*S*)-acid, as can be seen by the difference in conversion times, 45% in 6h and 78% in 14·5h. Excellent yields (96%) and stereoselectivity were obtained (99% ee).[96]

Given the suitability of 2-bromopropanoic acid for lipase esterification reactions in organic solvents, the esterification of this acid was chosen to study yeast catalysed esterification reactions.

4.2.1 Butyl Ester

4.2.1.1 Preparation of butyl 2-bromopropanoate

In order to follow the esterification reactions by gas chromatography and NMR, an authentic sample of racemic butyl 2-bromopropanoate **77** was prepared using *Candida cylindracea* lipase following the reported method of Kirchner *et al.*[96] A mixture of 2-bromopropanoic

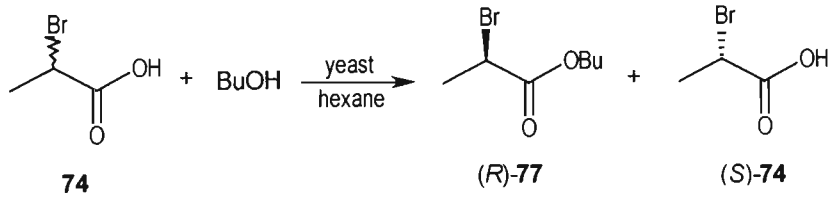
acid and a 3-fold molar excess of butanol were dissolved in hexane, and then powdered lipase was added. The suspension was stirred at room temperature for a total of 72h.

Samples were taken at 7 and 72h for analysis by gas chromatography. After 7h, 21% conversion of starting material to product had taken place and after 72h, 99% conversion had occurred. This showed that esterification was occurring at a slower rate than reported by Kirchner *et al.*[96] (45%, 6h).

Chiral gas chromatographic analysis of the reaction after 7h showed only 36% ee, whereas Kirchner *et al.*[96] reported 100% ee for the ester after 6h. The difference was most likely due to a variation in water content of the crude lipase preparation as water has been shown to affect both activity and stereoselectivity of this lipase.[97] Isolation of the product after 72h gave a high yield (83%) of a racemic product, which was used as a reference for chiral gas chromatography.

4.2.1.2 Yeast Reactions

Yeast catalysed esterification of 2-bromopropanoic acid **74** was carried out to determine if esterification would occur using whole cells instead of isolated enzymes. To facilitate direct comparisons, identical conditions to those that were used for the reaction catalysed by the isolated lipase were used for the yeast catalysed reaction. A mixture of racemic 2-bromopropanoic acid and a 3-fold molar excess of butanol were dissolved in hexane, to give a 2·75%(v/v) butanol solution, and then yeast was added in place of the lipase powder (Scheme 4-6).



74 (*R*)-**77** (*S*)-**74**

Scheme 4-6

Samples were removed every 24h for gas chromatographic analysis in order to follow the progress of the reaction (Table 4-1). The amount of ester increased slowly over the three-day period, but after 72h the amount of esterification achieved (8%) was low compared to the result obtained from the isolated enzyme reaction (99%). It has been found in other yeast reactions employing organic solvents that a small amount of water is required to activate the

yeast.[60, 56, 59] It was thought that the low activity of the yeast in this reaction might be due to the absence of water. However, the addition of 0·8ml of water to the reaction did not appear to activate the yeast and in fact, the amount of conversion fell slightly.

Table 4-1 Yeast esterification of 2-bromopropanoic acid with butanol

HOURS	CONVERSION*
24	2%
48	5%
72	8%
Addition of H_2O	
96	7%

*gas chromatographic ratio of ester to acid

Yeast is obviously a more complex system than an isolated enzyme and the conditions enabling esterification for one system are not necessarily going to be optimal for the other. It was important to optimise the conditions for the yeast catalysed esterification reaction, ie. ratio of substrates to yeast, amount of water present, etc.

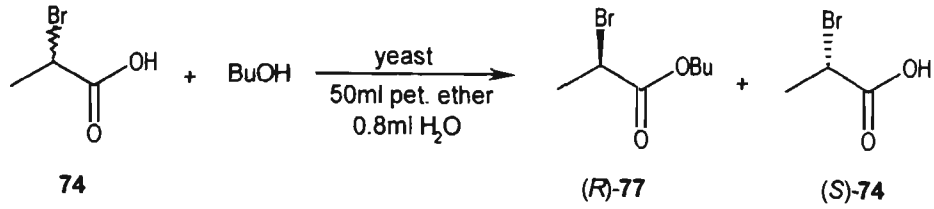
The actual quantity of active lipase enzyme present in the crude lipase is unknown. Kirchner *et al.*[96] mentions that the powder they used contained less than 1% of the enzyme and it is likely the lipase powder used for this study was of a similar purity. Dried bakers' yeast is similar in the sense that the majority of the weight is inert, however there appears to be even less information about the quantity of lipase available. It was shown in the present study (see *Chapter 2*) that with yeast catalysed reduction reactions, the maximum ratio of substrate to yeast which would permit complete reduction was 1mmole substrate/g yeast, however for many substrates more yeast is required for complete consumption of starting material. It is not surprising therefore that using 1g of yeast to esterify 1g of 2-bromopropanoic acid resulted in little reaction. It was useful nevertheless to establish that there was less lipase activity in dried bakers' yeast than in the crude lipase powder. A reaction was performed in duplicate using conditions previously optimised for yeast-mediated reduction reactions and the level of butanol was set at 5% since it had been shown that yeast retained its activity at this concentration.[93] One gram of yeast was added to a mixture of 1mmol of 2-bromopropanoic acid with 50ml petroleum ether, containing 5% butanol and 0·8ml water, and the reaction was stirred for 24h at room temperature. The reactions showed 15% (average) conversion by gas chromatography. A reaction was also carried out without yeast

and no ester was detected, indicating that the reaction was in fact yeast catalysed. It was promising however, that some esterification occurred with the yeast and water present.

The same reaction was performed in the absence of water and no ester was detected. Comparing this result to the initial result from the yeast reaction performed without water (Table 4-1), after 24h, 2% ester was expected. The main difference between the reactions was the amount of substrate, 1mmol (153mg) instead of 1g and assuming the degree of conversion was the same, the ester produced should have been detectable (3mg). The fact that none was detected appears to indicate that the reaction rate has slowed in the presence of the lesser amount of substrate.

Conducting the reaction with 5% (2·7mmol) butanol gave only a low conversion to ester. To determine whether altering the quantity of butanol would increase the conversion, a series of reactions with amounts of butanol varying from 0·5-20mmol was conducted. For these reactions, an internal standard (tetradecane) was added to allow ready comparison between different reaction conditions. Results are shown in Figure 4-2.

Inclusion of an internal standard showed the gas chromatographic response factor of the 2-bromopropanoic acid was *much* smaller than that of the butyl 2-bromopropanoate. The results shown in Table 4-1 were simply a gas chromatographic ratio of peak areas which resulted in a falsely high conversion. All future reactions were conducted with an internal standard enabling true conversions to be determined.



74 (*R*)-**77** (*S*)-**74**

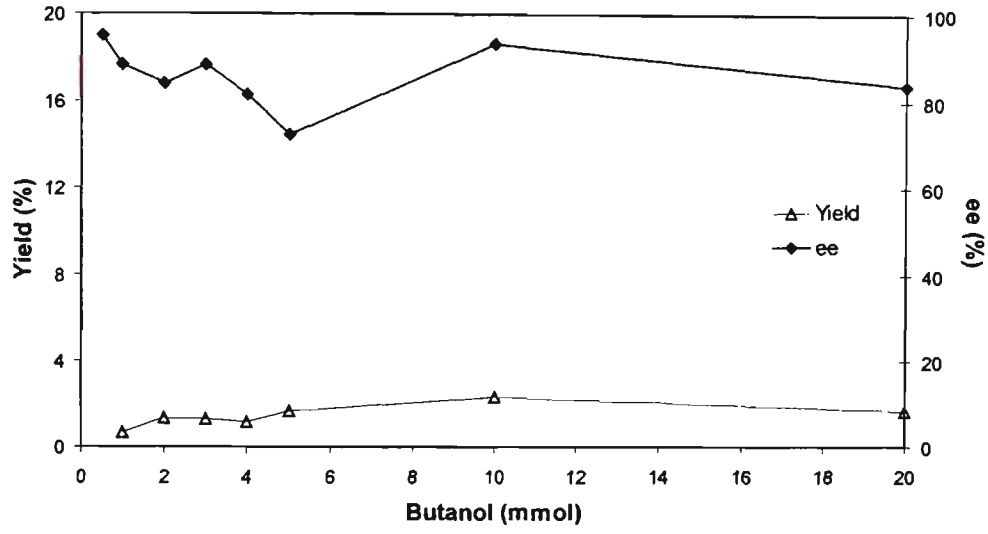


Figure 4-2 Yield and ee of the butanol optimisation of the yeast esterification of 2-bromopropanoic acid. Reaction contained 1mmol 2-bromopropanoic acid, 50ml petroleum ether, 0·8ml H_2O and 2µl tetradecane (internal standard). The mixture was stirred for 24h and gas chromatographic analysis was performed.

The yield of ester did not rise above 2·5% irrespective of the amount of butanol added to the reaction. Examination of the reaction mixture using chiral gas chromatography showed the ee lay in the range 72-95% indicating that there is no relationship between the quantity of butanol and the stereoselectivity of the reaction. Although somewhat variable, these results give a clear indication that yeast can behave stereoselectively when catalysing esterification reactions. The amount of ester produced was low compared to the isolated enzyme reaction and so further optimisation of the reaction conditions was undertaken.

Water has been shown to play an important role in enzyme reactions conducted in organic solvent systems and although 0·8ml water/g yeast has been found to be optimum for reduction reactions, it was necessary to determine the appropriate ratio for esterification reactions. The yeast catalysed esterification of 2-bromopropanoic acid was carried out at both 20mmol and 3mmol of butanol with varying amounts of water. The results are shown in Figure 4-3.

Again the yield of ester did not rise above 2·5% at either 3 mmol or at 20 mmol of butanol at all water concentrations. This indicates that under these conditions – that is, 1mmol 2-

bromopropanoic acid, 1g yeast, 50ml petroleum ether, the maximum amount of ester produced is 2·5%. Varying amounts of butanol or water did not improve ester production.

