THE YEAST MEDIATED SYNTHESIS OF THE l-EPHEDRINE PRECURSOR, l-PHENYLACETYLCARBINOL, IN AN ORGANIC SOLVENT

A Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

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STATEMENT BY THE CANDIDATE

This is to certify that the research contained in this thesis
is the sole work of the candidate. The work has not
been submitted for any other academic award.

M. M. KOSTRABY

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SPECIAL MENTIONS

To Mum and Dad, I am most grateful for all your assistance especially with looking after the children in the early days of my PhD studies.

To my children, Tristan and Kieran, a pair who are absolutely wonderful, kept their mum going through difficult times and were supportive and well behaved when they had to spend many a day in at the University with me whilst I studied. Tristan and Kieran you are wonderful children.

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PUBLICATION

Patent application (see Appendix)

Yeast mediated preparation of *l*-Phenylacetylcarbinol (*l*-PAC) in an organic solvent system.

Inventors: Margaret Del Giudice*, Andrew J. Smallridge, Maurie A. Trewhella.

*Maiden name of the author of this Thesis

SUMMARY

l-Ephedrine and *d*-pseudoephedrine are important pharmaceutical products commonly found in anti-asthmatic formulations, nasal decongestant mixtures and sinus preparations. *d*-Pseudoephedrine is the active ingredient in "Sudafed". *l*-Ephedrine is currently synthesised in a three step process. The first step utilises fermenting yeast in water to catalyse the acyloin condensation of benzaldehyde and acetaldehyde to form the *l*-ephedrine precursor, *l*-phenylacetylcarbinol (*l*-PAC). The second step involves the reaction of *l*-PAC with methylamine to form the corresponding N-methyl imine; the final step employs a metal catalyst to facilitate the reduction of the imine with hydrogen. This study is focused on the first step of the synthesis, which suffers from the drawback that two by-products, benzyl alcohol and 1-phenylpropan-1,2-diol are always formed. The study aims to improve the efficiency of the *l*-PAC formation by performing the reaction with dried yeast in an organic solvent.

In common with other yeast reaction systems, conducted in an organic solvent, a small amount of water (in this case citrate buffer, 1ml/g yeast) is required to initiate the yeast reaction. The yeast mediated acyloin condensation of benzaldehyde in an organic solvent was thoroughly investigated in order to optimise the production of *l*-PAC in high enantiomeric purity and to eliminate unwanted by-products. In aqueous fermenting systems, pyruvic acid is formed *in situ* from glucose and acts as the acyl donor; since an organic solvent does not support fermentation, pyruvate must be added to the system. In this study, both sodium pyruvate and pyruvic acid were investigated and it was found that only one quarter the quantity of pyruvic acid was required to produce *l*-PAC in a similar yield (24%) to that obtained when sodium pyruvate was used; additionally, *l*-PAC was obtained with higher enantiomeric purity with pyruvic acid (90% ee).

Other parameters investigated were pH, temperature and the addition of ethanol. It was found that the addition of a small quantity of ethanol, at a reaction temperature of 5°C and pH of 5.45, eliminated the production of by-products and resulted in a reasonable yield (24%) of *l*-PAC in high enantiomeric purity (90% ee).

In order to gain some understanding of the kinetics of the yeast mediated acyloin condensation of benzaldehyde in an organic solvent, the reaction was studied using ^{13}C labelled reagents under optimal reaction conditions. It was discovered that whilst excess sodium pyruvate was converted to ethanol, the ethanol was not incorporated into *l*-PAC. The effect of temperature on the rate of the reaction was examined with the aid of time lapse ^{13}C NMR. The reaction was much faster at a reaction temperature of 20°C compared with 5°C. However at 20°C the yeast was deactivated after 6h, whilst at 5°C the yeast was not deactivated, until after 55h, and a higher eventual yield of *l*-PAC was achieved.

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CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 Historical Background.

Ancient Chinese medicine utilised a plant called *Ma huang*, also known as *Ephedra sinica*, as an anti-asthmatic. Twigs of the plant were used to relieve bronchial spasms, hayfever, nettle rash, colds and chesty coughs.[1] Ephedrine was first isolated from *Ma huang* in 1887,[2] subsequently both Eli Lilly[2,3] and Merck[2] reported on the commercial extraction of ephedrine from the plant for medicinal purposes. In recent times, although about 30% of the world market is still supplied from these natural sources, the majority is commercially synthesised.[4,5,6] Today, ephedrine is one of the most potent vasopressor drugs known and has found widespread use for relieving congestion due to colds and allergies and as an agent to dilate the pupil.[7] This drug is also used to relieve symptoms of catalepsy, narcolepsy and hypotension.[8] Recent studies have shown that ephedrine in combination with caffeine has also proven to be useful in obesity control.[9]

1.2 The Commercial Preparation of *l*-Ephedrine

The commercial preparation of *l*-ephedrine (7) was first described in 1934 by Hildebrant and Klavehn[5] and involves three chemical steps, which are carried out in two parts. The first chemical step employs fermenting yeast to catalyse the acyloin condensation of benzaldehyde (1) and endogenous acetaldehyde (2) to give *l*-PAC (3) (Scheme 1.1). Two additional products, benzyl alcohol (4) and 1-phenylpropan-1,2-diol (5), are also obtained *via* the alcohol dehydrogenase (ADH) catalysed reduction of benzaldehyde (1) and *l*-PAC (3) respectively.[10]
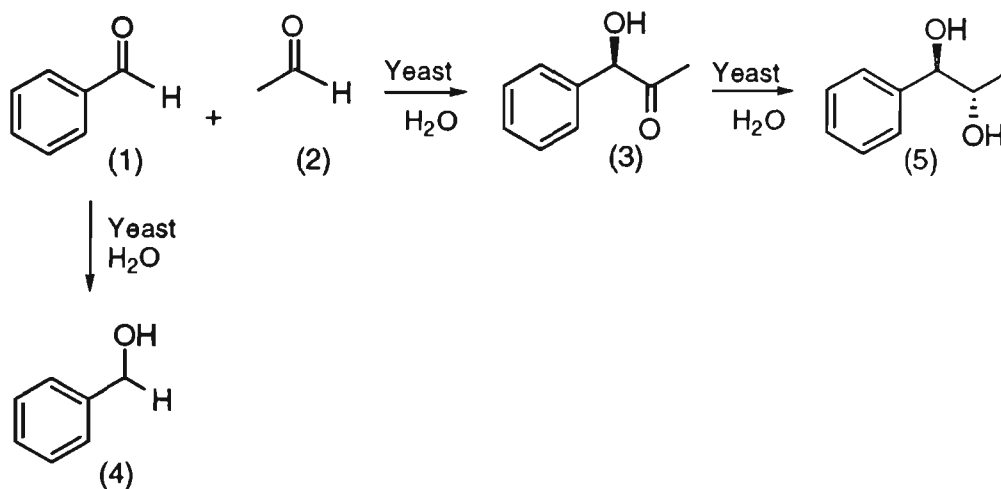
Scheme 1.1

The second part of the synthesis of *l*-ephedrine (7) involves the second and third chemical steps. The *l*-PAC (3) is extracted from the fermentation broth and then chemically converted to the imine (6) by reaction with methylamine (Scheme 1.2).[11]
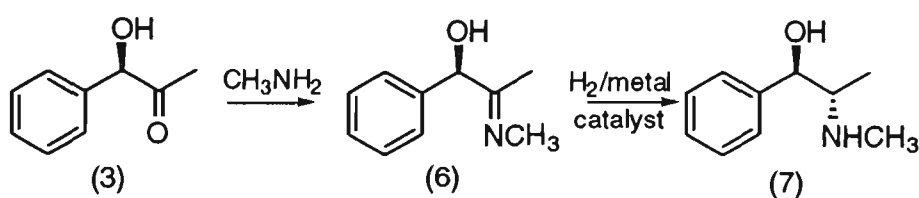
Scheme 1.2

The third chemical step involves the reduction of the imine (6) to *l*-ephedrine (7) using hydrogen and a metal catalyst.[5,7,10,11] *l*-Ephedrine (7) can also be produced in a one step process from *l*-PAC (3) by hydroamination in the presence of methylamine and hydrogen with a metal catalyst.[11]

Another commonly used vasopressor drug, *d*-pseudoephedrine (8), which is present in a number of nasal decongestants,[12] is formed by isomerisation of *l*-ephedrine (7) (Scheme 1.3).[11]
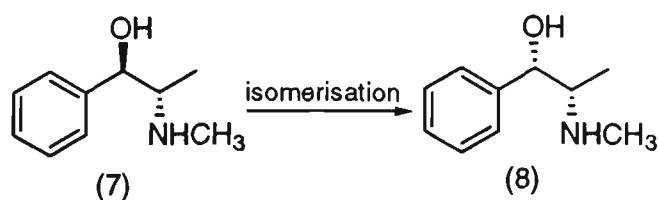
Scheme 1.3

An increase in the efficiency of any one of the three steps involved in the production of *l*-ephedrine (7) would make a significant contribution towards the commercial viability of the process.

1.3 The Biosynthesis of *l*-Phenylacetylcarbinol (*l*-PAC)

Early studies by Neuberg and co-workers[13,14,15,16] and by Discherl[17] revealed that particular yeasts were able to transform a number of substituted benzaldehydes into optically active phenylacetylcarbinols and benzyl alcohols (Scheme 1.4). Subsequently, the biotransformation of numerous aromatic aldehydes by fermenting yeast, *Saccharomyces cerevisiae*, has been reported (Table 1.1). [18,19]
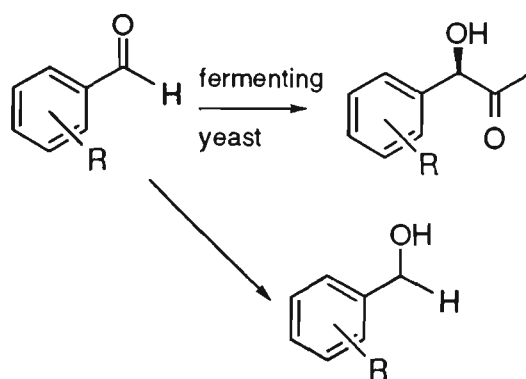
Scheme 1.4

Table 1.1. Aromatic aldehydes which have been converted to the corresponding optically active acyloin compounds and aromatic alcohols by *Saccharomyces cerevisiae*.[18,19]

Aromatic aldehyde	Acyloin product	Aromatic alcohol	Ref.
3-methoxy-4-hydroxy benzaldehyde	(1*R*)-3-methoxy-4-hydroxy phenylacetylcarbinol	3-methoxy-4-hydroxy benzyl alcohol	18
3,4-methylenedioxy benzaldehyde	(1*R*)-3,4-methylenedioxy phenylacetylcarbinol	3,4-methylenedioxy benzyl alcohol	18
benzaldehyde	phenylacetylcarbinol	benzyl alcohol	19
o-tolualdehyde	(1*R*)-2-methylphenylacetyl carbinol	2-methyl benzyl alcohol	19
m-tolualdehyde	(1*R*)-3-methylphenylacetyl carbinol	3-methylbenzyl alcohol	19
p-tolualdehyde	(1*R*)-4-methylphenylacetyl carbinol	4-methylbenzyl alcohol	19
2-chlorobenzaldehyde	(1*R*)-2-chlorophenylacetyl carbinol	2-chlorobenzyl alcohol	19
3-chlorobenzaldehyde	(1*R*)-3-chlorophenylacetyl carbinol	3-chlorobenzyl alcohol	19
4-chlorobenzaldehyde	(1*R*)-4-chlorophenylacetyl carbinol	4-chlorobenzyl alcohol	19
o-anisaldehyde	(1*R*)-2-methoxyphenylacetyl carbinol	2-methoxybenzyl alcohol	19
m-anisaldehyde	(1*R*)-3-methoxyphenylacetyl carbinol	3-methoxybenzyl alcohol	19
p-anisaldehyde	(1*R*)-4-methoxyphenylacetyl carbinol	4-methoxybenzyl alcohol	19
α,α,α-trifluoro-o-tolualdehyde	(1*R*)-2-(trifluoromethyl)-phenylacetylcarbinol	2-(trifluoromethyl)-benzyl alcohol	19
α,α,α-trifluoro-m-tolualdehyde	(1*R*)-3-(trifluoromethyl)-phenylacetylcarbinol	3-(trifluoromethyl)-benzyl alcohol	19
α,α,α-trifluoro-p-tolualdehyde	(1*R*)-4-(trifluoromethyl)-phenylacetylcarbinol	4-(trifluoromethyl)-benzyl alcohol	19

Neuberg and co-workers[13,14,15,16] proposed that the transformation of the aromatic aldehydes to the corresponding acyloin product occurred *via* the glycolytic pathway. This pathway is involved in the yeast mediated acyloin condensation of benzaldehyde (1) to form the *l*-ephedrine precursor, *l*-phenylacetylcarbinol (*l*-PAC (3)).