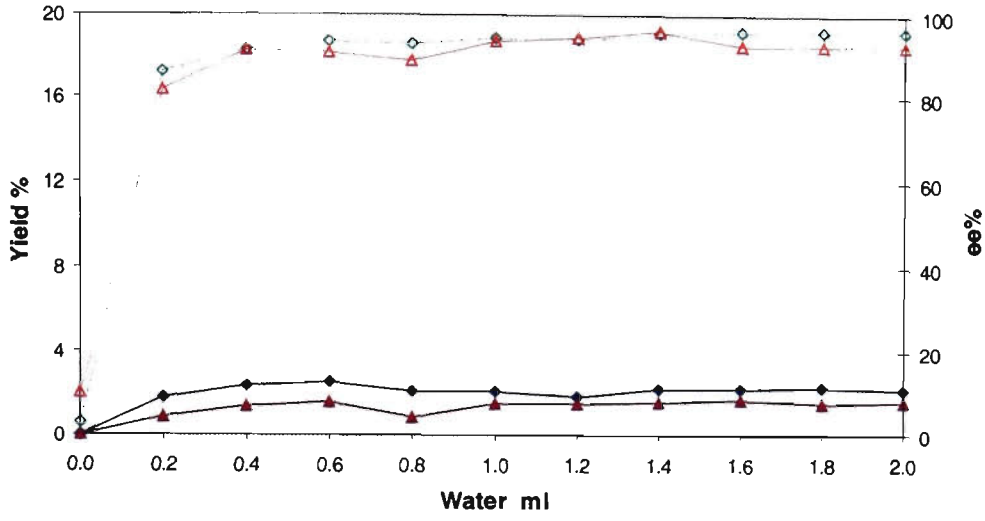
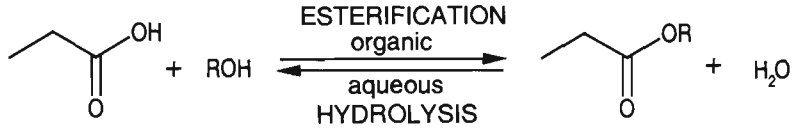


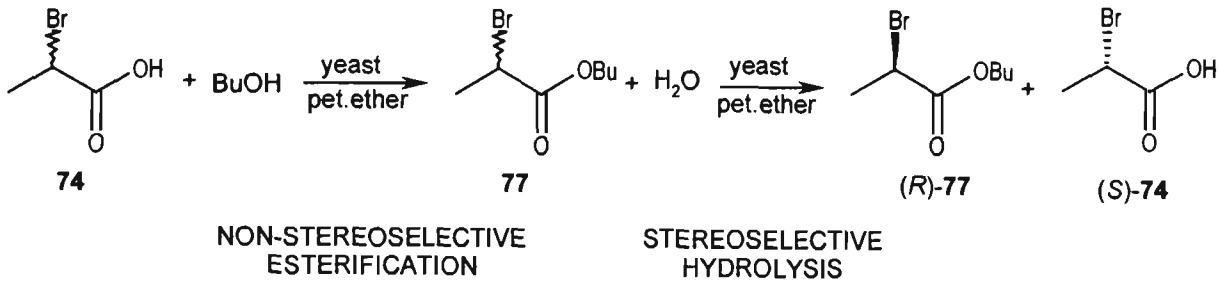
Figure 4-3 Yield(closed) and ee(open) of the water optimisation of the yeast esterification of 2-bromopropanoic acid with 3mmol(◆) and 20 mmol(▲) of butanol. Reaction contained 1mmole 2-bromopropanoic acid, 50ml petroleum ether, 3 or 20 mmol butanol and 2μl tetradecane (internal standard). The mixture was stirred for 24h and gas chromatographic analysis was performed.

Whilst altering the amount of water in the reaction had very little effect upon the yield of ester, it did significantly affect the stereoselectivity of the reaction. In the absence of water a racemic product was formed, and as the amount of water was increased the ee of the ester improved until it was close to 100%. The dependence of ee upon water quantity suggests that stereoselective hydrolysis may be occurring. It is known that lipase enzymes are capable of catalysing both esterification and hydrolysis reactions.[54] In an organic solvent environment, organic acids can be esterified and in an aqueous environment, esters are hydrolysed (Scheme 4-7).



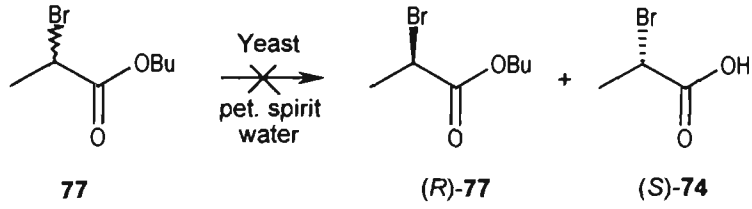
Scheme 4-7

If yeast is catalysing both esterification and hydrolysis, it follows that non-stereoselective esterification occurs because the ee of the product from the reaction containing no water is almost zero. As the quantity of water/g yeast is increased, the hydrolysis reaction becomes possible, but esterification continues, as the majority of the solvent is still organic. The ee of the product increases as the amount of water increases, suggesting that hydrolysis by yeast is a stereoselective process (Scheme 4-8).



Scheme 4-8

To test this hypothesis, racemic butyl 2-bromopropanoate was exposed to the yeast reaction system for 24h. No acid was detected, nor had the amount of ester decreased, indicating that hydrolysis of the ester, stereoselective or otherwise, was not occurring (Scheme 4-9).



Scheme 4-9

Another possible explanation of the change in ee with water quantity is that multiple enzymes are responsible for the esterification reaction, one set esterifying the (*R*)- and the other the (*S*)-enantiomer. Multiple enzymes have been known to catalyse the same reaction but with different stereoselectivity.[27] Nakamura *et al* isolated seven reductase enzymes from yeast and reacted them with various α-keto esters in water. They found that some enzymes produced the (*R*)-enantiomer of a particular substrate while other enzymes formed the (*S*)-enantiomer. It is likely that a similar mechanism is operative for the yeast catalysed esterification of 2-bromopropanoic acid. Yeast can be regarded as containing two enzymes, one that esterifies the (*R*)- and one that esterifies the (*S*)-acid. Close examination of the chiral gas chromatography results demonstrates the amount of the lesser enantiomer is unaffected by the amount of water in the reaction, and remains constant at a low level (Figure 4-4). The other enantiomer is produced more readily with increasing quantities of water. The enzyme or enzymes producing the dominant enantiomer are still working with no water present, but are greatly activated with the addition of water.

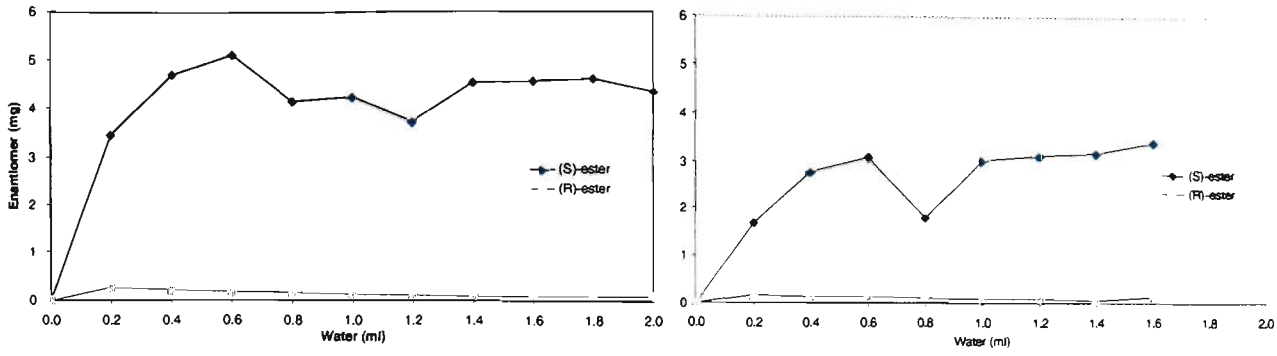


Figure 4-4 Comparison of the amount of each enantiomer of butyl 2-bromopropanoate formed over a range of water amounts in the yeast esterification reaction.

Enantioselectivity of the esterification of 2-bromopropanoic acid by *Candida cylindracea* is also affected by water content when performed in hexane.[97] Kitaguchi *et al.* found the addition of a small amount of water to the reaction improved the ee of the ester produced from 80% (no added water) to 95%. They hypothesised the enhanced stereoselectivity was due to the increased conformational flexibility gained from hydrogen bonding between the enzyme and the water. It has been determined that enzymes have the same conformation in an anhydrous organic solvent as they have in the dry, solid form, indicating that the enzyme is 'trapped' in a fixed conformation. It is thought that the rigid conformation of the enzymes cannot differentiate between enantiomers and is therefore not stereoselective. The addition of some water overcomes this rigid state and allows enantioselective esterification to occur. It is possible that this is also the case with yeast esterification, and that water is not only required for activity but also for effective enantioselectivity.

Overall, the production of butyl 2-bromopropanoate was not as high as expected. Varying the amounts of water or butanol added to the reaction did not improve the conversion. It has been shown in other yeast reactions that the extent of a reaction can be increased by using more yeast. A reaction containing 5g yeast instead of 1g was carried out and gas chromatographic analysis after 24h showed only a small amount of ester present although no 2-bromopropanoic acid remained. This was unusual as it shows the acid is being consumed but not converted to the ester. This suggests the low conversions obtained could be due to a decrease in the quantity of acid remaining in the solution after 24h.

4.2.1.3 Loss of 2-bromopropanoic acid

Re-examination of the gas chromatography traces from previous reactions indicated that under certain conditions, the amount of 2-bromopropanoic acid remaining in solution after 24h had decreased dramatically (Figure 4-5). The addition of just 0·2ml of water to the reaction resulted in over half the original amount of acid disappearing. Reactions containing

more water showed similar losses with the quantity of acid slightly decreasing as the amount of water present increased. This indicates the addition of water has a large impact on the amount of acid remaining in solution after 24h.

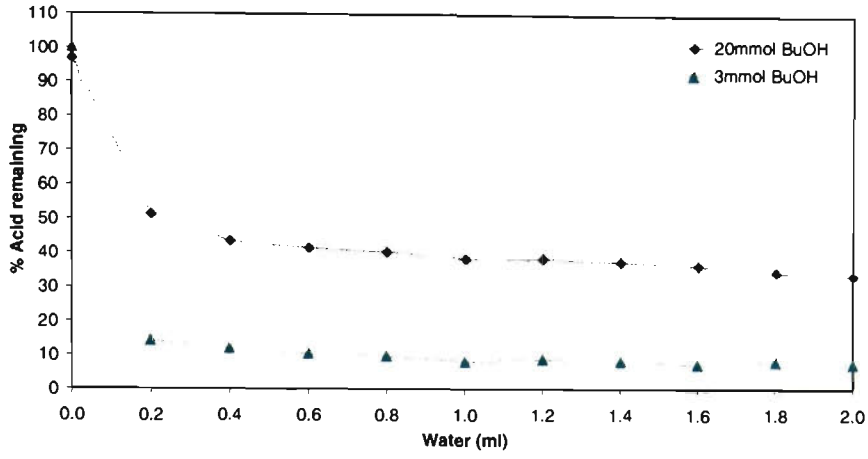


Figure 4-5 Amount of 2-bromopropanoic acid remaining in solution after 24h with 3mmol butanol (▲) and 20mmol butanol (◆). Reactions contained 1mmol 2-bromopropanoic acid, 50ml petroleum ether, 3 or 20 mmol butanol and 2μl tetradecane (internal standard). The mixture was stirred for 24h and gas chromatographic analysis was performed.

Conversely, increasing the amount of butanol in a reaction increased the amount of 2-bromopropanoic acid remaining in solution after the 24h period (Figure 4-6). A reaction was also performed in neat butanol which resulted in no loss of 2-bromopropanoic acid after 24h, however no ester was formed either.

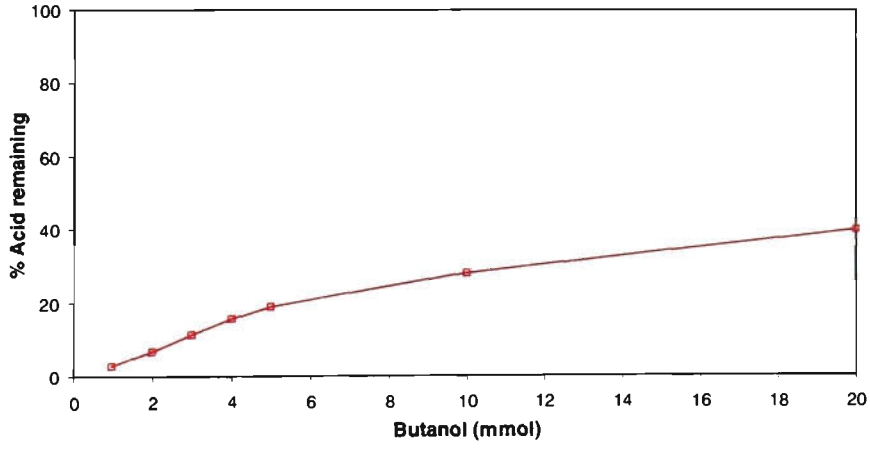


Figure 4-6 Amount of acid remaining in solution after 24h exposure to the yeast in the organic solvent system. Reaction contained 1mmol 2-bromopropanoic acid, 50ml petroleum ether, butanol, 0.8ml water and 2μl tetradecane (internal standard). The mixture was stirred for 24h and gas chromatographic analysis was performed.

These findings indicate that both butanol and water affect the amount of 2-bromopropanoic acid in solution after 24h. A series of reactions was carried out in order to eliminate other

components of the reaction system that may also be affecting the loss of acid (Table 4-2). Butanol was not included in these reactions for two reasons; to maximise the loss of acid as butanol inhibits loss, and to ensure that any loss of acid is not due to esterification.

Table 4-2 Amount of 2-bromopropanoic acid remaining after 24h exposure to an organic solvent under various reaction conditions.

	REACTION	% ACID REMAINING
1	Petroleum ether	100
2	Petroleum ether, yeast	55
3	Petroleum ether, water	60
4	Petroleum ether, water, yeast	3

All reactions contained 1mmol 2-bromopropanoic acid, 50ml petroleum ether and 2µl of internal standard. Reactions containing water had 1·0ml added and reactions containing yeast had 1g added.

2-Bromopropanoic acid is stable in petroleum ether as shown by line 1 in Table 4-3. Adding yeast to the petroleum ether reduced the amount of acid left in solution after 24h to almost one half (55%), indicating clearly the observed loss of acid is due to a mechanism other than esterification with the added alcohol. This is quite a different result from that observed in the reactions with 3mmol and 20mmol butanol, performed in the absence of water; 97 and 100% 2-bromopropanoic acid remaining after 24h, respectively (Figure 4-5, 1st points). This suggests that butanol inhibits any loss of 2-bromopropanoic acid that is due to interaction with yeast. The 2-bromopropanoic acid could potentially be adsorbed or esterified onto the yeast.

In the reaction containing water and petroleum ether, 60% of the acid remained after 24h. The water forms a separate layer at this concentration and only the petroleum ether layer is sampled for gas chromatographic analysis. This suggests that the loss is due to the acid partitioning into water.

When water and yeast were added in combination to the petroleum ether, very little acid remained in solution after 24h. The loss of 2-bromopropanoic acid appears to be related to yeast activity; once the yeast has been activated by the addition of water, the maximum amount of 2-bromopropanoic acid is lost.

In order to determine the extent to which these factors could be controlled a series of reactions were conducted (Table 4-3).

Table 4-3 Amount of 2-bromopropanoic acid remaining after 24h exposure to yeast in an organic solvent with various additives.

	Reaction	% Acid Remaining
1	Petroleum ether, dioxane/water, yeast	3
2	Petroleum ether, sat. NaCl solution, yeast	9
3	Petroleum ether, HCl, yeast	56

All reactions contained 1mmol 2-bromopropanoic acid, 50ml petroleum ether and 2µl of internal standard and 1g yeast. Reaction 1 also contained 1ml water and 1ml dioxane. Reactions 2 and 3 contained 1ml sat NaCl and 1ml of 2M HCl, respectively.

The simplest explanation is that the 2-bromopropanoic acid is partitioning into the water present in the reaction. To overcome this problem, dioxane was added along with the water to increase the miscibility of the water in petroleum ether. Analysis by gas chromatography after 24h showed there was no real difference in the quantity of 2-bromopropanoic acid lost compared with the reaction performed in the absence of dioxane. Addition of a saturated salt solution was another method of changing the amount of 2-bromopropanoic acid partitioning into the water, however that also proved ineffectual. These results suggest the loss of 2-bromopropanoic acid is not due to increased solubility in water compared to petroleum ether.

Yeast is mildly basic so that when water is added, the 2-bromopropanoic acid may potentially be converted to the carboxylate salt that would not be soluble in petroleum ether and therefore not be detected by gas chromatography. Addition of dilute acid to the reaction instead of water would decrease the pH, thus preventing salt formation and maintaining the 2-bromopropanoic acid in the petroleum ether. The addition of 2M HCl in place of water made the most significant difference to the amount of 2-bromopropanoic acid remaining after 24h, 50% compared to 5% remaining with the addition of just water. Formation of the carboxylate salt is therefore a possible reason for loss of half the 2-bromopropanoic acid in the yeast catalysed esterification reaction.

It was thought that the remainder of the acid could be bound to the solid yeast, either with the water associated with the yeast or with the solid yeast itself. To investigate these possibilities, a larger scale reaction was carried out and various isolation procedures were performed to see what was required to remove 2-bromopropanoic acid from the yeast. With normal extraction

procedures, washing the yeast with 3x50ml ethyl acetate, 25% (distilled) of the 2-bromopropanoic acid could be recovered. Since gas chromatography of the filtered reaction mixture revealed only 5% of the 2-bromopropanoic acid, clearly a significant amount of the material was bound to the hydrated yeast and easily washed out with an organic solvent.

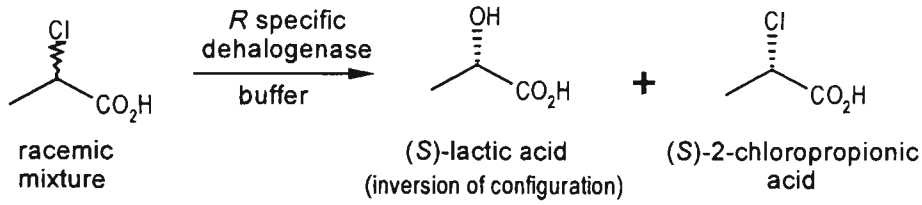
Further washing of the solid yeast with 2M HCl (300ml) followed by extraction of the aqueous phase with ether afforded a further 25% of the 2-bromopropanoic acid. In total, 50% of the 2-bromopropanoic acid could be recovered after 24h exposure to the yeast/solvent system with washing with ethyl acetate and then 2M HCl. This agreed with the gas chromatography result obtained when 2M HCl was used instead of water in the small scale reactions (Table 4-4, line 3). The whereabouts of the other 50% however remain unresolved.

The optical rotation of the recovered acid, in the absence of added butanol, was found to be zero, indicating that the loss of acid was not stereoselective.

The remaining 50% of the 2-bromopropanoic acid may be non-reversibly esterifying onto sites within the yeast, so by pretreating the yeast with another organic acid, acetic acid, any sites that could be esterified should be occupied, leaving the 2-bromopropanoic acid available to be esterified with butanol. Acetic acid (1 mmol) was added to the yeast, water and petroleum ether mixture and was stirred for 24h then 2-bromopropanoic acid (1mmol) was added and left to stir for a further 24h. Gas chromatographic analysis showed a small amount of 2-bromopropanoic acid (3-4%) remaining in solution. Addition of acetic acid and 2-bromopropanoic acid at the same time also gave a similar result. This approach was not preventing reaction from occurring, so instead of using a different organic acid, the amount of 2-bromopropanoic acid was doubled with the intention that 1 mmol would react with the yeast while the other would be left for esterification with butanol. However, this was not the case with only 13% of the acid left after 24h. This suggests the loss of 2-bromopropanoic acid is a far more complex than simply esterifying the yeast and the addition of even greater quantities of acid would be required for 1 mmol to be available for esterification.

Reactions performed with 2mmol of 2-bromopropanoic acid with butanol resulted in no esterification occurring. This was an unexpected result since 1mmol of acid forms some ester, albeit a rather small amount. The larger quantity of acid is having a detrimental effect on the activity of the yeast enzymes suggesting there is a pH limit for yeast enzyme activity.

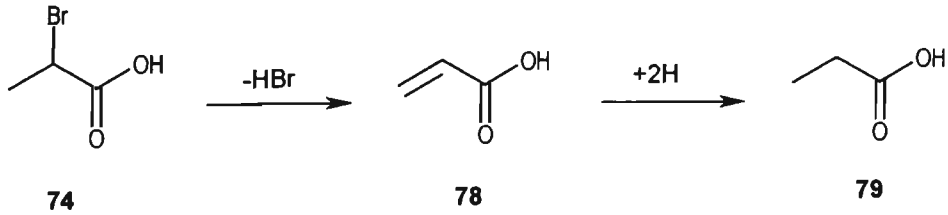
The 2-bromopropanoic acid may undergo other reactions catalysed by yeast. Generally in organic solvents, there has been little evidence of side reactions occurring, however yeast does contain multiple enzymes and unexplored reactions are possible. One of these alternative reactions could be dehalogenation. Racemic 2-chloropropanoic acid has been resolved by a dehalogenase enzyme from *Pseudomonas putida*[98] (Scheme 4-10).



Scheme 4-10

The resolution resulted in a mixture of (*S*)-lactic acid and (*S*)-2-chloropropanoic acid. If the same enzyme is at work in the yeast reaction with 2-bromopropanoic acid then the result would also be (*S*)-lactic acid and (*S*)-2-bromopropanoic acid. This type of resolution could explain the increased ee when water was added as dehalogenase enzyme activity may require water as do other enzymes present in yeast. No lactic acid was observed on the gas chromatograms of the reaction products.

Another possible scheme is the elimination of HBr to give acrylic acid **78** which may further react to give propanoic acid **79** (Scheme 4-11). No trace of either acrylic acid or propanoic acid could be detected by gas chromatography from a reaction sample.



Scheme 4-11

All attempts to determine the fate of the remaining 50% of the 2-bromopropanoic acid by changing reaction conditions have been unsuccessful. The reason for the low conversion of acid to ester must remain unanswered until the circumstances surrounding the loss of starting material are clarified. Further work would require more intrusive methods, such as labelling the substrate and following the reaction by NMR.

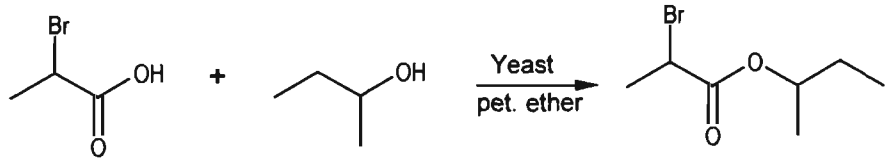
4.2.2 Reactions with Other Alcohols

Two other alcohols, *sec*-butanol and benzyl alcohol, were also investigated as substrates for the yeast catalysed esterification of 2-bromopropanoic acid. *sec*-Butanol was chosen because it is a simple, readily available alcohol which contains a chiral centre that could be used to test the stereoselectivity, with respect to the alcohol, of yeast catalysed esterifications. Benzyl alcohol was chosen because it has different electronic properties from either butanol or *sec*-butanol which may result in different reactivity.