The sequence of reactions of the glycolytic pathway was elucidated in the 1930s by Embden, Meyerhof and Warburg and has become known as the Embden-Meyerhof pathway.[20] The overall reaction of the glycolytic pathway from glucose (9) to pyruvic acid (10) is given in Scheme 1.5.
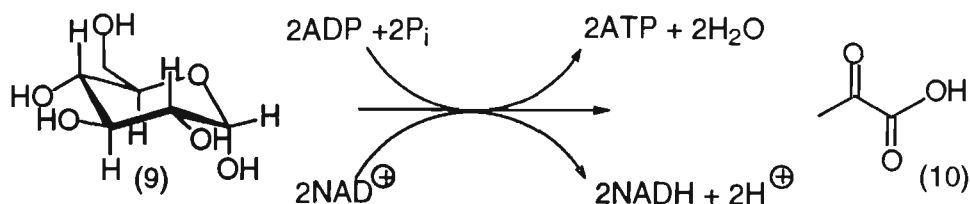
Scheme 1.5

1.3.1 *The reaction pathway for the formation of l-PAC (3)*

The reaction pathway for the formation of *l*-PAC (3) has been documented by a number of groups studying the reaction under fermentation conditions.[21,22,23] The pyruvic acid (10), which is formed from glucose (9), is converted *in situ* to acetaldehyde (2) by pyruvate decarboxylase (PDC) with thiamine pyrophosphate (TPP) as a bound cofactor. The acetyl intermediate (III) (TPP bound acetaldehyde) reacts with benzaldehyde to form *l*-PAC (3) (Scheme 1.6).[21] The overall pathway of *l*-PAC (3) formation is given in Scheme 1.7.
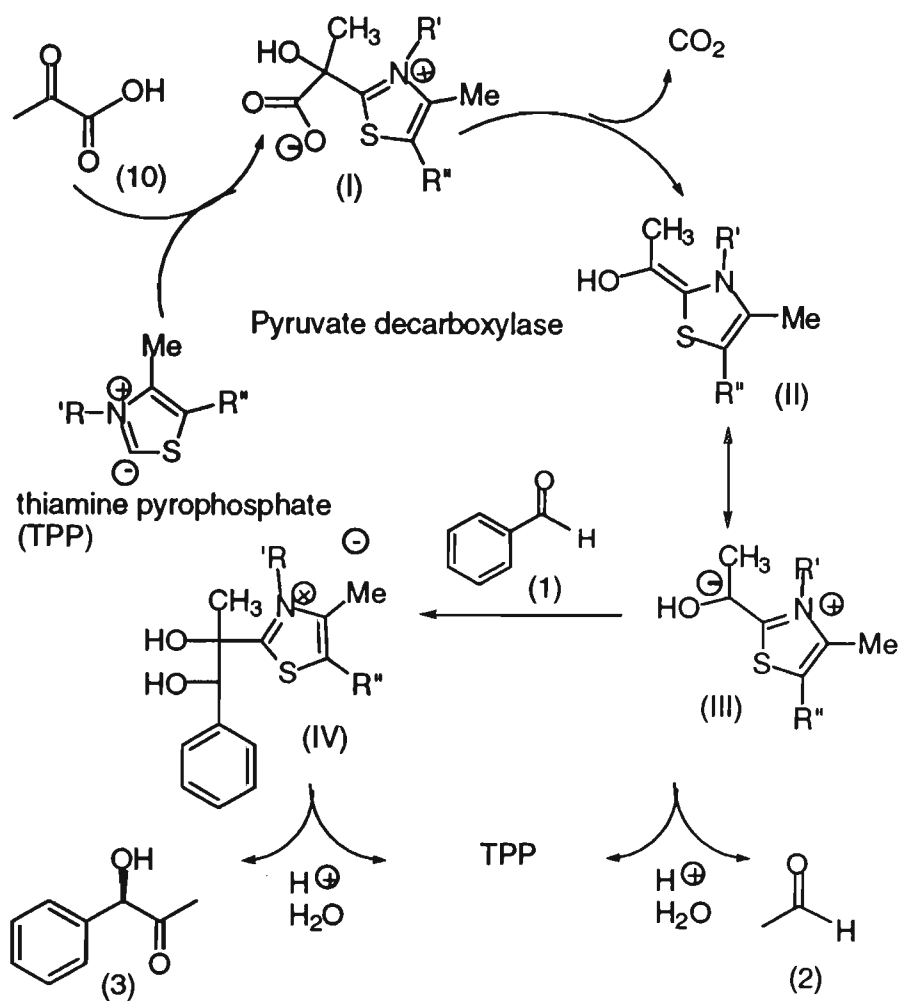
Scheme 1.6
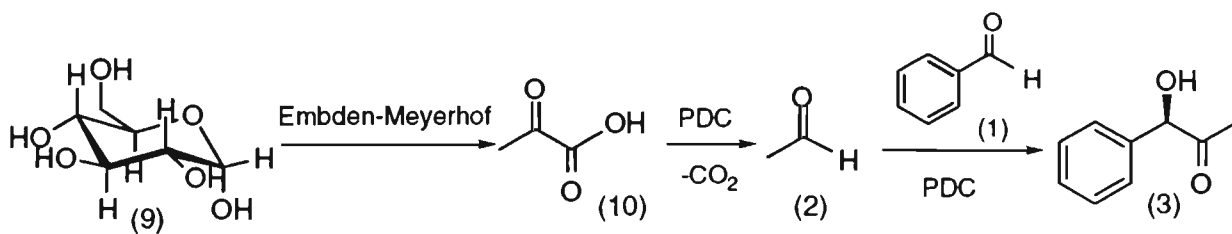
Scheme 1.7

The major by-product from this reaction, benzyl alcohol (4), is produced by alcohol dehydrogenase (ADH)[22] and/or other oxidoreductases[22,23] (Scheme 1.8). Minor products such as the diol (5) (Figure 1.1) are also formed as a result of similar enzyme activity.[10]
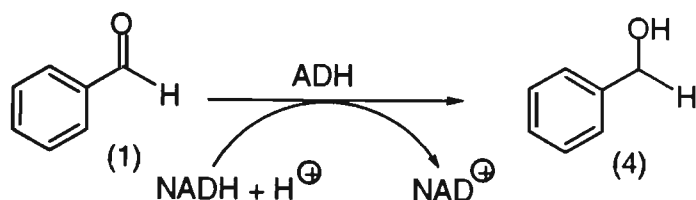
Scheme 1.8
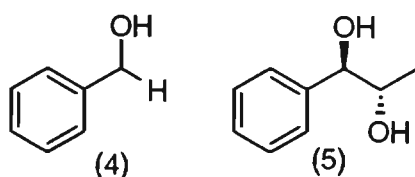
Figure 1.1 By-products ((4) and (5)) formed from the acyloin condensation of benzaldehyde (1) using fermenting yeast.

In order to confirm that PDC was the enzyme responsible for catalysing the condensation of aromatic aldehydes with pyruvate, Kren *et al.*[24] studied the acyloin condensation of a range of aromatic aldehydes. For comparison, the condensation reactions were carried out using both purified pyruvate decarboxylase and baker's yeast (*Saccharomyces cerevisiae*). The same optical isomer of PAC was obtained in both cases confirming that the enzyme, pyruvate decarboxylase, catalysed the condensation reaction. The results of these studies are given in Table 1.2.

Table 1.2 The optical isomers formed as a result of acyloin condensation reactions, which were carried out by Kren *et al*[24], with a range of aromatic aldehydes using purified pyruvate decarboxylase (a) and baker's yeast (b).

Aldehyde	Acetoin	% ee (a)	% ee (b)
benzaldehyde	(1R)-phenylacetylcarbinol	99	97
2-fluorobenzaldehyde	(1R)-(2-fluoro)-phenylacety carbinol	99	87
3-fluorobenzaldehyde	(1R)-(3-fluoro)-phenylacety carbinol	99	95
4-fluorobenzaldehyde	(1R)-(4-fluoro)-phenylacety carbinol	99	97
2,3-difluorobenzaldehyde	(1R)-(2,3-difluoro)-phenyl-acetylcarbinol	99	92
2-chlorobenzaldehyde	(1R)-(2-chloro)-phenylacety carbinol	98	81
3-chlorobenzaldehyde	(1R)-(3-chloro)-phenylacety carbinol	99	86
4-chlorobenzaldehyde	(1R)-(4-chloro)-phenylacety carbinol	99	77
2,6-difluorobenzaldehyde	(1R)-(2,6-difluoro)-phenyl-acetylcarbinol	92	87

1.3.2 *Minimisation of by-products*

The commercial synthesis of *l*-PAC (3) generally involves two stages; the first stage involves treating the yeast with a fermentation medium while in the second stage, or bioconversion stage, benzaldehyde (1) is added to the yeast and *l*-PAC (3) is produced. Due to the presence of other enzymes, such as alcohol dehydrogenase, in the yeast, unwanted by-products are formed.

The production of *l*-PAC (3) has been extensively studied[21-25] in order to understand the pathway of the reaction and consequently decrease the yield of unwanted by-products ((4) and (5), Figure 1.1) and improve the yield of *l*-PAC (3).

A recent patent[25] reported the condensation of benzaldehyde (1) with acetaldehyde (2) using yeast in an aqueous system. Although this condensation was reasonably successful the resulting *l*-PAC (3) was still contaminated with small amounts of by-products ((4) and (5) (Figure 1.1).

1.3.2.1 *Reaction conditions*

Various fermentation broths were used in an effort to reduce/prevent the production of the unwanted by-product, benzyl alcohol (4). [21] Mechanistic studies of the acyloin condensation of benzaldehyde (1) showed glucose (9), which was part of the fermentation broth, was converted to pyruvic acid (10). Although glucose (9) was the most common carbon source in the broth, [21] other sugars such as cane molasses and beet molasses, [26] and sucrose with added pyruvate, [27] were also used. Smith and Hendlin[28,29] added excess pyruvic acid (10) and acetone dried yeast powders to the fermentation broth containing cane molasses and adjusted the pH to 6.5. None of these attempts were successful; they neither enhanced the production of *l*-PAC (3) nor reduced the production of benzyl alcohol (4).

Extensive studies of the biotransformation of benzaldehyde (1) to *l*-PAC (3) have been carried out by Shin and Rogers[23,30] who used either immobilised yeast strains[30] or partially purified pyruvate decarboxylase (PDC). [23] Shin and Rogers[23] determined optimal reaction conditions in relation to temperature, pH, quantity of added ethanol, PDC activity, benzaldehyde (1) and pyruvate : benzaldehyde (1) ratio, in order to maximise *l*-PAC (3) production and minimise by-products. Partially purified PDC, which catalyses the production of *l*-PAC (3), was used in order to minimise the production of by-products which are formed in reaction mediums containing whole yeast cells due to the action of alcohol dehydrogenase (ADH) and oxidoreductase enzymes.

The optimal reaction conditions, which included the addition of sodium pyruvate in pH7 phosphate buffer, containing 2M ethanol, at 4°C and partially purified PDC, resulted in relatively high concentrations of *l*-PAC (3) (28.6g/L).[23] Although high yields of *l*-PAC (3) were obtained, the cost of using and recovering partially purified enzyme is considerably higher than the use of yeast in fermenting systems.

1.3.2.2 *Different yeasts*

Since varying the fermentation conditions had a limited effect on either the production of *l*-PAC (3) or the minimisation of by-products, attention was directed to the use of different yeasts.[31] Following earlier studies in which fresh brewer's yeast[13,14] was employed for the condensation of benzaldehyde (1) to form *l*-PAC (3), comparative studies were performed using a number of different yeast strains.[21] Some studies included using alcohol dehydrogenase (ADH) deficient strains of baker's yeast[22,32,33] in an attempt to ascertain the role of ADH in relation to the production of *l*-PAC (3). A summary of the yeast strains used is given in Table 1.3.[21] The findings of Long and Ward[22] and Nikolova and Ward[32,33] further confirmed that ADH, which is part of the suite of enzymes found in yeast, is responsible for the production of the unwanted by-product, benzyl alcohol (4).

Table 1.3 A summary of the various strains of yeast used to prepare *l*-PAC. *The greatest decarboxylase activity was found, by each of the research groups (1 - 10), with the given strains of yeast resulting in the highest yield of *l*-PAC (3).

Number	Microorganism	Strain Number	Ref.
1	Fresh brewer's yeast		13,14
2	*Hansenula anomala* *Brettanomyces vini* *Saccharomyces carlsbergensis* *Saccharomyces cerevisiae* *Saccharomyces ellipsoidence* *Torula utilis*	416 R XII	26
3	*Saccharomyces cerevisiae* *Saccharomyces carlsbergensis* *Saccharomyces fragilis* *Saccharomyces rouxii* *Saccharomyces latis* *Saccharomyces veronae* *Saccharomyces microellipsoides*	CBS 1171, NCYC 324 CBS 1485 ATCC 12424 CBS 732 NRRL Y 1549	27
4	*Candida* sp. *Saccharomyces* sp.		34
5	*Saccharomyces cerevisiae*		35
6	*Saccharomyces carlsbergensis* *Zymomonas mobilis*		36
7	*Saccharomyces cerevisiae* *Zygosaccharomyces rouxii* *Z. rouxii var. mellis*	ATCC 834 ATCC 2615 ATCC 10685	37-39
8	Commercial baker's yeast	Alcohol dehydrogenase deficient strain	22 32,33
9	*Candida flareri* *Saccharomyces cerevisiae*	Aldehyde resistant mutants	40
10	*Candida utilis*		30

1.3.2.3 *Additives*

Apart from some of the previously mentioned fermentation broths which contained either glucose (9),[21] cane molasses and beet molasses[26] or sucrose with added pyruvate,[27] other additives have also been included in the fermentation broth to enhance the production of *l*-PAC (3).[11]

Studies of the acyloin condensation of benzaldehyde (1) to form *l*-PAC (3) using fermenting yeast employed a number of different methods designed to optimise reaction conditions. A summary of the various methods used is given in Table 1.4.[21]

Table 1.4[21] A summary of the methods used for the fermentative biotransformation of benzaldehyde (1) to *l*-PAC (3).