In order to follow yeast catalysed esterifications, authentic samples of the esters were prepared using standard literature procedures.[99]

4.2.2.1 Yeast Esterifications with *sec*-Butanol

A preliminary reaction was carried out using the reaction conditions previously optimised for the butyl ester, 1mmol 2-bromopropanoic acid **74**, 10mmol *sec*-butanol (1·8%), 0·6ml water, 1g yeast in 50ml petroleum ether (Scheme 4-12). Analysis by gas chromatography showed only 2-bromopropanoic acid present after 24h. The esterification with butanol showed the amount of water present influenced both the activity and the stereoselectivity of the reaction. A series of reactions was carried out containing 0-2·0ml of water. No esterification was detectable by gas chromatography for any of the reactions.



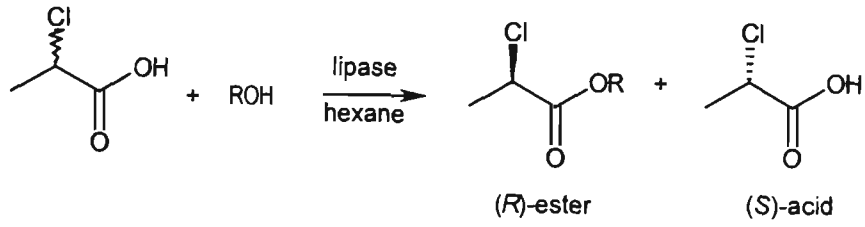
74 *sec*-butanol **80**

Scheme 4-12

To test whether the ester **80** was being hydrolysed back to 2-bromopropanoic acid **74** and alcohol, *sec*-butyl 2-bromopropanoate was exposed to the reaction conditions. No loss of ester was detected. Due to the apparent lack of reactivity of *sec*-butanol, esterification of 2-bromopropanoic acid with *sec*-butanol was not pursued further.

It was surprising to note the lack of reactivity of the *sec*-butanol with the 2-bromopropanoic acid with the yeast, compared to butanol. Bodnár *et al.*[7] found that the structure of the alcohol

had a significant effect of the reaction rate and enantioselectivity of the yeast lipase esterification of 2-chloropropanoic acid (Scheme 4-13).



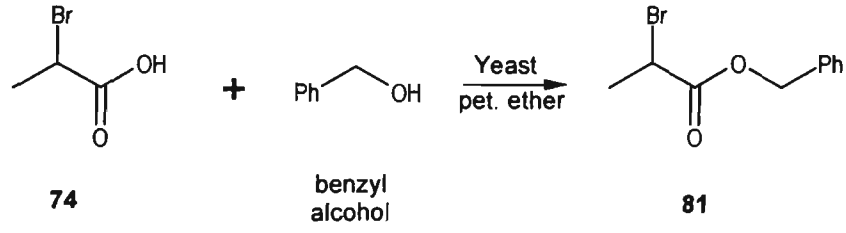
R = *sec*-butyl, butyl, 2-ethylhexanol

Scheme 4-13

Butanol reacted three times faster than *sec*-butanol (61% and 17·5% respectively). Bodnár *et al.* also tested other branched alcohols and since 2-ethylhexanol gave the same yield as butanol, it is difficult to speculate why some alcohols react and others do not.

4.2.2.2 Yeast Esterifications with Benzyl Alcohol

As with the attempted esterification of *sec*-butanol a preliminary reaction was performed with benzyl alcohol using conditions previously optimised for butanol (Scheme 4-14).



Scheme 4-14

A small amount of ester **81** was produced (<1%) and therefore a series of reactions were carried out with varying amounts of benzyl alcohol, 0·5-5·0%. Very little ester was formed in any of the reactions and therefore the attempted esterification of 2-bromopropanoic acid **74** with benzyl alcohol was also discontinued.

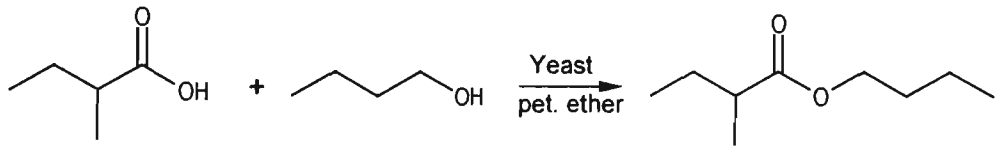
Whilst the yeast catalysed esterification with benzyl alcohol was somewhat more promising, the problem with the loss of 2-bromopropanoic acid must be solved before the difference in selectivity between alcohols can be determined.

In order to determine if the loss in the yeast reaction was specific to 2-bromopropanoic acid, other acids were tested.

4.3 ESTERIFICATION OF 2-METHYLBUTANOIC ACID

The resolution of 2-methylbutanoic acid has been successfully performed using *Candida cylindracea* lipase in cyclohexane.[100] Esterification with heptanol resulted in 85% ee of the (*R*) acid. Hence, 2-methylbutanoic acid was also used to study yeast catalysed esterification reactions.

A preliminary study was performed on 2-methylbutanoic acid to determine if it would be lost, as the 2-bromopropanoic acid had been in the yeast reaction. It was established that no loss was occurring so a series of yeast reactions with varying amounts of butanol (0·1-5·0%) was performed (Scheme 4-15).



75 **82**

Scheme 4-15

Analysis by gas chromatography showed no butyl ester **82** was formed. Another series of reactions was then carried out, this time varying the amount of water (0-2ml) in the reaction at two different levels of butanol, 0·5 and 5·0%. None of the reactions produced any butyl 2-methylbutanoate **82**.

To rule out the possibility that the ester **82** was being hydrolysed as soon as it was formed the ester **82** was exposed the same reaction conditions, however no acid **75** could be detected by gas chromatography.

Since no esterification was occurring, yeast reactions with 2-methylbutanoic acid and butanol were not pursued further.

4.4 ESTERIFICATION OF 2-PHENYLPROPANOIC ACID

A preliminary study was performed on 2-phenylpropanoic acid **76** to determine if it would be lost as the 2-bromopropanoic acid had in the yeast reaction. It was established that no loss was occurring so a series of yeast reactions with varying amounts of butanol (0·1-5·0%) was

CHAPTER 5

EXPERIMENTAL SECTION

5. Experimental Section

5.1 General

All gas chromatography was performed on a Shimadzu GC-17A with FID. The column used for the alkyl 3-oxobutanoates was an HP-1 (12m x 0·22 mm) with a thickness of 0·33μm and a non-polar, crosslinked, methylsiloxane phase. The column used for 2-bromopropanoic, 2-methylbutanoic, 2-phenylpropanoic acids and their esters was a BP-1 (15m x 0·22 mm) with a thickness of 0·25μm and a non-polar, bonded, dimethyl polysiloxane phase. The column used for acrylic and propanoic acid was a BP-10 (12m x 0·22 mm) with a thickness of 0·33μm and a moderately polar, bonded, 14% cyanopropylphenyl, 85% dimethyl polysiloxane phase. The column used for lactic acid was a BP-20 (12m x 0·22 mm) with a thickness of 0·33μm and a polar, bonded, polyethylene glycol phase.

Chiral gas chromatography was performed directly on butyl 2-bromopropanoate and on the trifluoroacetates of the reduced 3-oxobutanoates as described in the text. The chiral column was a Chiraldex G-TA (30m x 0·25 mm) with a thickness of 0·125μm and a cyclodextrin phase.

Bulb to bulb distillations were performed on a Buchi GKR-50.

300MHz ^{1}H and 75.43MHz ^{13}C NMR were recorded on a Bruker DPX300 instrument and refer to deuterochloroform solutions with tetramethylsilane as the internal reference (δ=0·00 ppm).

125.721MHz ^{13}C NMR were recorded on a Bruker DRX500 instrument and refer to hexane/ d_6-benzene (5% v/v) solutions with tetramethylsilane as the internal reference (δ=0·00 ppm).

Optical rotation measurements were carried out on a Optical Activity Polaar 2000-AA series polarimeter.

"Mauripan-Instant Dry Yeast" (*Saccharomyces cerevisiae*) was obtained from Mauri Foods Ltd, Australia and stored at room temperature with no precaution to preclude oxygen or water. No evidence of decreased activity was found with prolonged storage.

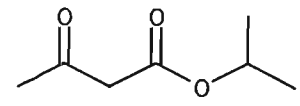
Petroleum ether refers to the fraction boiling at 40-60°C and was used without further purification.

Methyl 3-oxobutanoate **17**, benzyl 3-oxobutanoate **62**, *tert*-butyl 3-oxobutanoate **60**, (S)-(+)-2-butanol, 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one **63**, Mosher's acid **73**, 2-bromopropanoic acid **74**, 2-methylbutanoic acid **75** and 2-phenylpropanoic acid **76** were purchased from Sigma Aldrich and used without further purification. Ethyl 3-oxobutanoate-3-^{13}C and ethyl 3-oxobutanoate-1,3-^{13}C were purchased from ISOTECH.

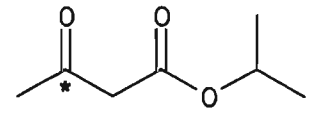
The alcohols used for the preparation of the labelled 3-oxobutanoates were dried used Mg/I, refluxed for 2h then distilled onto 3Å molecular sieves under nitrogen.[101]

Solid Superacid[85] **(Sulphated SnO_2)**: An aqueous solution of tin(II) chloride dihydrate (26g in 200ml) was prepared and adjusted to pH 8 with aqueous ammonium hydroxide (25ml). The yellowish precipitate was washed with water and dried at 110°C overnight to give tin(II) hydroxide (16.3g). To this powder (5g) sulfuric acid (25ml, 2N) was added, allowed to equilibrate for 2h, then evaporated to dryness and calcinated at 500°C for 4h to give the catalyst.

5.2 PREPARATION OF 3-OXOBUTANOATES



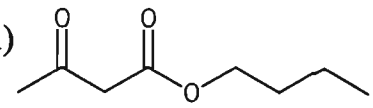
Isopropyl 3-oxobutanoate 59; 2,2,6-Trimethyl-4*H*-1,3-dioxin-4-one **63** (5g, 0·035 mol) was added to isopropanol (20 ml) with a catalytic quantity of *p*-toluenesulfonic acid and refluxed for 4h. Removal of isopropanol under reduced pressure gave a mixture of starting material and product. The product was isolated by forming the copper chelate followed by recrystallisation and regeneration of the desired 3-oxobutanoate using the procedure described by White *et al.*[73] Bulb to bulb distillation (100°/20mm) gave isopropyl 3-oxobutanoate (0·9g, 17%). lit.[102] b.p. 69°/11mm. ^{1}H NMR δ 1·28, d, *J* 6·2 Hz, $(CH_3)_2$; 2·28, s, H4; 3·43, s, H2; 5·08, sept, *J* 6·2 Hz, CH. ^{13}C NMR δ 21·08, $(CH_3)_2$; 29·47, C4; 49·88, C2; 68·41, CH; 166·06, C1; 200·19, C3.



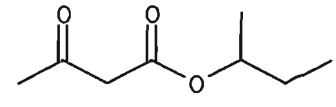
Isopropyl 3-oxobutanoate-3-^{13}C 59; Isopropyl alcohol (5ml) was added to ethyl 3-oxobutanoate-3-^{13}C (0·197g, mmol) with a catalytic quantity of solid superacid in a stainless steel sealed reaction vessel. The mixture was heated

at 145°C for 24h and purified using bulb-to-bulb distillation (80°C/20mm) to give isopropyl 3-oxobutanoate-3-^{13}C (0·136g, 65%). ^{1}H NMR δ 1·28, d, *J* 6·2 Hz, $(CH_3)_2$; 2·28, d, $J_{H\text{-}C}$ 6·0 Hz, H4; 3·44, d, $J_{H\text{-}C}$ 6·3 Hz, H2; 5·08, m, *J* 6 Hz, CH. The chemical shifts were identical to an authentic sample of unlabelled material.