Method of PAC production	Yield (g/l)	Yeast organism	Ref.
Batch cultivation with multiple doses of benzaldehyde and acetaldehyde	4.5	*S. cerevisiae*	26
Batch cultivation with benzaldehyde	5.2	*S. cerevisiae*	27
Sucrose, benzaldehyde and acetaldehyde added to the grown cells	6.3	*S. carlsbergensis*	34
Benzaldehyde, acetaldehyde and sodium pyruvate added to the reaction medium	10	*S. carlsbergensis*	41
Sodium pyruvate added to the reaction medium with benzaldehyde added at intervals	10.2	*S. cerevisiae*	19
Semicontinuous process with immobilized cells and benzaldehyde	10	*S. cerevisiae*	39
Fed-batch process with free cells and benzaldehyde	12	*S. cerevisiae*	42
Aldehyde resistant strain of yeast grown under oxygen limited or anaerobic conditions in the presence of benzaldehyde	12	*S. cerevisiae* *Candida flareri*	40
Benzaldehyde, pyruvate, TPP* and Mg2+	15	*S. cerevisiae*	43
Fed-batch process with benzaldehyde, glucose and using immobilised cells	15.2	*Candida utilis*	30
Partially purified PDC with pyruvate, ethanol and benzaldehyde	28.6	*Candida utilis*	23

*TPP thiamine pyrophosphate

In an attempt to devise a cleaner production of *l*-PAC (3) using fermenting yeast, Oliver *et al.*[11] studied the effect of various additives in the fermentation broth. The medium was based on that employed by Orica Australia (previously known as ICI) and contained a variety of complex materials including molasses, corn steep liquor, which was used as a source of nitrogen, as well as urea and potassium

dihydrogen phosphate. This was the standard medium for both the growth and inoculum and for the fermentation medium.

Oliver *et al.*[11] found that they were able to reduce the level of molasses in their broth by 40% whilst still maintaining productivity similar to the fermentations containing higher levels of molasses. As a result of these studies, they settled on a medium containing 40% molasses and 60% raw sugar for the bulk of their work. In order to reduce the number of additives in the fermentation broth, Oliver *et al.*[11] removed corn syrup liquor and found that the production of *l*-PAC (3) lay in a similar range (11.2 - 14.9g/l), but a 10% drop in the production of benzyl alcohol (4) resulted. Whey was added to fermentation broths as a possible source of lactic acid for conversion to *l*-PAC (3) or as a source of thiamine, a co-factor of PDC, however no benefit was observed from the inclusion of whey.[11]

Oliver *et al.*[11] examined the total carbohydrate content of the broth and concluded that because of the direct relationship with pyruvate levels, a substantial amount of carbohydrate must be maintained in order to maximise the production of *l*-PAC (3).

1.4 Biocatalytic Synthesis in Organic Solvents

The synthesis of *l*-PAC (3) using an aqueous fermenting system encountered a number of problems including solubility of the substrate in the fermentation medium, the production of the unwanted by-products, benzyl alcohol (4) and diol (5), and difficulties with the isolation of the desired product.[44] In an attempt to overcome these problems, organic solvents were employed in the yeast mediated acyloin condensation of benzaldehyde (1) to produce *l*-PAC (3).[33]

Enzymes have been widely explored as catalysts in organic synthesis[45-49] due to their specificity. A number of syntheses have been studied using enzymes in aqueous solutions since it was conventionally perceived that enzymes only worked in aqueous media. In an effort to eliminate a number of problems associated with the use of aqueous solutions in organic synthesis, studies revealed that many enzymes,

including oxidoreductases, hydrolases and isomerases, are catalytically active in a variety of organic solvents, provided that a small amount of water is present.[50-59] The use of organic solvents in enzymatic biotransformations has been applied to the synthesis of a number of chiral compounds and is thus of importance to food, pharmaceutical and specialty chemical industries.[59]

The use of organic solvents in biocatalytic transformations has been widely explored since it has a number of advantages compared with the use of aqueous systems. These advantages include increased solubility of substrate in organic media, prevention of hydrolysis of substrate/product, improved product recovery, improved stereoselectivity[57] and increased stability of the enzyme in an organic solvent.[50]

A number of enzyme catalysed biotransformations that were previously impossible to perform in aqueous solutions for kinetic or thermodymanic reasons[50,60] have been successfully accomplished using organic solvents. These enzymatic conversions used either purified or partially purified enzymes in organic solvents.[61,62] Reactions which have been successfully undertaken in organic solvents include lipase-catalysed regioselective acylation of glycols[63] and sugars,[64,65] esterification of fats,[66,67] lipase-catalysed stereoselective transesterifications and esterifications,[68,69] lipase-catalysed transesterification of alcocols,[55,61,71-73] polyphenol oxidase-catalysed oxidation of phenols,[74] alcohol dehydrogenase-catalysed stereoselective oxidoreductions[75,76] and peroxidase-catalysed oxidations using biosensors.[77,78] For example, the oxidation of 3-methyl-2-buten-1-ol (11) to the unsaturated aldehyde 3-methyl-2-butenal (12) in heptane has been optimised using yeast alcohol dehydrogenase (ADH) (Scheme 1.9).[76]
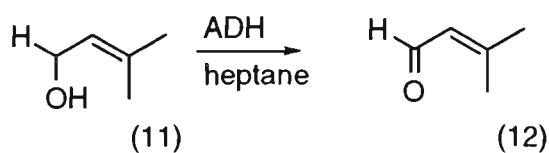
Scheme 1.9

Baker's yeast (*Saccharomyces cerevisiae*) is a cheap source of a wide variety of enzymes which can be utilised to catalyse the synthesis of a number of chiral compounds.[79-89] Product yields, from reactions employing fermenting baker's yeast,[13-17] are limited due to a range of problems;[87] solubility of the substrate in the fermentation medium, the production of unwanted by-products, and difficulties with the isolation of the desired product due to a large biomass.[44] A range of chiral organic compounds has been successfully synthesised with high optical purity and in high yield using dried baker's yeast in an organic solvent. Solubility and separation problems which were encountered when using fermenting yeast in an aqueous system have been overcome with this methodology.[44,79-92]

1.4.1 *Yeast mediated reduction in an organic solvent*

A number of yeast mediated reductions have been studied in an organic solvent system, including the reduction of keto esters to chiral hydroxy esters and the reduction of carbon-carbon double bonds in nitrostyrenes. These reactions were studied in order to improve both the isolated yield and the stereoselectivity.

1.4.1.1 *Reduction of keto esters using baker's yeast in an organic solvent*

Chiral hydroxy esters are important building blocks for the synthesis of a number of biologically active compounds.[93,94] In order to overcome problems, including insolubility of starting material and hydrolysis of the product, organic solvents have been successfully utilised in the yeast mediated reduction of a range of keto esters.[80,81,85,86,93-98]

It was originally thought that an organic solvent would seriously damage the yeast cell membrane and denature the enzymes decreasing the productivity of the reaction by releasing denatured enzymes into the solvent. The yeast cells were therefore immobilised since this was known to enhance the stability of enzymes in organic solvents.[86]

The reduction of a number of α-keto esters was studied using immobilised baker's yeast in an organic solvent in order to investigate whether changes in reaction conditions appreciably affected the stereochemistry of the product. The results were compared with those obtained using free and immobilised baker's yeast in water.[93,94] A series of α-keto esters, ethyl-2-oxoalkanoates were reduced using immobilised baker's yeast in hexane (Scheme 1.10). These studies showed little difference in the yield and stereoselectivity of the products obtained for R= methyl ethyl or propyl when compared with either of the aqueous systems. In all cases the (S)-isomer was obtained. Reversal of the stereochemistry was observed in reactions involving longer chain alkyl groups, R= butyl or pentyl; in hexane the (R)-isomer was obtained whilst in both aqueous systems, the (S)-isomer resulted.
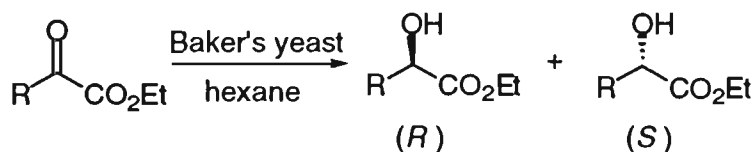
Scheme 1.10

The reversal of stereochemistry is probably due to a yeast mediated enantioselective decomposition. In water the (R)-hydroxy ester is hydrolysed to the corresponding acid leaving the (S)-hydroxy ester as the sole product (Scheme 1.11).[96]
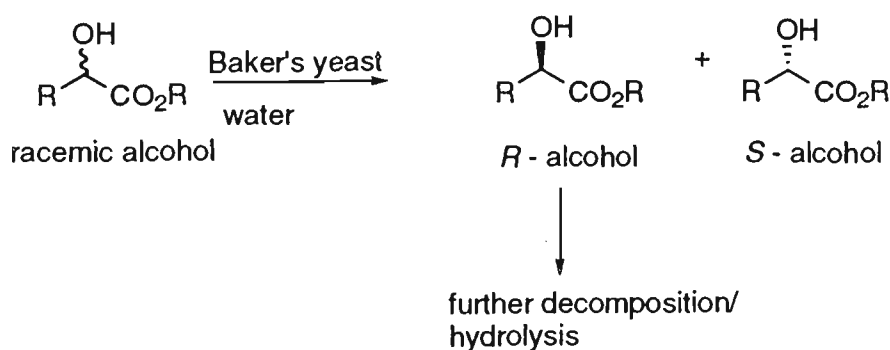
Scheme 1.11

A further series of α-keto esters, alkyl 3-methyl-2-oxobutanoates, was studied using immobilised baker's yeast in an organic solvent[94] and the (R)-hydroxy ester was obtained in each case (Scheme 1.12). The (R)-enantiomer was also obtained from reductions employing free or immobilised baker's yeast in water.[94] Higher stereoselectivity was obtained in the hexane system than in either of the aqueous

systems. It was also observed that as the alkyl chain length increased from methyl to butyl, the ee of the product increased with the highest ee (93%) being observed for the butyl derivative. The enhancement in stereoselectivity in this system appears to be due to the nature of the solvent since the hydrolysis reaction was not observed.[94]
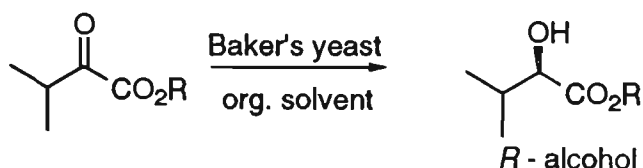
Scheme 1.12

Studies of the reduction of some β-keto esters in hexane (Scheme 1.13), have found that the stereochemistry of the product can be controlled by employing immobilised baker's yeast with the inclusion of additives such as alcohols, dimethyl sulphoxide (DMSO), thioacetamide or adenine, instead of glucose.[99] Although the reaction conditions were not optimised Naoshima et al.[99] found the additives to be either L-selective or D-selective. Reduction of the ester (13) in hexane with the inclusion of additives such as saturated alcohols (C_{1-4}), DMSO, thioacetamide and adenine, gave the L-hydroxy ester with an enantiomeric purity of 21 - 43%. This was in contrast to adding allyl alcohol or quinine which resulted in the D-isomer, with 55% and 36% ee respectively. Reduction of the ester (14) with the inclusion of saturated alcohols, thioacetamide or adenine, as additives resulted in the L-isomer with 18 - 55% ee. In contrast, the addition of allyl alcohol gave D-isomer product with 64% ee. Reduction of the ester (15) gave the D-isomer irrespective of the additive used.

1. R = CH_2Cl
2. R = CH_3CH_2
3. R = $CH_3(CH_2)_2$
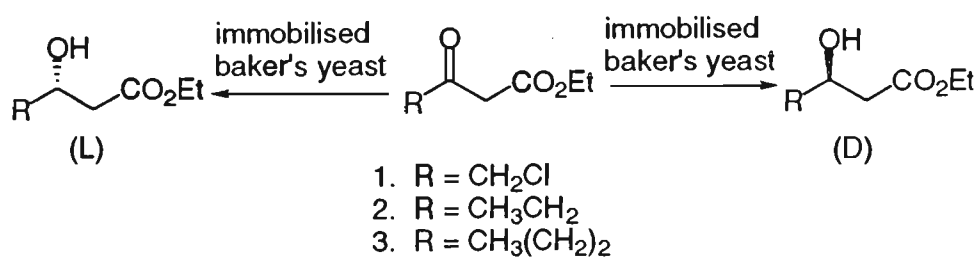
Scheme 1.13

Following the success of immobilisation of yeast in organic solvents, the reduction of keto esters was studied using free baker's yeast in organic solvents.[80,81,85,86,92-98] These studies were successful and showed that immobilisation was not required when using yeast in an organic solvent. Studies involving the reduction of keto esters using non-immobilised yeast in an organic solvent revealed that 0.2 - 1.2ml H_2O/ g yeast was required and if more than 1.2ml of H_2O/ g yeast was used, the reduction was supressed.[81,85]

The reduction of ethyl 2-oxoheptanoate (16: R = Et) using baker's yeast in various hydrophobic organic solvents was studied in order to obtain the highest enantioselectivity and chemical yield of the corresponding chiral alcohol (17: R = Et) (Scheme 1.14).[86] The best results were achieved using benzene as the solvent. A series of α-keto esters was reduced in benzene and it was found the enantiselectivity of the product shifted to the (R)-isomer in contrast with reductions in water which resulted in a predominance of the (S)-isomer, with the exception of (16: R = Me) which gave the (S)-isomer in both systems.[86,95] It was also observed as the size of the alkyl group increased, the ee also improved (13-86%).[86]
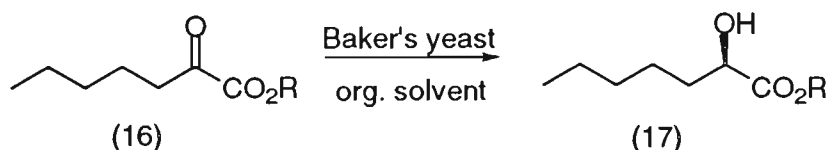
Scheme 1.14

The (R)-isomer is the major product in benzene since unlike the aqueous systems, hydrolysis of the product does not occur.

The stereoselective reduction of a series of β-keto esters using free baker's yeast in an organic solvent has also been investigated (Scheme 1.15).[80,81,92,95,98] (S)-3-Hydroxy butanoates were prepared in high isolated yield (56-96%) and with high enantioselectivity (>94% ee) from their corresponding β-keto esters using yeast in light petroleum spirit.[92] The isolated yield and optical purity were generally higher than those reported for the reductions with fermenting yeast in water[100-103] and in the

organic systems reported by North[98] who obtained isolated yields of 15-39%. The yeast mediated reduction of ethyl acetoacetate (18: R = Me, R' = Et) was studied using a variety of organic solvents including petroleum spirit, toluene, carbon tetrachloride and diethyl ether.[81,95] The optimum reaction conditions, 0.8ml H_2O/ g of yeast and 1 -2g yeast/ mmol substrate, gave ethyl (S)-3-hydroxybutyrate (19: R = Me, R' = Et), with a conversion of 90-100%, isolated yield of 53-58% with 96-98% ee. The best stereoselectivity was achieved using 1g yeast/mmol substrate in light petroleum spirit.[81] These results are superior to those obtained by Rotthaus et al.[95] who used 5g yeast/ 0.5mmol substrate in hexane, toluene, diethyl ether and ethyl acetate and achieved conversion rates of 80-100% with 57-100% ee.[95] The reduction of the chlorinated β-keto ester, ethyl 4-chloroacetoacetate (18: R =ClCH$_2$, R' = Et) in toluene resulted in the (R)-isomer with an optical purity of 73%, compared with the reaction in water which resulted in the (S)-isomer with an optical purity of 14%.[95]
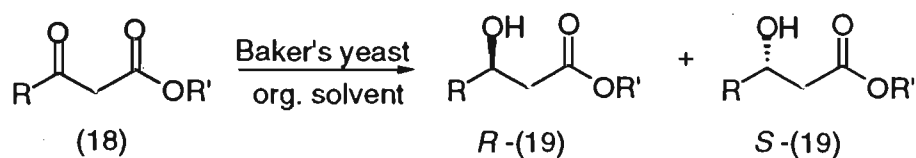
Scheme 1.15

1.4.1.2 Reduction of diones using yeast in an organic solvent

The microencapsulation of the yeast cells was studied as an alternative to immobilisation of yeast. Microencapsulated yeast was used to catalyse the reduction of the diketone, 1-phenyl-1,2-propanedione (20) to 2-hydroxy-1-phenyl-1-propanone (21) (Scheme 1.16).[104] A range of organic solvents including octane, butyl acetate, dodecane and decane was used for the reaction with the highest yield (46%) obtained in decane.[104]
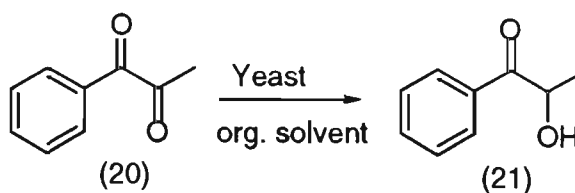
Scheme 1.16

1.4.1.3 *Reduction of nitrostyrenes using yeast in an organic solvent*

The yeast mediated reduction of β-substituted nitrostyrenes in aqueous systems invariably resulted in racemic mixtures due to the racemisation of the product under the mildly basic conditions.[105] A series of β-nitrostyrenes were reduced using baker's yeast in an organic solvent (Scheme 1.17) and whilst racemic mixtures were also obtained but the corresponding nitroalkanes were produced in good yield.[84] It was found that racemisation was not occuring in the organic solvent system[84] and mechanistic studies indicated that a reversible non-stereoselective protonation occured at the β-centre followed by a stereoselective addition of hydride at the α-position.[85] In an organic system nitrostyrenes with electron-donating groups attached to the aromatic ring resulted in higher yields of nitroalkanes than those containing electron-withdrawing groups; this was also observed in aqueous systems.[84]
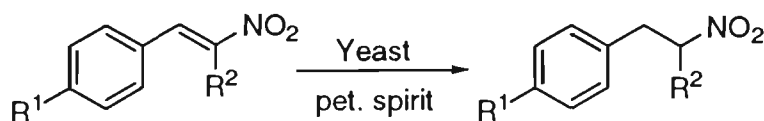
Scheme 1.17

1.4.2 *The biotransformation of hydrazones and oximes to aldehydes and ketones using baker's yeast in organic solvents*

In the synthesis of some complex organic molecules, hydrazones and oximes are employed as protecting groups for ketones and aldehydes. The regeneration of carbonyl compounds from hydrazones and oximes generally requires strongly oxidative or reducing conditions and entails the use of expensive reagents or tedious proceedures. Transformations employing milder conditions are desirable. Kamal and Praveen Reddy[106] have explored the enzymatic regeneration of aldehydes and ketones from hydrazones and oximes using baker's yeast in organic solvents (Scheme 1.18). The mild transformation conditions which they employed were found to be suitable for aldehydes and ketones and offer a biocatalytic method of practical importance to the synthetic chemist. Kamal and Praveen Reddy[106] found that no transformation occurred in benzene unless a small amount of water was added. This requirement was also observed in other enzyme catalysed reactions in organic media.[44,50,57,59,80-89]
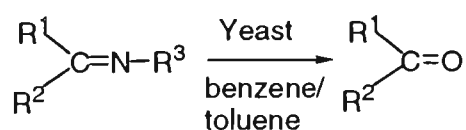
Scheme 1.18

1.4.3 *Alkylation of α-cyanoketones using baker's yeast in an organic solvent*

The versatility of baker's yeast as a biocatalytic reagent in an organic solvent is continually being explored and its use expanded. Initial studies involving the reaction of 3-oxo-3-phenylpropanenitrile (22) with baker's yeast in an organic solvent resulted in both a racemic alkylated product, 2-cyano-1-phenylbutanone (23) (40%), and a low yield of (*S*)-3-hydroxy-3-phenylpropanenitrile (24) (10%) (Scheme 1.19). The addition of a small quantity of acetaldehyde to the reaction resulted in the exclusive formation of 2-cyano-1-phenylbutanone (18) as a racemate in good yield (71%) (Scheme 1.20). These reaction conditions were used to alkylate a series of α-

cyanoketones and resulted in the formation of racemic alkylated product in relatively good yield.[107]
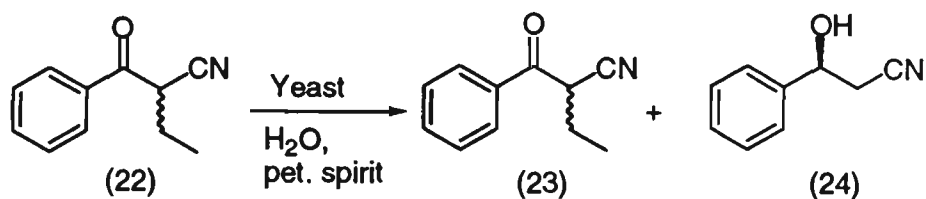
Scheme 1.19
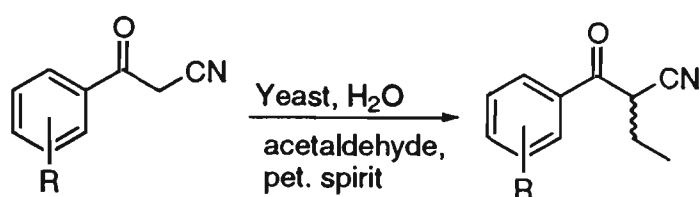
Scheme 1.20

1.4.4 *Synthesis of l-PAC (3) in organic solvents*

Studies by Nikolova and Ward[33] found the yeast reduction of benzaldehyde (1) to benzyl alcohol (4) (Scheme 1.21) in aqueous media resulted in 2 to 3 times more product than was obtained in an organic solvent, such as hexane, containing 2% of an aqueous solution.[33,80-82] The results of these studies suggested that using an organic solvent in place of water for the yeast mediated acyloin condensation of benzaldehyde (1) to form *l*-PAC (3) would reduce the amount of benzyl alcohol (4) produced.
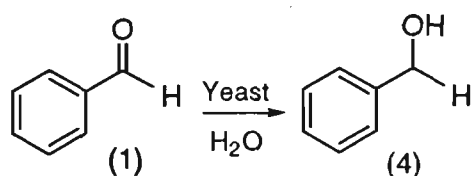
Scheme 1.21

Biotransformation activity, a measure of the rate of the yeast-induced condensation of benzaldehyde (1) and acetaldehyde (2), was observed using the organic solvents; hexane, hexadecane, chloroform and toluene.[88] The highest biotransformation activities were observed using hexane and hexadecane. Although the isolated yield and enantiomeric purity of the *l*-PAC (3) formed were not quoted, maximum biotransformation activity was observed when the hexane : moisture content was 90:10.[89] The biotransformation activity, given as mmol/h/g, was measured using GC analysis of the reaction medium.[88]

Nikolova and Ward[32,33] have investigated the biotransformation of benzaldehyde (1) and pyruvate (10) to *l*-PAC (3) using whole cell yeast in a two-phase aqueous-organic reaction medium. They employed a method which involved pre-treatment of the yeast with buffer and subsequent lyophilisation. Celite was then mixed with the lyophilised cells, in order to immobilise the yeast, the mix was then resuspended in buffer and relyophilised. The lyophilised biocatalyst was then suspended in the biotransformation medium which consisted of hexane, that had been saturated with sodium citrate buffer (pH 6) and additional buffer at a level of 0.5-20% v/v. For example, a 10% moisture level was prepared by mixing 10ml of buffer with 90ml of the presaturated organic solvent. Sodium pyruvate (10b) was then added as substrate followed by benzaldehyde (1) (Scheme 1.22). The reactions were conducted on an orbital shaker at 28°C and the amount of *l*-PAC (3) formed was determined by GC.
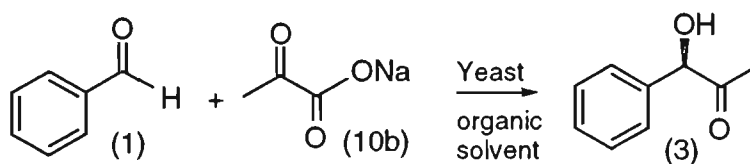
Scheme 1.22

Product yield was not specifically stated by Nikolova and Ward but analysis of their graphical data revealed that they had only obtained 4.5% conversion of benzaldehyde (1) to *l*-PAC (3).[89] Their results indicate that *l*-PAC (3) can be synthesised in an organic solvent, but the system would require further development in order to attain higher yields of *l*-PAC (3) with high enantiomeric excess (ee).

1.5 The Significance of Improving the Production of *l*-PAC (3)

The world market for *l*-ephedrine (7) and the related compound, *d*-pseudoephedrine (8) (Scheme 1.3), is over 1000 tonnes per annum with *d*-pseudoephedrine (8) making up the bulk of the sales.[4] The market value of these drugs is about $80/kg which amounts to more than $80M per annum. Approximately 30% of the world market is supplied from natural sources and the remainder supplied with synthetic product manufactured by traditional processes.[4,5,6] The importance of the development of a low cost method for the production of *l*-ephedrine (7) is highlighted by the fact that Orica Australia (previously ICI Australia) recently completed and then prematurely closed a $70M ephedrine plant due to the marginal economic viability of these current processes.[4]

1.6 Aim of the Project

This project involves an investigation of the yeast-mediated acyloin condensation of benzaldehyde (1) to form *l*-PAC (3) in an organic solvent, with the aim of:

(i) optimising the production of *l*-PAC (3) by increasing the conversion of benzaldehyde (1) to the product, by increasing the ease of isolation of the product from the reaction mixture, by eliminating the formation of by-products, and by improving enantiomeric purity of the product.

(ii) establishing the role of pyruvic acid (10) and acetaldehyde (2) in this acyloin reaction

(iii) increasing the level of understanding of the mechanism of this reaction through NMR studies of reactions involving ^{13}C labelled reagents and reactions carried out at different temperatures.

CHAPTER 2

STUDIES OF THE EFFECT OF SODIUM PYRUVATE ON THE YEAST MEDIATED ACYLOIN CONDENSATION OF BENZALDEHYDE.

STUDIES OF THE EFFECT OF SODIUM PYRUVATE ON THE YEAST MEDIATED ACYLOIN CONDENSATION OF BENZALDEHYDE.

2.1 Introduction.

Pyruvate is an important substrate in the yeast mediated acyloin condensation of benzaldehyde (1) to form *l*-phenylacetylcarbinol (*l*-PAC) (3).[22,23,32,33] In the fermenting system, glucose (9), which is part of the fermentation broth, is converted to pyruvic acid (10) *via* the Embden-Meyerhof Pathway.[20] The pyruvic acid (10) condenses with added benzaldehyde (1) to form *l*-PAC (3) (Scheme 2.1).
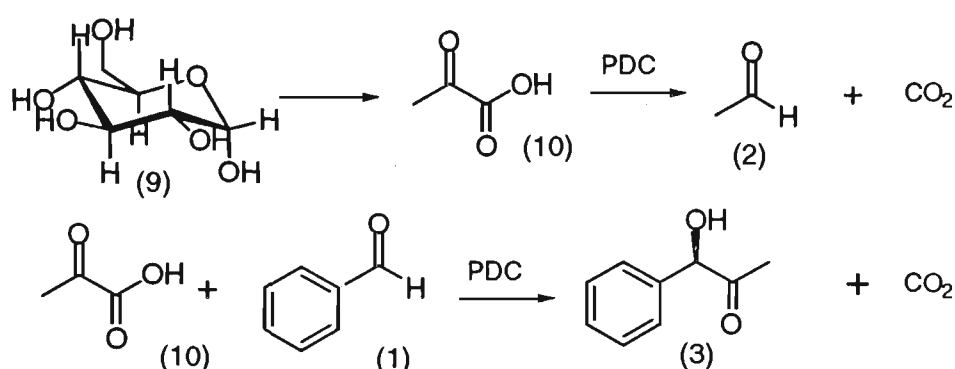
Scheme 2.1

The biotransformation of benzaldehyde (1) to *l*-PAC (3), in a system using fermenting yeast does not require the addition of pyruvate since this is produced *in situ* from glucose (9) (Scheme 2.1) but in systems using partially purified PDC, pyruvate was added to the reaction medium since the substrate was no longer produced.[23] In an organic medium using non-fermenting yeast, it is also necessary to add sodium pyruvate (10b) to the reaction.[89,90]

Nikolova and Ward[89,90] used the following procedure for the production of *l*-PAC (3) in an organic system. Freshly pressed baker's yeast was suspended in a pH6 sodium citrate buffer, lyophilised and mixed with celite in order to immobilise the yeast. The immobilised yeast was then resuspended in buffer and again lyophilised. The condensation reactions were carried out in a two phase organic/aqueous system obtained by saturating hexane (90ml) with buffer (10ml).