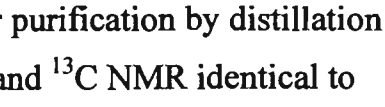
Butyl 3-oxobutanoate 60; Ethyl 3-oxobutanoate **53** (15g, 0·125 mol) was refluxed in butanol (50ml) and *p*-toluenesulfonic acid (100mg) for 2hrs. Removal of the butanol gave a mixture of butyl 3-oxobutanoate and the enol ether. Bulb to bulb distillation (110°C/20mm) also returned a mixture of butyl 3-oxobutanoate and the enol ether. It was not possible to readily separate these compounds so the 3-oxobutanoate was used without further purification (4·8g, 27%). lit.[103] b.p. 81°/5mm. ^{1}H NMR δ 0·95, t, *J* 7·3 Hz, H4'; 1·41, m, H3'; 1·65, m, H2'; 2·28, s, H4; 3·45, s, H2; 4·16, t, *J* 6·5 Hz, H1'. ^{13}C NMR δ 12·97, C4'; 18·41, C3'; 29·43, C2'; 29·89, C4; 49·52, C2; 64·65, C1'; 166·6, C1; 199·9, C3.



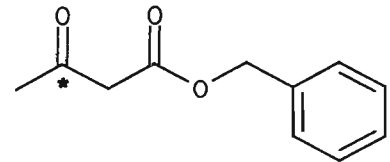
(±)-*sec*-Butyl 3-oxobutanoate 61; Using a similar procedure to that previously described 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one **63** (5g, 0·035mol) and *sec*-butanol (20 ml) gave the title compound after purification by distillation (120°/20mm), (3·94g, 72%). ^{1}H NMR δ 0·924, t, *J* 7·5Hz, H4'; 1·25, d, *J* 6·2Hz, H1'; 1·59, m, H3'; 2·29, s, H4; 3·45, s, H2; 4·91, m, H2'. ^{13}C NMR δ 8·9, C4'; 18·7, C1'; 28·2, C3'; 29·4, C4; 49·8, C2; 72·9, C2'; 166·2, C1; 200·0 C3. The NMR data is identical to that previously reported.[75]



(*S*)-(+)-*sec*-Butyl 3-oxobutanoate 61; Using a similar procedure 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one **63** (0·9g, 6·3mmol) and (*S*)-(+)-*sec*-butanol (0·5g, 6·7mmol) gave the title compound after purification by distillation (120°/20mm) (0·4 g, $[\alpha]_D$ 47%). +19·1° (c=1, $CHCl_3$). ^{1}H and ^{13}C NMR identical to racemic sample.



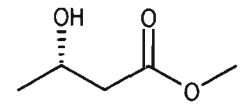
Benzyl 3-oxobutanoate-3-^{13}C 62; Benzyl alcohol (0·226g) was added to ethyl 3-oxobutanoate-3-^{13}C (0·200g, mmol) with catalytic quantities of solid superacid in a one-piece vigeroux distillation unit and refluxed for 24h at 135°C. Filtration and



distillation (175°C /1mm) yielded benzyl 3-oxobutanoate-3-^{13}C (88%, 0·260g). ^{1}H NMR δ 2·273, d, $J_{H\text{-}C}$ 6·0 Hz, H4; 3·515, d, $J_{H\text{-}C}$ 6·3 Hz, H2; 5·204, s, H1'; 7·382, s, Ph. The chemical shifts were identical to an authentic sample of unlabelled material.

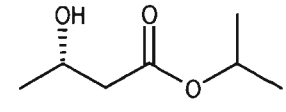
5.3 YEAST REDUCTION OF 3-OXOBUTANOATES

(*S*)-(+)-Methyl 3-hydroxybutanoate 66; Methyl 3-oxobutanoate **17** (1g, 8·6 mmol) was added to a 500ml round-bottom flask with yeast (8·6g), water (6·9ml) and petroleum ether (300ml) and stirred at room temperature for 24hrs. The reaction mixture was filtered and the yeast washed with ethyl acetate (100ml). The solvent was then removed under reduced pressure, and bulb to bulb distillation (bp. 170°) gave the desired product (0·56g, 57%). lit.[104] b.p. 71-73°/17mm. ^{1}H NMR δ($CDCl_3$) 1·25, d, *J* 6·3 Hz, H4; 2·44, dd, *J* 8·4, 16·5Hz, H2; 2·53, dd, *J* 16·5, 3·9Hz, H2; 3·73, s, CH_3; 4·22, m, H3. ^{13}C NMR δ21·81, C4; 41·93, C2; 51·07, CH_3; 63·63, C2; 172·6, C1. $[\alpha]_D$ +46·89° ($CHCl_3$, c=**1**), lit.[105] +33·3° ($CHCl_3$, c=1·2).

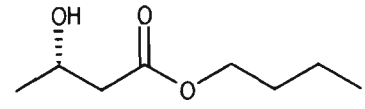


The trifluoroacetate was prepared by dissolving methyl-3-hydroxybutanoate **67** (ca. 5mg) in dichloromethane (0·2ml) and adding freshly distilled trifluoroacetic anhydride (0·25ml). After leaving this to stand at room temperature for 30 minutes the solution was gently heated to remove excess reagent. Ethanol was added to the vial and chiral gas chromatography showed a ratio of 99:1 (98% ee).

Isopropyl (*S*)-(+)-3-hydroxybutanoate 68; Using a similar procedure isopropyl 3-oxobutanoate **59** (1g, 6·9 mmol) was reacted with yeast (13·7g) and water (10·95ml) in petroleum ether. Bulb to bulb distillation (100°/20mm) gave the desired product. (0·95g, 96%). lit.[106] b.p. 78-79°/21mm. ^{1}H NMR δ($CDCl_3$) 1·23, d, *J* 6·3 Hz, H4; 1·27, d, *J* 6·3Hz, $(CH_3)_2$; 2·40, dd, *J* 16·2, 8·4Hz, H2; 2·48, dd, *J* 16·2, 3·6, H2; 2·66, s(br), OH; 4·21, m, H3; 5·07, sept, *J* 6·3 Hz, CH. ^{13}C NMR δ21·1, C4; 21·8, $(CH_3)_2$; 42·5, C2; 63·7, C3; 67·5, CH; 171·8, C1. $[\alpha]_D$ +38·89° ($CHCl_3$, c=1). The trifluoroacetate of the product was prepared as described previously and chiral gas chromatography showed a ratio of 98·4:1·6 (97% ee).



Butyl (*S*)-(+)-3-hydroxybutanoate 69; Using a similar procedure butyl 3-oxobutanoate **60** (1g, 6·3 mmol) was reacted with yeast (18·9g) and water (15·1ml)in petroleum ether. Bulb to bulb distillation



(100°/1mm) followed by radial chromatography (petroleum ether: ether, 90:10) gave the desired product (0·9g, 89%). lit.[15] b.p. 81°/5mm. ^{1}H NMR δ ($CDCl_3$) 0·94, t, *J* 7·5 Hz, H4'; 1·23, d, *J* 6·3 Hz, H4; 1·36, m, H3'; 1·62, m, H2'; 2·41, dd, *J* 16·5, 8·4Hz, H2; 2·49, dd, *J* 16·5, 3·6 Hz, H2; 2·95, d, *J* 3·9 Hz, OH; 4·12, t, *J* 6·6 Hz, H1'; 4·18, m, H3. ^{13}C NMR δ13·0, C4'; 18·5, C3'; 21·8, C4; 30·0, C2'; 42·2, C2; 63·6, C3; 63·9, C1'; 172·3, C1. $[\alpha]_D$ +36·70° ($CHCl_3$, c=1), lit.[77] $[\alpha]_D$ +35·1° (solvent not cited) (97%ee). The trifluoroacetate of the product was prepared as described previously and chiral gas chromatography showed only one enantiomer (>99% ee).

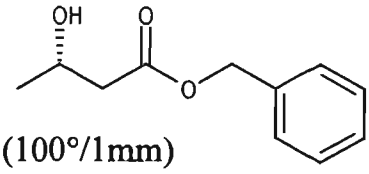
***tert*-Butyl (*S*)-(+)-3-hydroxybutanoate 71**; Using a similar procedure *tert*-butyl 3-oxobutanoate **55** (0·6g, 3·8mmol) was reacted with yeast (42g) and water (33·4ml) in petroleum ether. Bulb to bulb distillation (100°/1mm) gave the desired product. (0·41g, 68%). lit.[12] b.p. 74-79°/11mm. ^{1}H NMR δ ($CDCl_3$) 1·23, d, J 6·3Hz, H4; 1·49, s, *tert*-Bu; 2·34, dd, *J* 16·2, 8·4, H2; 2·43, dd, *J* 16·2, 3·6Hz, H2; 3·12, s(br), OH; 4·15, m, H3. ^{13}C NMR δ21·7, C4; 27·5, $(CH_3)_3$; 43·2, C2; 63·7, C3; 80·6, C1'; 171·7, C1. $[\alpha]_D$ + 34·05° ($CHCl_3$, c=1), lit[12] $[\alpha]_D$ + 30·8° ($CHCl_3$, c=1). The trifluoroacetate of the product was prepared as described previously and chiral gas chromatography showed a ratio of 99:1 (98% ee).

***sec*-Butyl (S)-(+)-3-hydroxybutanoate 70**; Using a similar procedure *sec*-butyl 3-oxobutanoate **61** (1g, 6·3mmol) was reacted with yeast (25·3g) and water (20·2ml) in petroleum ether. Bulb to bulb distillation (140°/20mm) gave the desired product as a mixture of diastereomers. (0·9g, 89%). ^{1}H NMR δ($CDCl_3$) 0·92, t, *J* 7·5 Hz, H4'; 1·240, dd, *J* 6·3Hz, H1' (RS); 1·242, d, *J* 6·3 Hz, H1' (SS); 1·245, d, *J* 6·3Hz, H4; 1·6, m, H3'; 2·409, dd, *J* 16·2, 5·4Hz, H2 (RS); 2·413, dd, *J* 16·2, 5·4Hz, H2 (SS); 2·499, dd, *J* 16·2, 3·9Hz, H2 (SS); 2·501, dd, *J* 16·2, 3·9Hz, H2 (RS); 3·027, s(br), OH; 4·2, m, H3; 4·9, sextet, *J* 6·3 Hz, H2'. ^{13}C NMR δ9·0, C4'; 18·8, C1'; 21·8, C4; 28·1, C3'; 42·4, C2; 63·7, C3; 72·1, C2'; 172·0, C1. $[\alpha]_D$ +31·11°, ($CHCl_3$, c=1), lit.[12] $[\alpha]_D$ +13·7°, ($CHCl_3$, c=1). The trifluoroacetate of the product was prepared as described previously and chiral gas chromatography showed a ratio of 98·7:1·3 (97% ee) at C2.

***(S)-sec*-Butyl (*S*)-(+)-3-hydroxybutanoate 70**; Using a similar procedure (S)-*sec*-butyl 3-oxobutanoate **61** (0·158g, 1mmol) was reacted with yeast (4g) and water (3·2ml)in petroleum ether. ^{1}H NMR δ($CDCl_3$) 0·92, t, *J* 7·5 Hz, H4'; 1·242, d, *J* 6·3 Hz, H1'; 1·245, d, *J* 6·3Hz, H4; 1·6, m, H3'; 2·413, dd, *J* 16·2, 5·4Hz,

H2; 2·499, dd, *J* 16·2, 3·9Hz, H2; 3·027, s(br), OH; 4·2, m, H3; 4·9, sextet, *J* 6·3 Hz, H2'. ^{13}C NMR δ9·0, C4'; 18·8, C1'; 21·8, C4; 28·1, C3'; 42·4, C2; 63·7, C3; 72·1, C2'; 172·0, C1. $[\alpha]_D$ +52·3°, ($CHCl_3$, c=1). The trifluoroacetate of the product was prepared as described previously and chiral gas chromatography showed a ratio of 98:2 (96% ee at C3, 100% ee at C2').

Benzyl (S)-(+)-3-hydroxybutanoate 72; Using a similar procedure benzyl 3-oxobutanoate **62** (1g, 5·2 mmol) was reacted with yeast (26g) and water (28·8ml)in petroleum ether. Bulb to bulb distillation (100°/1mm) gave the desired product. (0·72g, 72%). ^1H NMR δ($CDCl_3$) 1·25, d, *J* 6·3 Hz, H4; 2·49, dd, *J* 16·8, 8·4Hz, H2; 2·58, dd, *J* 16·8, 3·6Hz, H2; 4·24, m, H3; 5·18, s, H1'; 7·38, s, Ph. ^{13}C NMR δ21·9, C4; 42·3, C2; 63·7, C3; 65·9, C1'; 127·6, 128·0, (*o, m*); 127·8 (*p*); 135·0, (*i*); 172·0, C1. The NMR data is identical to that previously reported.[107] $[\alpha]_D$ +29·0° ($CHCl_3$, c=1). The trifluoroacetate of the product was prepared as described previously and chiral gas chromatography showed a ratio of 97:3 (94% ee).



5.4 NMR STUDIES

5.4.1 Techniques

Dried bakers yeast (0·1g) was placed into a 10mm NMR tube with hexane (2·5ml), benzene-d_6 (125µl) as a lock substance, the 3-oxobutanoate-3-^{13}C (0·1mmol) and water (80µl). The tube was prepared with a vortex plug and placed in a Bruker 500 MHz NMR spectrometer operating at 125·72 MHz for ^{13}C at 20°C. The tube was spun at 20 Hz and the autoshim program was set to Z^1, Z^2 and Z^3 with an increment of 2. Each spectrum was derived from 128 scans with a total time of about 7 mins. A ^{13}C NMR spectrum was run every hour over a period of 60h, which allowed the steady conversion of substrate to product to be observed. Each accumulated data set was Fourier transformed after application of line broadening of 1 Hz.

After each 60 hour period, the supernatant was decanted and gas chromatographic analysis was performed to determine the extent of reduction.

The intensity of the product peak (63 ppm) was measured relative to the benzene-d_6 and these values were converted to Excel graphs.

5.4.2 Pretreatment Reactions

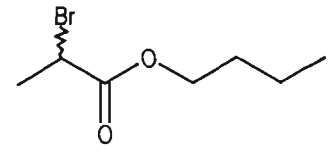
A series of experiments were carried out involving pretreating the yeast for successively longer periods. Dried bakers yeast (0·1g) was placed into a 10mm NMR tube with hexane (2·5ml), benzene-d_6 (125µl) as a lock substance and the tube was placed inside the NMR spectrometer for 3h. The sample was spinning during this time and the temperature was held constant at 22°C. Ethyl 3-oxobutanoate-3-^{13}C (0·1mmol) and water (80µl) were then added to the tube and the experiment was carried out as described earlier for a further 60h. This procedure was repeated with three pretreatment times of 6, 12 and 24h and the plots of relative intensity *vs* time for each experiment were prepared. After each experiment a gas chromatographic analysis was performed on the supernatant (Table 5-1).

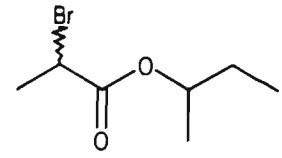
Table 5-1 Measured conversions for the yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane in an NMR tube after 60h with pretreatment.

PRETREATMENT TIME	GC CONVERSION
24h	6%
12h	39%
6h	63%
3h	60%

5.5 PREPARATION OF ESTERS OF 2-BROMOPROPANOIC ACID

Butyl 2-bromopropanoate 77; To a suspension of *Candida cylindracea* lipase (1·35g) in hexane (100ml), 2-bromopropanoic acid **74** (1·5g, 0·01mol) was added with a three-fold molar excess of butanol (2·75ml, 0·03mol) and stirred at room temperature for 72h. The suspension was filtered and the hexane removed under vacuum and distilled (110°C/20mm, lit 84-86°C/14mm) to give butyl-2-bromopropanoate (1·72g, 83%). ^{1}H NMR δ 0·97, t, *J* 7·2Hz, H4'; 1·43, m, *J* 7·5Hz, H3'; 1·68, m, *J* 6·9Hz, H2'; 1·85, d, *J* 6·9Hz, H3; 4·15-4·23, m, H1'; 4·38, q, *J* 6·9Hz, H2. ^{13}C NMR δ 13·0, C3'; 18·4, C4'; 21·1, C2'; 29·8, C3; 39·6, C1'; 65·2, C2; 161·7, C1.



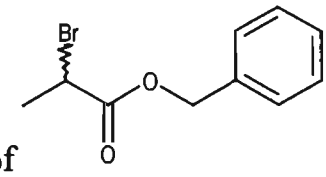


***sec*-Butyl 2-bromopropanoate 80**; To a suspension of *Candida cylindracea*lipase (1·35g) in hexane (100ml), 2-bromopropanoic acid **74** (1·5g, 0·01mol) was added with a three-fold molar excess of *sec*-butanol (2·75ml, 0·03 mol) and stirred for 5 days. The suspension was filtered and washed with petroleum ether (60ml) and the filtrate washed with $NaHCO_3$ (10%) to remove any unreacted acid, dried over Na_2SO_4 and the petroleum ether removed under vacuum. Excess *sec*-butanol was removed gently with the bulb-to-bulb at 100°C/20mm. No product was recovered.

Following the method set out by Hassner and Alexanian,[99] 2-bromopropanoic acid (1·53g, 0·010mol), *N,N*-dicyclohexylcarbodiimide (2·27g, 0·011mol), *sec*-butanol (0·82g, 0·011mol) and dimethylaminopyridine (0·12g, 0·001mol) were added to ether (25ml) and stirred for 3h. The reaction was filtered and the filtrate washed with water (3x50ml), 5% CH_3COOH (50ml) and again with water (50ml). The ether layer was dried over Na_2SO_3 and the ether removed under vacuum. Bulb-to-bulb distillation (105°C/20mm) gave the title compound as a mixture of diastereomers. (0·84g, 40%).

Diastereomer 1: ^{1}H NMR δ 0·94, t, *J* 7·5, H4'; 1·259, d, *J* 6·3, H1'; 1·63, m, H3'; 4·358, q, *J* 6·9, H2; 4·907, sextet, *J* 6·3 Hz, H2'. ^{13}C NMR δ 8.89, C4'; 18.32, C1'; 20.95, C3; 27.99, C3'; 39.99, C2'; 73.41, C2; 169.2, C1. '

Diastereomer 2: ^{1}H NMR δ 0·96, t, *J* 7·5, H4'; 1·260, d, *J* 6·0, H1'; 1·63, m, H3'; 4·361, q, *J* 6·9, H2; 4·912, sextet, *J* 6·3 Hz, H2'. ^{13}C NMR δ 8.94, C4'; 18.58, C1'; 21.04, C3; 27.99, C3'; 40.15, C2'; 73.46, C2; 169.2, C1. Please check assignment. 13C cf H



Benzyl 2-bromopropanoate 81; To a suspension of *Candida cylindracea* lipase (1·35g) in hexane (100ml), 2-bromopropanoic acid **74** (1·5g, 0·01mol) was added with a three-fold molar excess of benzyl alcohol (3·4ml, 0·03 mol) and stirred for 5 days. The suspension was filtered and washed with petroleum ether (60ml) and the filtrate washed with $NaHCO_3$ (10%) to remove any unreacted acid, dried over Na_2SO_4 and the petroleum ether removed under vacuum. Excess benzyl alcohol was removed using bulb-to-bulb distillation at 100°C/5mm. No product was recovered.

Following the method set out by Hassner and Alexanian,[99] 2-bromopropanoic acid (1·53g, 0·010mol), *N,N*-dicyclohexylcarbodiimide (2·27g, 0·011mol), benzyl alcohol (1·2g,

0·011mol) and dimethylaminopyridine (0·12g, 0·001mol) were added to ether (25ml) and stirred for 3h. The reaction was filtered and the filtrate washed with water (3x50ml), 5% CH_3COOH (50ml) and again with water (50ml). The ether layer was dried over Na_2SO_3 and removed under vacuum. Bulb-to-bulb distillation gave multiple compounds.

2-Bromopropanoic acid (1·53g, 0·010mol) and thionyl chloride (1·5g) were heated gently with stirring under reflux until gas evolution ceased. Benzyl alcohol (4·3g, 0·04mol) in ether (10ml) was carefully added. The reaction was washed with 5% NaOH (2x5ml), 10% HCl (5ml) and water (10ml). The ether layer was dried over Na_2SO_3 and removed under vacuum. Bulb-to-bulb distillation (120°C/5mm) gave the title product (0·54g, 28%). ^{1}H NMR δ 1·88, d, *J* 6·9, H3; 4·43, q, *J* 6·9, H2; 5·23, s, H1'; 7·40, s, Ph. ^{13}C NMR δ 21·0, C3; 39·4, C2; 67·0, C1'; 127·6, 127·9, 128·0, Ph; 161·7, C1.

5.6 YEAST REACTIONS WITH 2-BROMOPROPANOIC ACID AND BUTANOL

5.6.1 Yeast-mediated preparation of butyl 2-bromopropanoic acid

Yeast (1g) was stirred with 2-bromopropanoic acid (1·5g, 0·01mol) and butanol (2·75ml, 0·03mol) in petroleum ether for 72h. Samples were removed at 24, 48 and 72h. Water (0·8ml) was then added and the reaction was allowed to stir for another 24h (Table 5-2). The amount of conversion is calculated as the ratio of areas of 2-bromopropanoic acid and the butyl ester from gas chromatograms.

Table 5-2 Results from yeast catalysed esterification of 2-bromopropanoic acid and butanol.

TIME (h)	CONVERSION (%)
24	2
48	5
72	8
Addition of water	
96	7

Yeast (1g) was stirred with 2-bromopropanoic acid (0·15g, 0·001mol) in petroleum ether (50ml) with butanol (2·5ml, 0·027mol) and water(0·8ml) for 24h. Gas chromatography

conversion showed 15% esterification. Two variations of this reaction were performed, one excluding the water and another excluding the yeast. The butyl ester could not be detected in the gas chromatograms of either of these reactions.