The pre-treated yeast, sodium pyruvate (10b) and benzaldehyde (1) were added to the biotransformation medium and the mixture was agitated on an orbital shaker at 28°C for a total period of 26h. Although a specific yield was not quoted, graphical data presented indicated a yield of about 4.5%.[89]

The basic reaction conditions employed by Nikolova and Ward[89] were studied in this work with the general aim of developing a more efficient synthesis of *l*-PAC (3), the precursor of *l*-ephedrine (7). The specific objective was to eliminate the need for immobilisation of the yeast and to develop a less cumbersome process involving fewer manipulations and with enhanced commercial viability.

2.2 Preparation of Racemic Phenylacetylcarbinol (PAC (3)) and 1-Phenylpropan-1,2-diol (5).

In order to facilitate the detection and identification of PAC (3) and possible by-products in the reaction mixture authentic samples of PAC (3) and its reduction product, 1-phenylpropan-1,2-diol (5), were prepared.

Racemic PAC (3) was synthesised *via* a three step process in low overall yield (6%) (Scheme 2.2).[91]
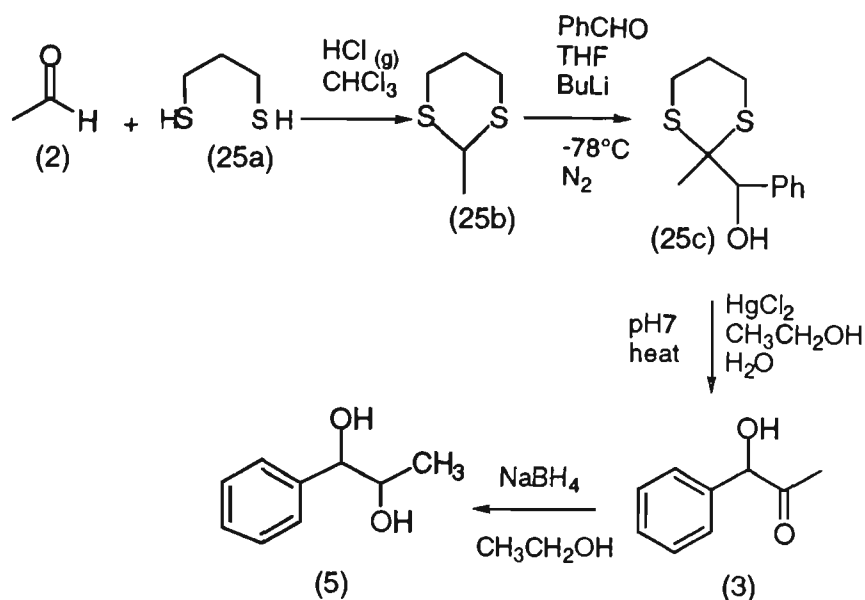
Scheme 2.2

Acetaldehyde (2) and propan-1,3-dithiol (25a) were condensed *via* the method of Corey and Erickson[91] to produce 2-methyl-1,3-dithiane (25b) in a 32% yield. The dithiane (11b) was then reacted with butyl lithium to form the tertiary carbanion *in situ* followed by the addition of benzaldehyde (1) to produce 2-methyl-α-phenyl-1,3-dithiane-2-methanol (25c) in a 28% yield. Hydrolysis with mercuric chloride in aqueous ethanol afforded PAC (3) in a 64% yield. The PAC (3) was then reduced using sodium borohydride to form 1-phenylpropan-1,2-diol (5).

An authentic sample of 1-phenylpropan-1,2-dione (20), the other possible reaction by-product, was synthesised by a colleague, Abilio Ten.

2.3 Yeast Mediated Acyloin Condensation of Benzaldehyde

The yeast mediated acyloin condensation of benzaldehyde (1) was undertaken in petroleum spirit using dried baker's yeast, rather than the immobilised yeast described previously.[89,90] The reaction system contained 5g yeast, 5ml pH6 citrate buffer, 1mmol benzaldehyde (1) and 1g sodium pyruvate (10b) (Scheme 2.3). The reaction mixture was suspended in petroleum spirit and stirred at 20°C. After 24h gas

chromatographic (GC) analysis indicated that the reaction mixture was composed of 70% benzaldehyde (1), 21% benzyl alcohol (4), 8% PAC (3) and 1% diketone (20).
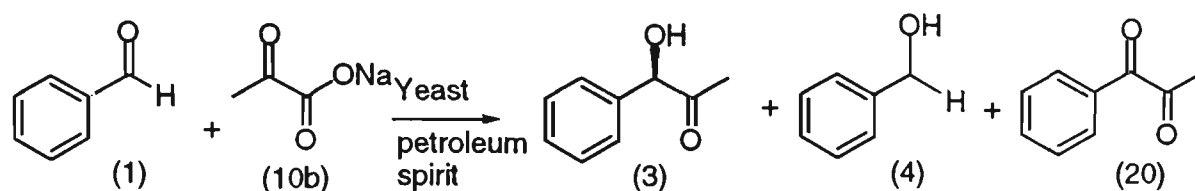
Scheme 2.3

Whilst *l*-PAC (3) was formed by the yeast mediated acyloin condensation of benzaldehyde (1) and sodium pyruvate (10b), side reactions involving the reduction of benzaldehyde (1) to benzyl alcohol (4) and the oxidation of *l*-PAC (3) to the diketone (20) were also occurring. In order to reduce the activity of the yeast oxidoreductase enzymes involved in the production of benzyl alcohol (4) and the diketone (20), ethanol, was added to the reaction medium since it has been shown to inhibit reactions of this kind.[92]

The yeast mediated acyloin condensation was carried out using the conditions described above with the inclusion of ethanol (0.5ml). GC analysis of the reaction mixture after 24h showed only benzaldehyde (1) and *l*-PAC (3) in a ratio of 88 : 12. No benzyl alcohol (4) or diketone (20) was formed under these conditions.

Although the addition of ethanol to the reaction medium successfully eliminated the production of the unwanted by-products, benzyl alcohol (4) and the diketone (20), the reaction required optimisation to increase the amount of *l*-PAC (3) produced.

2.4 Effect of Sodium Pyruvate

In an aqueous system, using partially purified PDC from *Candida utilis*, which had been treated in a fermentor in a medium containing glucose, Shin and Rogers[23] examined the effect of adding sodium pyruvate (10b) to the reaction system in order to achieve higher concentrations of *l*-PAC (3). It should be noted that the pyruvate added to their reaction system is in addition to that formed *in situ* from glucose and thus the exact amount of pyruvate in the system is not known. Shin and Rogers[23] obtained an optimal concentration of *l*-PAC (3) of 28.6g/l (190.6mM) compared with 15.2g/l in their system without added sodium pyruvate.

In order to examine the effect of sodium pyruvate (10b) on the production of *l*-PAC (3) in an organic medium, the yeast mediated acyloin condensation of benzaldehyde (1) was carried out using varying quantities of sodium pyruvate (10b) (0.5 -3.0g). The formation of *l*-PAC (3) was monitored using GC and the results are plotted in Figure 2.1.

When the reaction contained only 0.5g of sodium pyruvate, no *l*-PAC (3) and only a small amount of benzyl alcohol (4) was formed. As the quantity of sodium pyruvate (10b) was increased the amount of *l*-PAC (3) formed also increased. The best result, 31%, was obtained with 2.5g sodium pyruvate. A saturation point of 2.5g of sodium pyruvate/5ml of citrate buffer was obtained and thus at 3g not all the sodium pyruvate (10b) was soluble. The yield of *l*-PAC (3) decreased slightly, from 31% to 28%, and by-products, the diol (5), which is formed as a result of the reduction of *l*-PAC (3), and the diketone (20) were formed. Due to solubility problems, for all remaining reactions the sodium pyruvate (10b) was firstly dissolved in the pH6 citrate buffer before the buffer was added to the yeast.
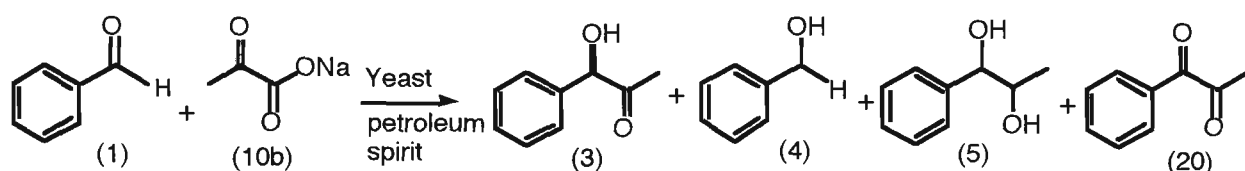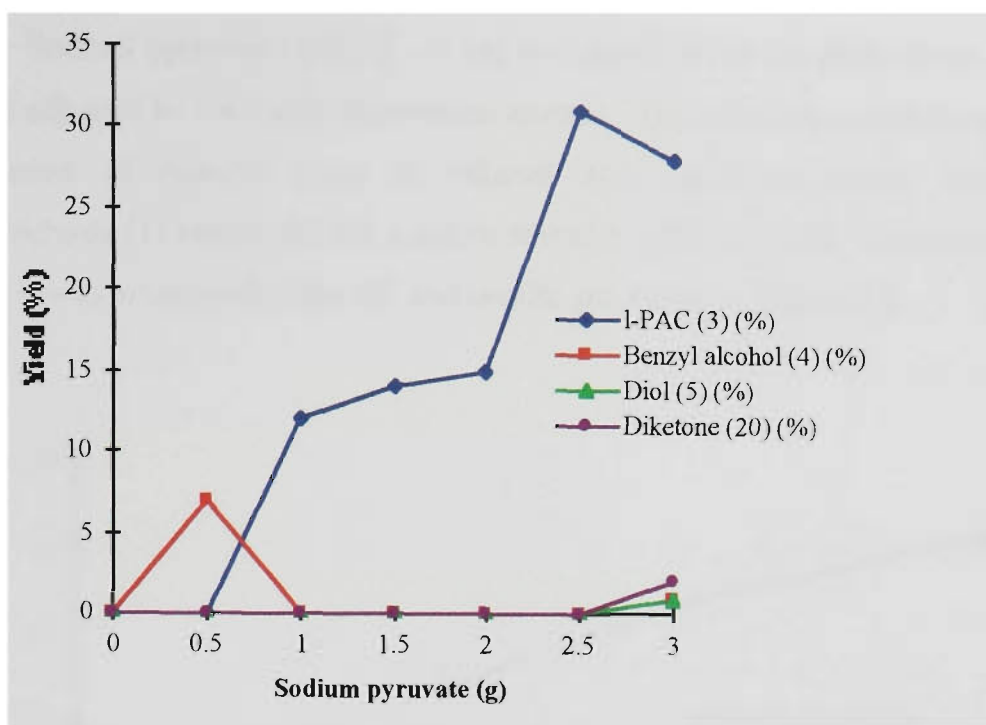
Figure 2.1 The effect of sodium pyruvate (10b) on the production of *l*-PAC (3) and various other by-products from the yeast mediated acyloin condensation of benzaldehyde. All reactions contained 5g of yeast, 5ml buffer, 45ml petroleum spirit, 0.5ml of ethanol, 1mmol of benzaldehyde and were stirred for 24h at 20°C.

These results suggest that a large excess of sodium pyruvate (10b) (23mmol) compared with benzaldehyde (1) (1mmol) is necessary for the reaction to take place. It was also observed that the sodium pyruvate (10b) lowered the pH of the citrate buffer and it therefore seemed possible that the altered pH of the reaction system may have been responsible for the increase in the amount of *l*-PAC (3) produced.

The highest amount of *l*-PAC (3) (31%) was obtained using 2.5g of sodium pyruvate (10b) in 5ml of the pH6 citrate buffer, which resulted in a final pH of 5.45. In order to determine whether the sodium pyruvate (10b) or the pH was the more significant factor, the quantity of sodium pyruvate (10b) added to the reaction was varied with the pH held constant at 5.45 by the addition of ammonium acetate.