5.6.2 Optimisation of butanol content

2-Bromopropanoic acid (0·153g, 1·0 mmol), petroleum ether (50ml), tetradecane (100µl) and butanol (0·5-20mmol) were added and a sample removed for gas chromatography. Yeast (1g) and water (0·8ml) were added and the reaction stirred for 24h. A gas chromatography sample was taken and the amount of ester was calculated by using the ratio of the areas of the ester and tetradecane peak and comparing to a standard curve (Table 5-3). GC retention times 8·0min acid, 10·5min ester, 14min $C_{14}H_{30}$.

Table 5-3 Measured conversions and ee of the yeast-mediated esterification of 2-bromopropanoic acid in petroleum ether with different amounts of butanol.

BUTANOL (mmol)	CONVERSION (%)	ee (%)
0·5	<0·5	95
1	0·7	88
2	1·4	84
3	1·3	88
4	1·1	81
5	1·7	72
10	2·6	93
20	1·6	83

5.6.3 Optimisation of water content

2-Bromopropanoic acid (1·0 mmol, 0·153g), petroleum ether (50ml), tetradecane (internal standard, 100µl) and butanol (3mmol or 20mmol) were added and a sample removed for gas chromatography. Yeast (1g) and water (0-2·0ml) were added and the reaction stirred for 24h. A gas chromatography sample was taken and the amount of both acid and ester was compared to the tetradecane peak. Chiral gas chromatography was also performed on the reactions. The results are shown in Table 5-4.

Table 5-4 Measured conversion and ee for the yeast-mediated esterification of 2-bromopropanoic acid in petroleum ether at two amounts of butanol varying the amount of water between 0-2ml.

WATER (ml)	3mmol BUTANOL CONVERSION (%)	ee(%)	20mmol BUTANOL CONVERSION (%)	ee(%)
0	<0·5	10	<0·5	2.8
0·2	0·89	82	1·78	86
0·4	1·40	91	2·36	91
0·6	1·55	91	2·53	93
0·8	0·90	89	2·06	93
1·0	1·50	94	2·08	95
1·2	1·53	95	1·85	95
1·4	1·55	96	2·21	96
1·6	1·69	93	2·23	96
1·8	1·53	93	2·25	96
2·0	1·55	93	2·12	96

5.6.4 Hydrolysis Reaction

Butyl-2-bromopropanoate (1mmol), yeast (1g), water (0·8ml) and tetradecane (2μl) were added to petroleum ether (50ml) and allowed to stir for 24h. Gas chromatography showed only ester present and no decrease in the amount of ester compared to the internal standard.

5.6.5 Reactions investigating the loss of 2-bromopropanoic acid

5.6.5.1 **Variations in amount of water.**

The amount of water was varied from 0-2ml for two quantities of butanol, 3 and 20mmol. The general reaction conditions are as follows: 2-Bromopropanoic acid (0·153g, 1mmol), butanol (3 or 20 mmol) and internal standard (100μl) were added to petroleum ether (50ml) and a sample removed for gas chromatographic analysis. Yeast (1g) and water were then added and the reaction was allowed to stir at room temperature for 24h. The amount of acid remaining was calculated by comparison of the peak areas relative to the internal standard prior to the reaction and after 24h (Table 5-5).

Table 5-5 Amount of 2-bromopropanoic acid remaining after 24h exposure to yeast in petroleum ether at two amounts of butanol varying the amount of water between 0-2ml.

WATER (ml)	3mmol BUTANOL % REMAINING	20mmol BUTANOL % REMAINING
0	100	97
0.2	14	51
0.4	12	44
0.6	10	42
0.8	10	41
1	8	39
1.2	9	39
1.4	8	38
1.6	7	37
1.8	8	35
2	7	34

5.6.5.2 Variations in amount of butanol.

The amount of butanol was varied from 0-20mmol. The general reaction conditions are as follows: 2-Bromopropanoic acid (0·153g, 1mmol), butanol, and internal standard (100µl) were added to petroleum ether (50ml) and a sample removed for gas chromatographic analysis. Yeast (1g) and water (0·8ml) were then added and the reaction allowed to stir at room temperature for 24h. The amount of acid remaining was calculated by comparing the peak areas relative to the internal standard prior to reaction and after 24h.

Table 5-6 Amount of 2-bromopropanoic acid remaining after 24h exposure to yeast in petroleum ether at varying amounts of butanol between 0-20mmol.

BUTANOL (mmol)	ACID REMAINING (%)
0	-
0·5	-
1	3
2	7
3	12
4	16
5	19
10	28
20	40

5.6.5.3 **Addition of dioxane.**

2-Bromopropanoic acid (0·153g, 1mmol) and internal standard (100μl) were added to petroleum ether (50ml) and a sample removed for gas chromatographic analysis. Yeast (1g), water (1·0ml) and dioxane (1ml) were then added and the reaction allowed to stir at room temperature for 24h. The amount of acid remaining was calculated by comparing the peak areas relative to the internal standard prior to the reaction and after 24h. 3% 2-bromopropanoic acid remained.

5.6.5.4 **Addition of NaCl.**

A similar reaction was carried out with the addition of saturated sodium chloride solution (1·0ml) instead of the water and dioxane. After 24h 9% of the 2-bromopropanoic acid remained.

5.6.5.5 **Addition of 2M HCl.**

A similar reaction was carried out with the addition of 2M hydrochloric acid (1·0ml) instead of the saturated sodium chloride solution. After 24h 56% of the 2-bromopropanoic acid remained.

5.6.5.6 **Isolation of 2-bromopropanoic acid by washing the yeast in 2M HCl.**

2-Bromopropanoic acid (1g), yeast (1g) and water (6·5ml) were added to petroleum ether (350ml) and the reaction allowed to stir at room temperature for 24h. The reaction was filtered and the filtrate evaporated. Bulb-to-bulb distillation (120°C/20mm) gave 2-bromopropanoic acid (0·32g, 32%). The yeast was washed with ethyl acetate (2x100ml) and removal of the solvent gave a further quantity of 2-bromopropanoic acid (0·21g, 21%). The yeast was then suspended in 2M hydrochloric acid (200ml) and filtered with Filter Aid. Ethyl acetate was used to extract the acid from the filtrate to give more 2-bromopropanoic acid (0·25-0·30g). $[\alpha]_D$=0 (c=1, $CHCl_3$).

5.6.5.7 **Pretreatment with acetic acid.**

Yeast (1g) and glacial acetic acid (1mmol) were added to petroleum ether (50ml) and allowed to stir at room temperature for 24h. 2-Bromopropanoic acid (1mmol) and internal standard (100µl) were then added, a sample was removed for gas chromatographic analysis, and the reaction allowed to stir at room temperature for a further 24h. The amount of acid remaining was calculated by comparing the peak areas relative to the internal standard before and after 24h. After 24h 2% of the 2-bromopropanoic acid remained.

5.6.5.8 **Addition of acetic acid.**

2-Bromopropanoic acid (1mmol), glacial acetic acid (1mmol) and internal standard (100µl) were added to petroleum ether (50ml) and a sample removed for gas chromatographic analysis. Yeast (1g) and water (1·6ml) were then added and the reaction allowed to stir at room temperature for 24h. The amount of acid remaining was calculated by comparing the peak areas relative to the internal standard before and after 24h. After 24h 3% of the 2-bromopropanoic acid remained.

5.6.5.9 **Addition of extra 2-bromopropanoic acid.**

2-Bromopropanoic acid (0·306g, 2mmol), butanol (1mmol), yeast (1g) and water (1·6ml) were added to petroleum ether (50ml) and the reaction allowed to stir at room temperature for 24h. The amount of acid lost was calculated by comparing the peak areas relative to the internal standard before and after 24h. After 24h 3% of the 2-bromopropanoic acid remained.

5.7 YEAST REACTIONS WITH 2-BROMOPROPANOIC ACID AND SEC-BUTANOL.

5.7.1 Yeast-mediated preparation of sec*-butyl 2-bromopropanoate*

2-Bromopropanoic acid (0·153g, 1mmol), *sec*-butanol (0·74g, 10mmol), yeast (1g) and water (1ml) were added to petroleum ether (50ml) and the reaction allowed to stir at room temperature for 24h. No ester was detected by gas chromatography.

5.7.2 Optimisation of water content

A series of reaction involving 2-bromopropanoic acid (0·153g, 1mmol), *sec*-butanol (0·74g, 10mmol), yeast (1g) and water (0-2·0ml) were added to petroleum ether (50ml) were conducted at room temperature for 24h. No ester was detected by gas chromatography in any reaction.

5.8 YEAST REACTIONS WITH 2-BROMOPROPANOIC ACID AND BENZYL ALCOHOL.

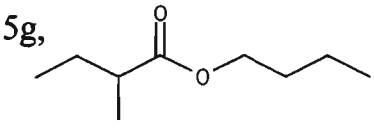
5.8.1 Preparation of benzyl 2-bromopropanoate

2-Bromopropanoic acid (0·153g, 1mmol), benzyl alcohol (1·08g, 10mmol), yeast (1g) and water (1ml) were added to petroleum ether (50ml) and the reaction allowed to stir at room temperature for 24h. A small amount of ester (<1%) was detected by gas chromatography.

5.8.2 Benzyl alcohol optimisation

A series of reaction involving 2-bromopropanoic acid (0·153g, 1mmol), benzyl alcohol (0·25-2·5ml), yeast (1g) and water (1ml) were added to petroleum ether (50ml), were conducted at room temperature for 24h. Again only a small amount of ester (<1%) was detected by gas chromatography.

5.9 PREPARATION OF BUTYL 2-METHYLBUTANOATE



2-Methylbutanoic acid **75** (1g, 0·010mol) and thionyl chloride (1·5g, 0·011mol) were heated gently with stirring under reflux until gas evolution ceased. Dry ether (10ml) was added and then butanol (2·5ml, 0·04mol) in ether (5ml) was carefully added dropwise. The reaction was washed with 5% NaOH (2x5ml), 10% HCl (5ml) and water (10ml). The ether layer was dried over Na_2SO_3 and removed under vacuum. Bulb-to-bulb distillation at 100°C/20mm gave the title product **82** (0·1g, 6%). ^{1}H NMR δ 0·92, t, *J* 7·2Hz, H4 or H4'; 0·96, t, *J* 7·2Hz, H4 or H4'; 1·16, d, *J*

6·9Hz, H5; 1·39-1·55, m, H3a & H3'; 1·59-1·77, m, H3b & H2'; 2·37, pent, *J* 6·9Hz, H2; 4·09, t, J 6·6Hz, H1'.

5.10 Yeast Reactions with 2-Methylbutanoic Acid

5.10.1 Yeast-mediated preparation of butyl 2-methylbutanoate

2-Methylbutanoic acid **75** (0·102g, 1mmol), butanol (1·08g, 10mmol), yeast (1g) and water (1ml) were added to petroleum ether (50ml) and the reaction allowed to stir at room temperature for 24h. No ester was detected by gas chromatography.

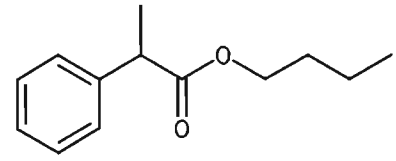
5.10.2 Optimisation of butanol content

A series of reactions involving 2-methylbutanoic acid **75** (0·102g, 1mmol), butanol (0·25-2·5ml), yeast (1g) and water (1ml) were added to petroleum ether (50ml) were conducted at room temperature for 24h. No ester was detected by gas chromatography in any reaction.

5.10.3 Hydrolysis Reaction

As previously except with butyl 2-methylbutanoate (1mmol) instead of butyl 2-bromopropanoate (*Section 5.6.4*). No acid was detected.

5.11 Preparation of Butyl 2-Phenylpropanoate



2-Phenylpropanoic acid (1·5g, 0·010mol) and thionyl chloride (1·5g, 0·011mol) were heated gently with stirring under reflux until gas evolution ceased. Dry ether (10ml) was added and then butanol (2·5ml, 0·04mol) in ether (5ml) was carefully added dropwise. The reaction was washed with 5% NaOH (20ml), 10% HCl (20ml) and water (20ml). The ether layer was dried over Na_2SO_3 and removed under vacuum to give the title product. (1·7g, 83%). ^{1}H NMR δ 0·90, t, *J* 7·2Hz, H4'; 1·339, hex, *J* 7·5Hz, H3'; 1·52, d, *J* 7·2Hz, H3; 1·58, pent, *J* 6·9Hz, H2'; 3·73, q, *J* 7·2Hz, H2; 4·09, t, *J* 6·6Hz, H1'. ^{13}C NMR δ13·0, C4'; 17·9, C3; 18·4, C3'; 30·0, C2'; 45·0, C2; 64·0, C1'; 126·4, 127·9, *(o, m)*; 126·9, *(p)*; 140·1, *(i)*; 174·0, C1.

5.12 YEAST REACTIONS WITH 2-PHENYLPROPANOIC ACID

5.12.1 Yeast-mediated preparation of butyl 2-phenylpropanoate

2-Phenylpropanoic acid (0·15g, 1mmol), butanol (1·08g, 10mmol), yeast (1g) and water (1ml) were added to petroleum ether (50ml) and the reaction allowed to stir at room temperature for 24h. No ester was detected by gas chromatography.

5.12.2 Optimisation of butanol content

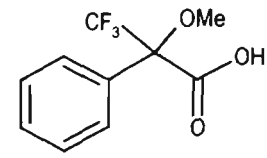
A series of reactions containing 2-phenylpropanoic acid **76** (0·15g, 1mmol), butanol (0·25-2·5ml), yeast (1g) and water (1ml) were added to petroleum ether (50ml) were conducted at room temperature for 24h. No ester was detected by gas chromatography in any reaction.

5.12.3 Hydrolysis Reaction

As previously except with butyl 2-methylbutanoate (1mmol) instead of butyl 2-phenylpropanoate (Section 5.6.4). No acid was detected by gas chromatography.

5.13 YEAST REACTIONS WITH MOSHER'S ACID

5.13.1 Yeast-mediated preparation of butyl 2-methoxy-2-trifluoromethylphenylacetic acid



2-Methoxy-2-trifluoromethylphenylacetic acid **73** (0·23g, 1mmol), butanol (1·08g, 10mmol), yeast (1g) and water (1ml) were added to petroleum ether (50ml) and the reaction allowed to stir at room temperature for 24h. No ester was detected by gas chromatography.

CHAPTER 6

APPENDIX

6.1 LISTING OF THE ONE ENZYME SYSTEM VISUAL BASIC PROGRAM USED IN CHAPTER 3.

Comments are highlighted in blue.

```
'Enz_Act Macro
'by Andrew Smallridge and Caroline Medson
'from a program written by Bruce Coller and Matthew Coller

Sub Enz_Act()
'*****************************
    cSstd = 152          'Standard concentration of the substrate
    Rtstd = 8.1          'Standard rate
    KM = 99.8            'Michaelis Menton Constant
    KP = 1.78            'Product inhibition Constant
    DI = 1.5             'initial delay
    dt = 0.1             'time increment
    A = 1.5              'time constant for enzyme activation by water
    actF = 1.2           'activation factor
    cE0 = 1              'initial enzyme activity
    cP0 = 0              'initial product concentration
    tD = 19              'start of enzyme deactivation
    D = 11               'time constant for enzyme deactivation
'*****************************
Set pretreat_cell = Worksheets("Macro results").Cells(2, 1) 'pretreatment is in A2
Set cS0 = Worksheets("Macro results").Cells(2, 2)           'Initial Substrate concentration is
in B2
Worksheets("Macro results").Cells(3, 1) = "Time (H)"        'set row heading
Set t_cell = Worksheets("Macro results").Cells(3, 2)        'Time starts at B3
Worksheets("Macro results").Cells(4, 1) = "Enzyme Activity" 'Set row heading
Set E_cell = Worksheets("Macro results").Cells(4, 2)        'Enzyme activty starts at B4
Worksheets("Macro results").Cells(5, 1) = "product"         'Set row heading
Set P_cell = Worksheets("Macro results").Cells(5, 2)        'Product starts at B5
Set SSq_cell = Worksheets("Macro results").Cells(2, 3)      'Result in C2
Set resid_cell = Worksheets("Macro results").Cells(11, 2)   'Squares start in B11
'*****************************
'Initialise Values
    cS = cS0
    cE = cE0
    cP = cP0
    tPrt = pretreat_cell.Value
    t = 0
    counter = 1
```

```
    SSq = 0
*****************************
'Enzyme kinetics
    While t < 600
    tim = t / 10

'total time yeast exposed to system
    ttot = tim + tPrt

    'ENZYME ACTIVITY
    'calculate enzyme activity during activation period
    cE = ((actF * (1 - Exp(-(tim + tPrt) / A))))

    'calculate enzyme activity if past deactivation time
    If ttot > tD Then cE = (actF * (Exp(-(ttot - tD) / D)))

    'Enzyme activity = 0 during initial delay: no reaction
    If tim < DI Then cE = 0


    'RELATIVE RATE
    'calculate relative rate
    RtRel = 0.935 * cS / cSstd * (cSstd + KM) / (cS + KM + (KP * cP))

    'Calculate rate
    Rt = RtRel * Rtstd * cE

    'Calculate increment of Substrate to product
    dStoP = Rt * dt
    cS = cS - dStoP: If cS < 0 Then cS = 0      'decrease substrate
    cP = cP + dStoP: If cP < 0 Then cP = 0      'increase product
      t = t + 1
 *********************************
    'Insert in excel worksheet
    t_cell.Value = t / 10
    P_cell.Value = dStoP
    E_cell.Value = cE
*********************************
    'Increment cells every hour
    If t = counter * 10 Then
    P_cell.Value = cP - cP0
    counter = counter + 1
    Set t_cell = Worksheets("Macro results").Cells(3, counter + 1)
    Set E_cell = Worksheets("Macro results").Cells(4, counter + 1)
    Set P_cell = Worksheets("Macro results").Cells(5, counter + 1)

    'Calculate sum of the residuals squared
    mvalue = Worksheets("Macro results").Cells(5, counter)
    Evalue = Worksheets("Macro results").Cells(6, counter)
    Set resid_cell = Worksheets("Macro results").Cells(11, counter)
```

```
    Diff = mvalue - Evalue
    SqDiff = Diff * Diff
    SSq = SSq + SqDiff
    resid_cell.Value = SqDiff
End If
Wend
End Sub
```

6.2 Listing of the Two Enzyme System Visual Basic program used in Chapter 3.

Comments are highlighted in blue.

```
'Enz_Act Macro
'by Andrew Smallridge and Caroline Medson
'from a program written by Bruce Coller and Matthew Coller

Sub Enz_Act()
'*****************************
    cSstd = 145          'Standard concentration of the substrate
    Rtstd = 12.8         'Standard rate
    KM = 99.8            'Michaelis Menton Constant
    KP = 1.78            'Product inhibition Constant
    DI = 0.7             'initial delay 1.5 hours
    dt = 0.1             'time increment 0.1 hours
    A = 1.5              'time constant for enzyme activation by water
    actF = 1.2           'activation factor
    cE0 = 1              'initial enzyme activity
    cP0 = 0              'initial product concentration
    tD = 4               'start of 1st enzyme deactivation
    D = 10               'time constant for 1st enzyme deactivation
    tD2 = 23             'start of 2nd enzyme deactivation
    D2 = 18              'time constant for 2nd enzyme deactivation
'*****************************
Set pretreat_cell = Worksheets("Macro results").Cells(2, 1) 'pretreatment is in A2
Set cS0 = Worksheets("Macro results").Cells(2, 2)           'initial Substrate concentration is
in B2
Worksheets("Macro results").Cells(3, 1) = "Time (H)"        'set row heading
Set t_cell = Worksheets("Macro results").Cells(3, 2)        'Time starts at B3
Worksheets("Macro results").Cells(4, 1) = "Enzyme Activity" 'set row heading
Set E_cell = Worksheets("Macro results").Cells(4, 2)        'Enzyme activty starts at B4
Worksheets("Macro results").Cells(5, 1) = "product"         'set row heading
Set P_cell = Worksheets("Macro results").Cells(5, 2)        'Product starts at B5
Set SSq_cell = Worksheets("Macro results").Cells(2, 3)      'Result in C2
Set resid_cell = Worksheets("Macro results").Cells(11, 2)   'Squares start in B11
'*****************************
'Initialise Values
```

```
    cS = cS0
    cE = cE0
    cE2 = cE0
    cP = cP0
    tPrt = pretreat_cell.Value
    t = 0
    counter = 1
    SSq = 0
*****************************
'Enzyme kinetics

    While t < 600
    tim = t / 10

'total time yeast exposed to system
    ttot = tim + tPrt

    'ENZYME ACTIVITY
    'calculate enzyme activity during activation period
    cE = ((actF * (1 - Exp(-(tim + tPrt) / A))))

    'calculate enzyme activity if past deactivation time
    If ttot > tD Then cE = (actF * (Exp(-(ttot - tD) / D)))
    If ttot > tD2 Then cE2 = (actF * (Exp(-(ttot - tD2) / D2)))

    'Enzyme activity = 0 during initial delay: no reaction
    If tim < DI Then cE = 0
    If tim < DI Then cE2 = 0

    'RELATIVE RATE
    'calculate relative rate
    RtRel = 0.935 * cS / cSstd * (cSstd + KM) / (cS + KM + (KP * cP))
    cEE2 = (cE * 0.8) + (cE2 * 0.2)

    'Calculate rate
    Rt = RtRel * Rtstd * cEE2

    'Calculate increment of Substrate to product
    dStoP = Rt * dt
    cS = cS - dStoP: If cS < 0 Then cS = 0  'decrease substrate
    cP = cP + dStoP: If cP < 0 Then cP = 0  'Increase product
        t = t + 1
*******************************
    'Insert in excel worksheet
    t_cell.Value = t / 10
    P_cell.Value = dStoP
    E_cell.Value = cE
*******************************
    'Increment cells every hour
    If t = counter * 10 Then
```

```
    t_cell.Value = t
    P_cell.Value = cP - cP0
    counter = counter + 1
    Set t_cell = Worksheets("Macro results").Cells(3, counter + 1)
    Set E_cell = Worksheets("Macro results").Cells(4, counter + 1)
    Set P_cell = Worksheets("Macro results").Cells(5, counter + 1)

    'Calculate sum of the residuals squared
    mvalue = Worksheets("Macro results").Cells(5, counter)
    Evalue = Worksheets("Macro results").Cells(16, counter)
    Set resid_cell = Worksheets("Macro results").Cells(11, counter)     'Result in c2
    Diff = mvalue - Evalue
    SqDiff = Diff * Diff
    SSq = SSq + SqDiff
    resid_cell.Value = SqDiff
End If
Wend
End Sub
```

CHAPTER 7

REFERENCES

7. References

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