Sodium pyruvate (10b) (1 - 2.5g) was dissolved in the pH6 citrate buffer and the pH adjusted to 5.45 with ammonium acetate. This solution was then added to the suspension of baker's yeast in ethanol and petroleum spirit, the substrate benzaldehyde (1) added and the mixture stirred at 20°C for 24h. The formation of *l*-PAC (3) was monitored using GC and results are shown in Figure 2.2.
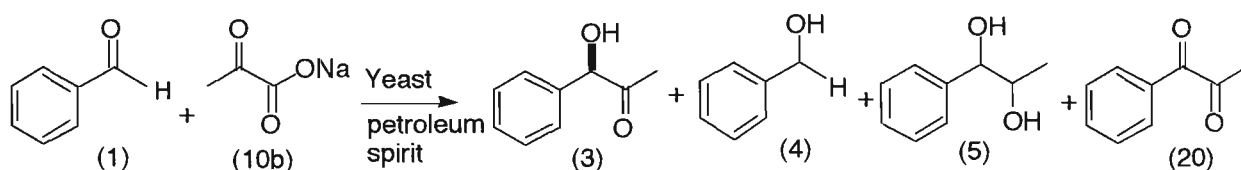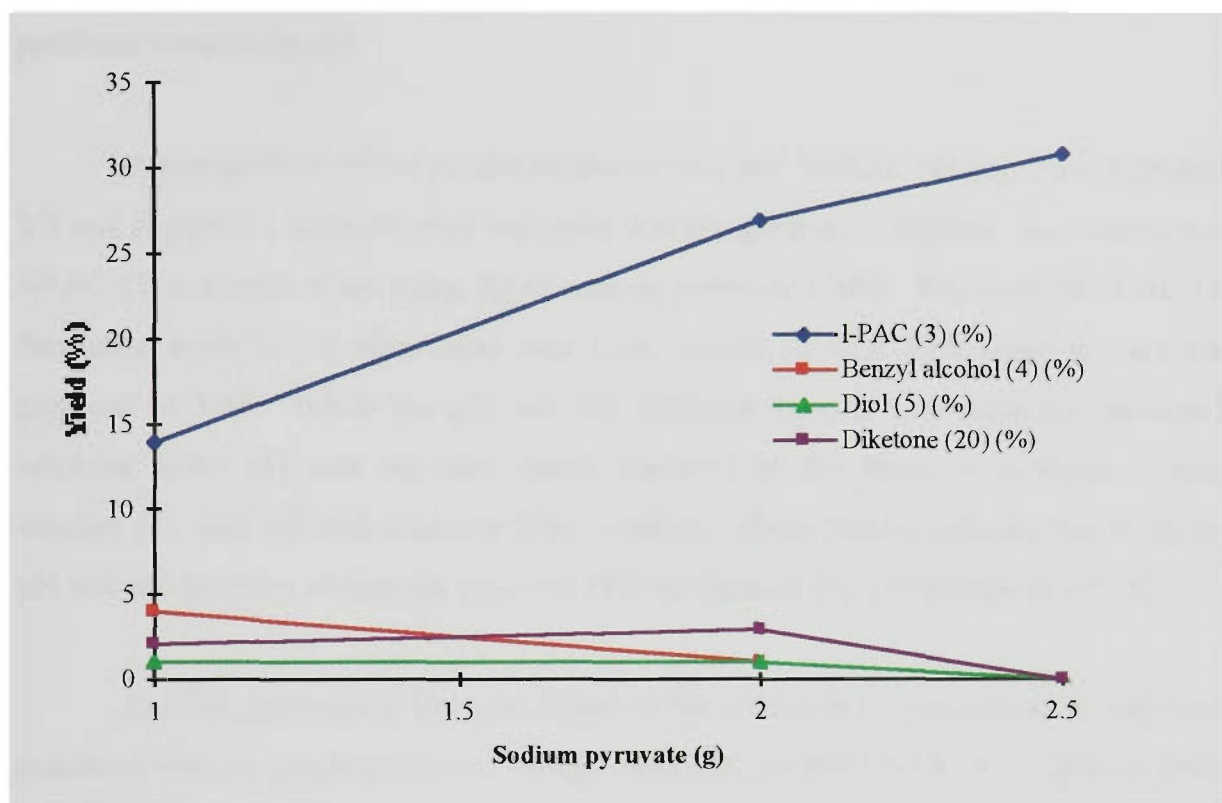
Figure 2.2 The effect of sodium pyruvate (10b) on the production of *l*-PAC (3) and various other by-products from the yeast mediated acyloin condensation of benzaldehyde with the pH adjusted to 5.45 by the addition of ammonium acetate. All reactions contained 5g of yeast, 5ml buffer, 45ml petroleum spirit, 0.5ml of ethanol and 1mmol of benzaldehyde and were stirred for 24h at 20°C.

When only 1g of sodium pyruvate (10b) was used in the acyloin condensation of benzaldehyde (1) the yield of *l*-PAC (3) was only 14% and significant amounts of the by-products, benzyl alcohol (4), diol (5) and diketone (20) were formed. As the quantity of sodium pyruvate (10b) was increased the GC results showed the yield of *l*-PAC (3) increased and the by-products decreased. The highest yield of *l*-PAC (3) (31%) was again obtained using 2.5g of sodium pyruvate (10b) and again no by-products were observed.

A comparison of the results obtained with and without pH adjustment (Figure 2.2 and Figure 2.1 respectively) indicates that the greatest difference in conversion to *l*-PAC (3) occurred when using 2g of sodium pyruvate (10b). The level of *l*-PAC (3) formation without pH adjustment was 15%, compared with 27% when the pH was adjusted to 5.45. When the pH was not adjusted, by-products were not observed, whereas when pH was adjusted, small amounts of the three by-products, benzyl alcohol (4), diol (5) and diketone (20), resulted These results indicate that both the pH and the quantity of sodium pyruvate (10b) influence the production of *l*-PAC (3).

Sodium pyruvate (10b) was found to be a necessary ingredient in the yeast mediated acyloin condensation of benzaldehyde (1) to form *l*-PAC (3). Results from the above reactions indicate that between 2g and 2.5g (18-23mmol) of sodium pyruvate (10b) is required per mmol of benzaldehyde (1). This is a large excess of sodium pyruvate (10b) and before the reaction could form the basis for a commercial process, the reaction conditions would need to be further optimised in order to minimise the use of this expensive reagent.

In an aqueous system, under fermentation conditions, using partially purified PDC,[23] the addition of sodium pyruvate (10b) enhanced the production of *l*-PAC (3), this was also found to be the case in an organic system. Although the ratio of benzaldehyde (1) : sodium pyruvate (10b) added in an aqueous system was much lower (1:10) than in an organic system (1:23), in an aqueous fermenting system some pyruvate is formed *in situ* from glucose (9), but glucose (9) is not included in the organic medium. In both an organic system and fermenting system, a relatively large quantity of sodium pyruvate (10b) is required for the conversion to *l*-PAC (3).

2.5 Addition of Ethanol

Consistent with the well known ability of ethanol to deactivate yeast enzymes[57,108] the inclusion of a small quantity of ethanol in the condensation reaction medium eliminated the production of the unwanted by-products, (4), (5) and (20), presumably through the inhibition of oxidoreductase enzymes.

To determine whether the ethanol was also inhibiting the enzyme(s) catalysing the condensation reaction, a series of reactions was performed using 1-2.5g of sodium pyruvate (10b) with the pH adjusted to 5.45, but in the absence of ethanol. The results, plotted in Figure 2.3, show that as the quantity of sodium pyruvate (10b) increases, the yield of benzyl alcohol (4) and the diketone (20) decreases until with 2.5g of sodium pyruvate (10b) *l*-PAC (3) is the only product. The results indicate that sodium pyruvate (10b) also affects the unwanted oxidation/reduction reactions that produce the by-products.

A comparison of the results obtained with and without ethanol (Figure 2.2 and 2.3), using 2.5g of sodium pyruvate, shows the extent of *l*-PAC (3) produced as 31% and 8%, respectively. Thus the inclusion of ethanol not only resulted in the reduction of side reactions, it also led to an increased amount of *l*-PAC (3).
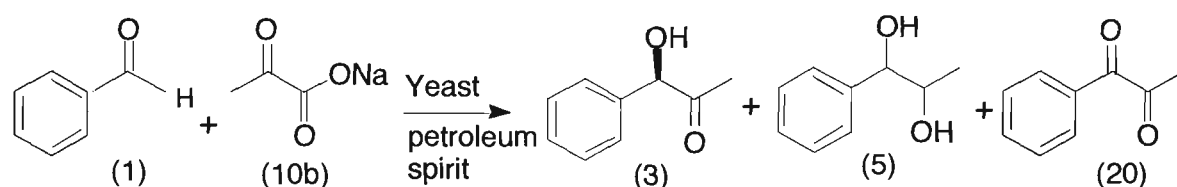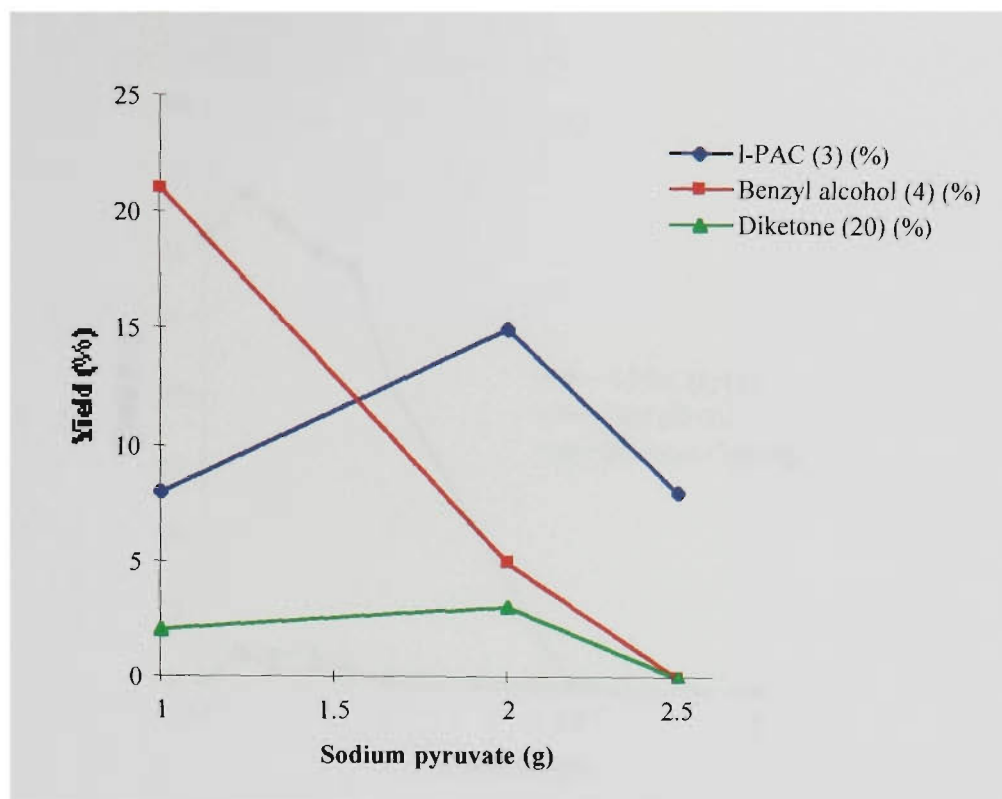
Figure 2.3 The production of *l*-PAC (3) and various other by-products from the yeast mediated acyloin condensation of benzaldehyde (1) with the pH adjusted to 5.45 by the addition of ammonium acetate following the addition of sodium pyruvate (10b) (1-2.5g). All reactions contained 5g of yeast, 5ml buffer, 45ml petroleum spirit and 1mmol of benzaldehyde (1) and were stirred for 24h at 20°C.

In order to determine the optimal quantity of ethanol a series of yeast mediated acyloin condensation reactions was conducted with 0.5 - 2ml of ethanol and using the conditions determined to be optimal in the previous section, i.e. 2.5g of sodium pyruvate, 5g of yeast and 1mmol benzaldehyde. The results are presented in Figure 2.4.
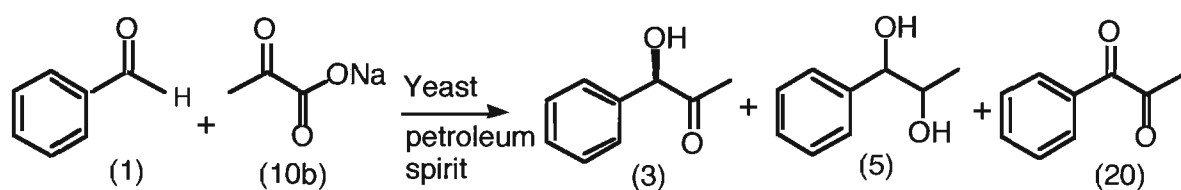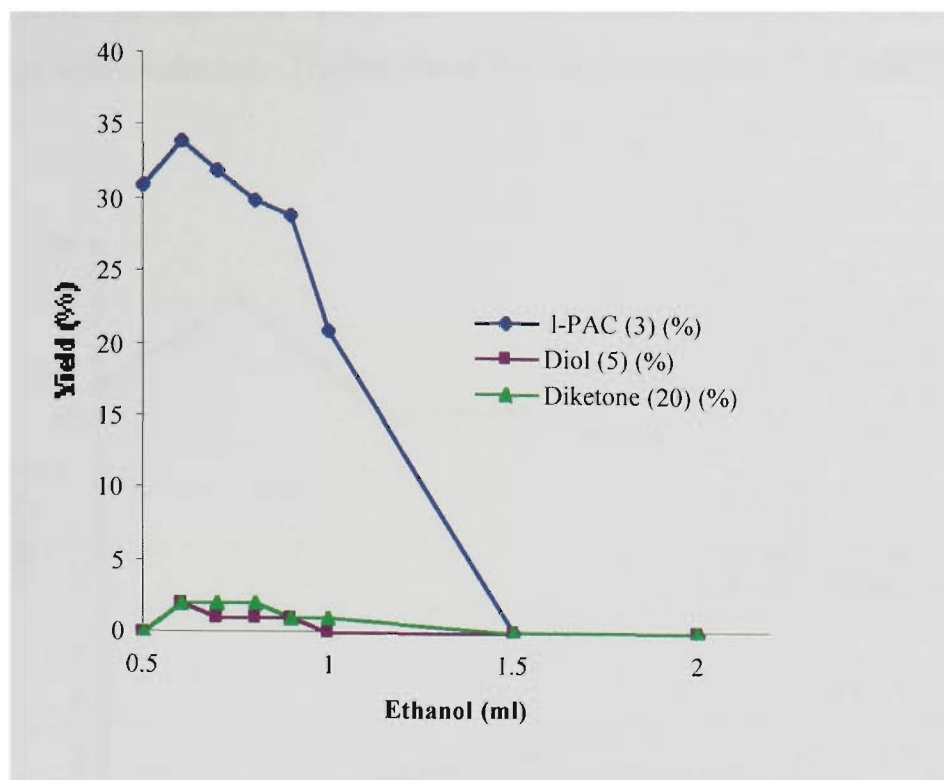
Figure 2.4 Variation in the quantity of ethanol (0.5 - 2ml) added to the reaction medium. All reactions contained 5 g of yeast, 5ml buffer (pH6), 45ml petroleum spirit, 2.5g of sodium pyruvate (10b) and 1.2 – 1.3mmol benzaldehyde (1) and were stirred for 24h at 20°C.

Very little variation in the yield of *l*-PAC (3), (31 - 34 %) was observed for amounts of ethanol between 0.5 and 0.9ml. The addition of between 0.6ml and 0.9ml ethanol led to the formation of a very small quantity of by-products (5) and (12). Addition of 1ml of ethanol led to a drop in the yield of *l*-PAC (3) (21%), whilst the addition of 1.5 - 2ml of ethanol completely halted the reaction. The highest yield of *l*-PAC (3) (34%) was observed with 0.6ml ethanol, however small amounts of by-products (5 and 12) were also obtained.

Since both ethanol and sodium pyruvate (10b) were found to influence the formation of *l*-PAC (3) and by-products, it was logical to attempt to reduce the amount of sodium pyruvate (10b), which is expensive, and increase the amount of

ethanol. A series of reactions using 0.5 - 0.9ml ethanol and only 2g of sodium pyruvate (10b) was conducted. The results of this study are plotted in Figure 2.5.
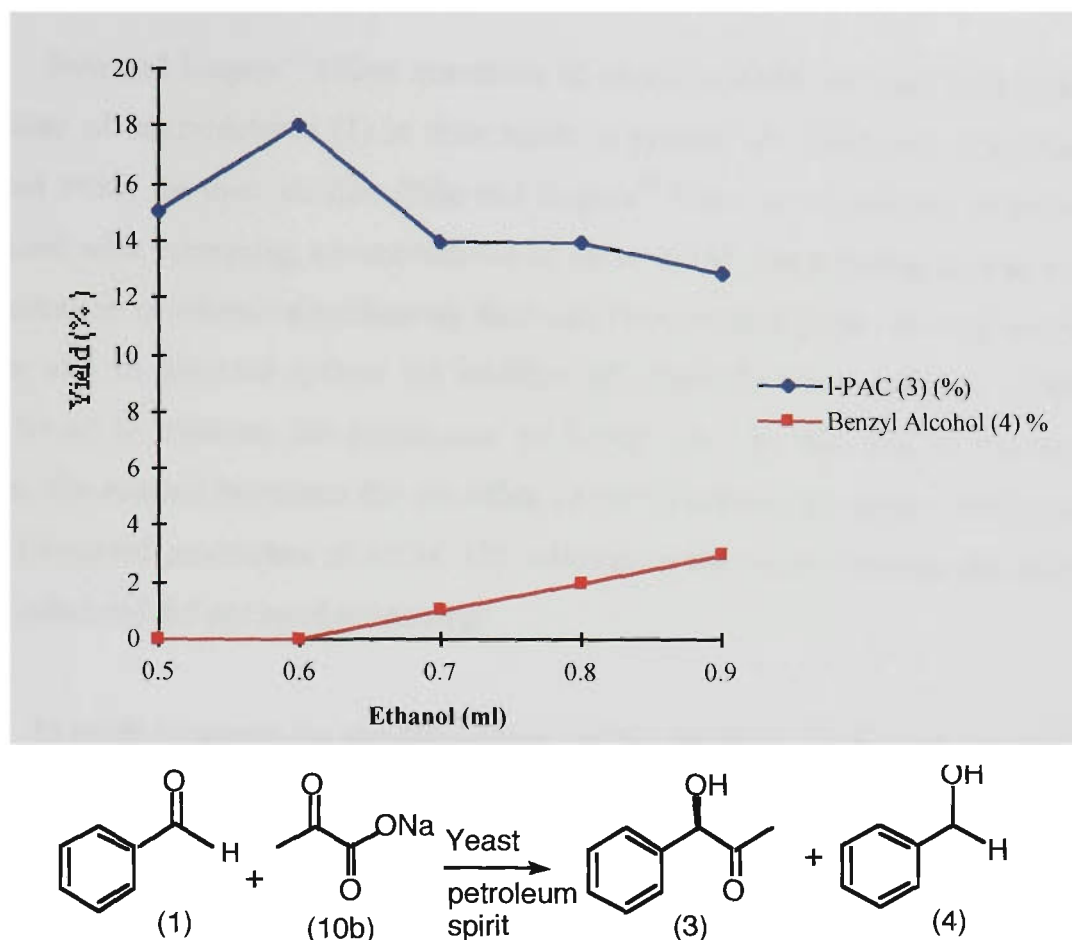
Figure 2.5 Variation in the quantity of ethanol (0.5–0.9ml) added to the reaction medium using only 2g of sodium pyruvate (10b). All reactions contained 5g of yeast, 5ml buffer (pH6), 45ml petroleum spirit and 1.2mmol benzaldehyde (1) and were stirred for 24h at 20°C.

With 2g of sodium pyruvate (10b) amounts of *l*-PAC (3) in the range of 13 - 18% were obtained. The highest yield was obtained when 0.6ml of ethanol was added to the reaction medium; the addition of 0.7 - 0.9ml of ethanol led to the formation of small amounts of benzyl alcohol (4). Under these conditions the only by-product formed was benzyl alcohol (4); neither the diol (5) nor the diketone (20) were observed.

Overall, when the quantity of sodium pyruvate (10b) was decreased to only 2g, with the inclusion of ethanol in the reaction medium, the production of *l*-PAC (3)

small amounts of ethanol seems to play a role in the production of *l*-PAC (3). The addition of ethanol appears not only to inhibit the reduction of benzaldehyde (1) to benzyl alcohol (4) but also to increase the amount of *l*-PAC (3) produced.

Shin and Rogers[23] added quantities of ethanol (0-6M) in order to increase the solubility of benzaldehyde (1) in their aqueous system, in which they used partially purified PDC. In their studies, Shin and Rogers[23] found the formation of *l*-PAC (3) increased with increasing concentrations of up to 2-3M, but a further increase in the concentration of ethanol significantly decreased the reaction rates. In both an organic system and an aqueous system the addition of relatively small amounts of ethanol were found to enhance the production of *l*-PAC (3). In the case of the aqueous system, the ethanol increased the solubility of the substrate and consequently resulted in the increased production of *l*-PAC (3), whereas in the organic system the solubility of the substrate did not need enhancing.

In order to assess the stereoselectivity of the reaction, *l*-PAC (3) was prepared and isolated in the following manner. Sodium pyruvate (10b) was dissolved in the pH6 citrate buffer before being added to the yeast. Ethanol, petroleum spirit and benzaldehyde (1) were added and the reaction mixture was stirred at 20°C for 24h. The yeast was then filtered and washed with diethyl ether. Distillation of the filtrate and washings gave *l*-PAC (3) in low isolated yield (16%). The optical rotation of the product was measured as $[\alpha]_D$ = -239.2° (c =0.64, CHCl$_3$), (Lit.[109], $[\alpha]_D$ = -408.7°, c = 1.1, CHCl$_3$) indicating it was the desired *l*-enantiomer. In order to obtain an accurate measure of the optical purity of the product, it was derivatised using trifluoroacetic anhydride and analysis using chiral GC indicated that the *l*-PAC (3) had been formed in an enantiomeric ratio of 87:13 (74% ee) (Figure 2.6). The difference between the enantiomeric excess as found by optical rotation compared with chiral gc can be attributed to the fact the optical rotation of PAC (3) is not linearly related to concentration.[109]
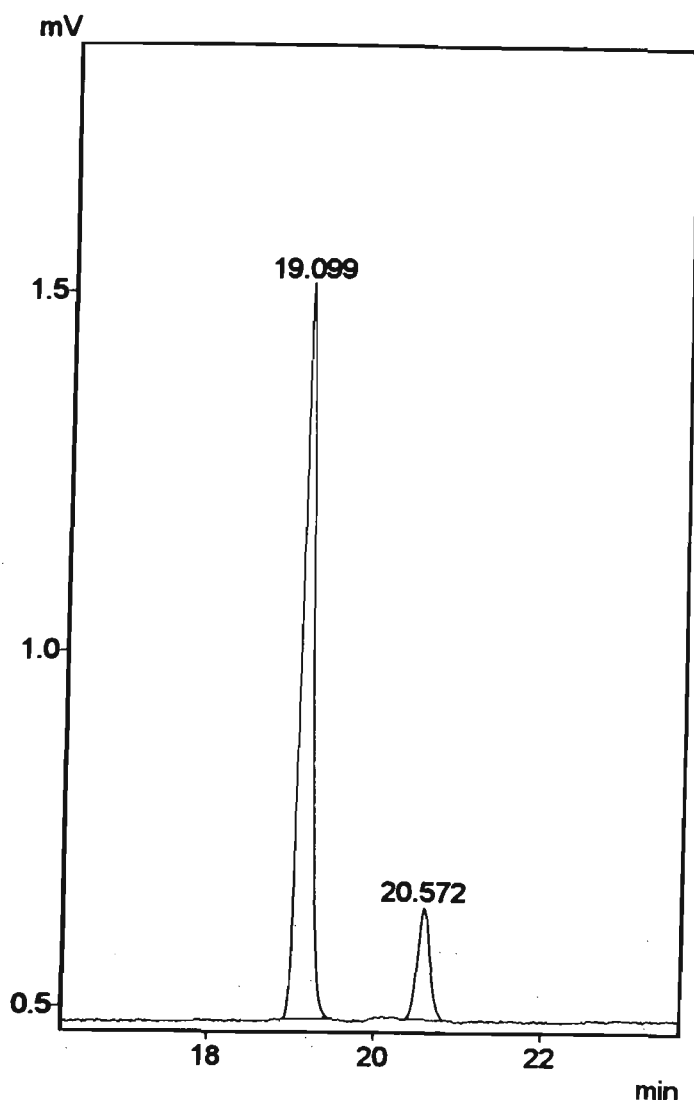
Figure 2.6 The chiral GC of the product derivatised using trifluoroacetic anhydride shows *l*-PAC (3) had been formed in an enantiomeric ratio of 87:13 (74% ee)

2.6 Acetaldehyde

Becvarova *et al.*[26,110] observed an increase in the production of *l*-PAC (3) when acetaldehyde (2) was added to the fermenting reaction medium. They proposed that the acetaldehyde (2) 'blocked' the yeast enzymes responsible for the reduction of benzaldehyde (1) to benzyl alcohol (4) consequently increasing the yield of *l*-PAC (3). It was postulated in the present study that if the yeast was treated with acetaldehyde (2) prior to the addition of the other reactants, the acetaldehyde (2) would be reduced to ethanol thereby consuming the NADPH responsible for the reduction of benzaldehyde (1) to benzyl alcohol (4) and thus decreasing or eliminating the production of benzyl alcohol (4) and increasing the overall production

of *l*-PAC (3). Since an organic system rather than an aqueous fermenting system was being used, the NADPH would not be replenished *via* the normal biochemical processes. In addition, it was hypothesised that the acetaldehyde (2) could act as an acyl donor, in place of sodium pyruvate (10b). Consequently, the yeast was pre-treated with acetaldehyde (2) for a 24h period before adding the benzaldehyde (1).

Sodium pyruvate (10b) (2.5g) was added to baker's yeast which had been activated with 0.05M sodium citrate and suspended in petroleum spirit. Acetaldehyde (2) was added and the mixture stirred at 20°C for 24h prior to the addition of benzaldehyde (1). The reaction mixture was analysed, using GC, after a further 24h and showed small amounts of *l*-PAC (3) (4%) and benzyl alcohol (4) (2%).

It was thought that the low yield of *l*-PAC (3) may have been due to the deactivation of the yeast enzymes involved in the condensation of benzaldehyde (1) to form *l*-PAC (3) since this was found to be the case in yeast mediated reduction reactions after 24h in organic solvents.[108] In an attempt to improve the yield of *l*-PAC (3) the pre-treatment time was varied from 1 - 12h and the results of this study are presented in Figure 2.7.

It was found that pre-treating the yeast at 20°C for periods of 1 - 12h resulted in the formation of only small amounts of *l*-PAC (3) (4 - 9%). For all further reactions involving pre-treatment of the yeast with acetaldehyde (2), 3h was deemed optimal since small quantities of by-products were formed if longer times were used.

For the above reactions the sodium pyruvate (10b) was added prior to the acetaldehyde (2) pre-treatment and a low conversion to *l*-PAC (3) resulted. It was found that if the sodium pyruvate (10b) was added to the reaction medium after the yeast was pre-treated with acetaldehyde (2), significantly more *l*-PAC (3) (30%) was produced.
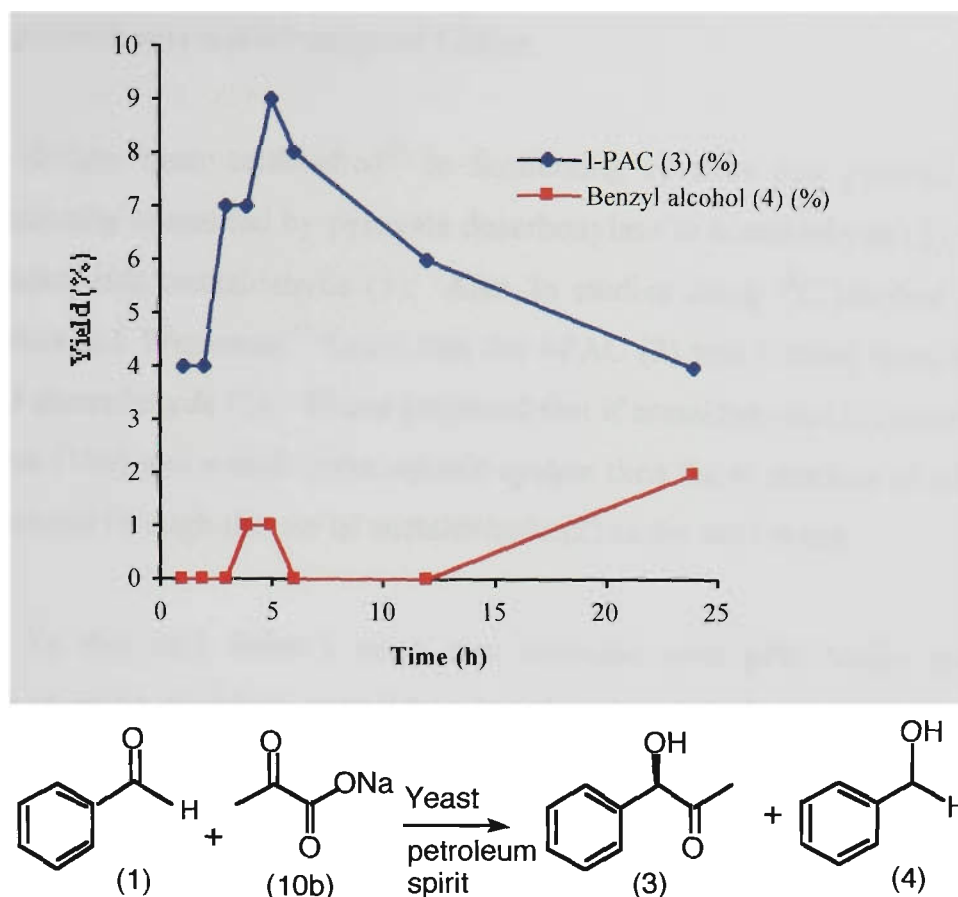
Figure 2.7 Pre-treatment of the yeast with acetaldehyde (2) for between 1 - 24h prior to the addition of benzaldehyde (1). All reactions contained 5g of yeast, 5ml buffer, 45ml petroleum spirit, 2.5g of sodium pyruvate (10b), which was added prior to acetaldehyde (2) pre-treatment, and 1mmol benzaldehyde (1) and were stirred for 24h at 20°C.

In an organic system, the acetaldehyde (2) is reduced to ethanol and consumes the NADPH thus preventing further reduction reactions. In experiments described earlier, ethanol was used to eliminate the production of the unwanted by-product, benzyl alcohol (4). Reactions utilising acetaldehyde (2) were carried out without ethanol since the acetaldehyde (2) added during the pre-treatment is subsequently converted to ethanol. It was decided to investigate the addition of further acetaldehyde (2) to the reaction medium to see if this reagent could also act as an acyl donor.

Sodium pyruvate (10b) was dissolved in 0.05M sodium citrate and added to baker's yeast. Petroleum spirit and acetaldehyde (2) were then added and the reaction stirred at 20°C for 3h prior to the addition of benzaldehyde (1) and further acetaldehyde (2). GC analysis of the reaction mixture after a further 24h showed that

a small quantity (4.5%) of benzyl alcohol (4) and no *l*-PAC (3) had been produced. This approach was not investigated further.

It has been established[21] in fermenting systems that pyruvic acid (10) is enzymatically converted by pyruvate decarboxylase to acetaldehyde (2) which in turn condenses with benzaldehyde (1). Also, in studies using ^{13}C labelled acetaldehyde (2), Gross and Werkman[111] found that the *l*-PAC (3) was formed from benzaldehyde (1) and acetaldehyde (2). It was proposed that if acetaldehyde (2) rather than sodium pyruvate (10b) was added to the organic system then the production of *l*-PAC (3) may be enhanced through the use of acetaldehyde (2) as the acyl donor.

To this end, baker's yeast was activated with pH6 buffer and stirred in petroleum spirit, to which benzaldehyde (1) and acetaldehyde (2) were added. The mixture was then stirred at 20°C for 24h. This approach also proved to be unsuccessful since only starting material was in evidence.

2.7 The Addition of Benzaldehyde

Studies of the acyloin condensation of benzaldehyde (1) to form *l*-PAC (3), using fermenting yeast, indicated that benzaldehyde (1) has a toxic effect on the yeast cells and that this is a limiting factor in the production of *l*-PAC (3).[11,30,34] A recent review[21] indicates that the method of addition of the substrate, benzaldehyde (1), to the reaction medium, influences the biotransformation process. In aqueous systems, it was found[30,38,39] that the production of *l*-PAC (3) increased when benzaldehyde (1) was slowly added to the reaction medium. The reaction system employed by Vojtisek and Netrval[112] involved adding benzaldehyde (1) in batches to the immobilised yeast cells resulting in yields of *l*-PAC (3) in the range of 10-12 g/l. Similar results were obtained by Mahmoud *et al.*[38,39] who added the benzaldehyde (1) to the immobilised yeast cells in a semi-continuous flow. Shin and Rogers[30] who had also used immobilised yeast cells improved the yields of *l*-PAC (3) by adding the benzaldehyde (1) to the reaction medium at a level of 2g/l in a batch feed process resulting in a final concentration of 15.2g/l.

Since in the fermenting system the gradual addition of benzaldehyde (1) to the reaction medium improved the yield of *l*-PAC (3), it was therefore of interest to investigate if the production of *l*-PAC (3) in an organic system would improve if the benzaldehyde (1) was gradually added.

Sodium pyruvate (10b), pH6 citrate buffer, ethanol and petroleum spirit were stirred at 20°C and then baker's yeast added. The benzaldehyde (1), in petroleum spirit, was slowly added to the stirred reaction mixture, using a syringe pump, over a period of 7h and the reaction stirred for a further 17h. GC analysis of the reaction mixture indicated only limited conversion to *l*-PAC (3) (7%).

Since the slow addition of benzaldehyde (1) gave a low yield of *l*-PAC (3), the benzaldehyde (1) was added in two portions. Benzaldehyde (1) was added to the reaction medium and stirred at 20°C for a period of 6h. Analysis by GC showed a 14% conversion to *l*-PAC (3). An equivalent amount of benzaldehyde (1) was then added to the reaction mixture which was then stirred for a further 18h. There was no further increase in the amount of *l*-PAC (3).

In contrast to reports of improved *l*-PAC (3) formation in aqueous systems, as a result of a slow addition of benzaldehyde (1),[23,30,38,39,112] the present work has shown that, in an organic solvent, the production of *l*-PAC (3) is dramatically decreased from 31% to 7%, when benzaldehyde (1) is slowly added to the reaction.

2.8 pH Studies

The standard reaction conditions which were established in experiments described in the previous sections, included activating the yeast with a pH6 citrate buffer, a technique also employed by Nikolova and Ward[32] for the yeast mediated acyloin condensation of benzaldehyde (1). The pH of the reaction system was varied in order to examine the effect of pH on the production of *l*-PAC (3).

Baker's yeast was activated with citrate buffer and petroleum spirit, ethanol and sodium pyruvate (10b) added. This mixture was stirred at 20°C for 0.5h, then benzaldehyde (1) was added and the mixture stirred at 20°C for 24h. The production of *l*-PAC (3) was monitored using GC and the results are plotted in Figure 2.8.
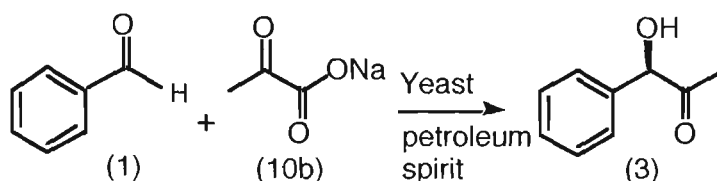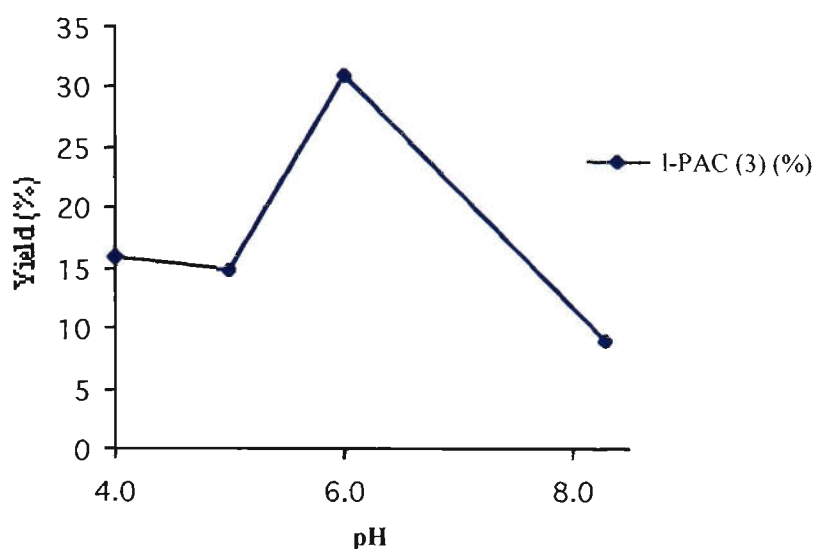

Figure 2.8 Variation in the pH (4-8.3) of the buffer solution. All reactions contained 5g of yeast, 5ml buffer, 45ml petroleum spirit, 2.5g of sodium pyruvate (10b), 0.5ml of ethanol and 1mmol of benzaldehyde (1) and were stirred for 24h at 20°C.

Citrate buffers ranging from pH 4 - 8.3 were used in the reaction medium to activate the yeast. It should be noted for these reactions that the sodium pyruvate (10b) was not dissolved in the buffer before activating the yeast and therefore the pH values refer to the pH of the buffer solutions prior to the addition of sodium pyruvate (10b).

Changing the pH of the citrate buffer from pH 4 - 5 resulted in very little change in the conversion of benzaldehyde (1) to *l*-PAC (3). The highest conversion (31%) was obtained at pH 6. If the pH was slightly basic, the amount of *l*-PAC (3)

fell below 10 %. The result of these experiments was that the pH of the buffer system employed remained as recommended by Nikolova and Ward,[32] a pH6 citrate buffer was added in all reactions involving sodium pyruvate (10b).

The pH proved to be an important factor in the production of *l*-PAC (3). The importance of a buffer was underlined when reactions were conducted using water rather than buffer to activate the yeast; very little *l*-PAC (3) was obtained, the product was mainly benzyl alcohol (4).

In an aqueous system, Shin and Rogers,[23] examined the effect of pH (4 -8) at a reaction temperature of 4°C and an optimised production of *l*-PAC (3) at pH7. They used a mixed citrate/sodium phosphate buffer solution and adjusted the pH accordingly. By comparison, in an organic system, *l*-PAC (3) production was optimised at pH6 using a citrate buffer.

2.9 Temperature and time

Shin and Rogers[23] studied the effect of temperature on *l*-PAC (3) formation in an aqueous medium using partially purified PDC. Their reactions were carried out at 4°C, 10°C and 25°C with 70mM benzaldehyde (1), 70mM sodium pyruvate (10b), and 7U/ml PDC enzyme in 40mM pH6 phosphate buffer with 30μM thiamine pyrophosphate (TPP), which is an enzyme cofactor[23]. Results of their studies showed the highest formation of *l*-PAC (3) at 4°C.

It has been shown that yeast in an organic solvent system is deactivated after 24h at 20°C, whilst at 10°C or less the yeast remains active for periods in excess of 70h,[108] consequently the effect of temperature on the production of *l*-PAC (3) was studied.

Using optimal conditions, the pH6 citrate buffer containing sodium pyruvate (10b) (2.5g) was added to baker's yeast (5g) followed by the addition of ethanol (0.5ml) and benzaldehyde (1) (1.2mmol) in petroleum spirit and the reaction mixture was stirred at approximately 5°C for 48h. After 24h, GC showed a 24% conversion

to *l*-PAC (3) compared to a slightly higher value (30%) at 20°C. After 48h very little increase in the amount of *l*-PAC (3) was observed but some of the diol (5) and diketone (20) (Figure 2.9) were produced. Consequently, further reactions at 5°C were only stirred for 24h.
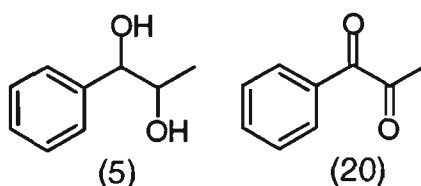
Figure 2.9 By-products formed after a 24h reaction time at 5°C

The product of the reaction at 5°C was isolated and purified by distillation to give *l*-PAC (3) in low yield (24%). The optical rotation of this product was found to be -375.8° (Lit.[109], $[\alpha]_D$ = -408.7°, c = 1.1, CHCl$_3$) indicating that it was the *l*-enantiomer; GC of the trifluoroacetyl derivative indicated a high enantiomeric purity (86% ee).

A comparison of the results obtained from the two reaction temperatures (an isolated yield of 16% and 74% ee at 20°C compared with 24% and 86% ee at 5°C) indicates that the lower temperature resulted in both a higher isolated yield and higher stereoselectivity.

Shin and Rogers[23] achieved their highest concentration of *l*-PAC (3) (28.6g/l (190.6mM)) in a system containing 200mM benzaldehyde (1) with 2M sodium pyruvate (10b) in a phosphate buffer (pH7) containing 2M ethanol at 4°C. Hence in both the organic system used in this study and the aqueous system employed by Shin and Rogers[23] lowering the reaction temperature resulted in a higher yield of *l*-PAC (3).

Acetaldehyde (2) pre-treatment of the yeast was shown to eliminate the unwanted by-products and facilitate a relatively high conversion (30%) to *l*-PAC (3), provided that the sodium pyruvate (10b) was added to the reaction mixture after the

pre-treatment. This conversion is similar to that found (31%) when using the standard reaction conditions at 20°C. Thus it was of interest to investigate whether reactions conducted at a low temperature and including an acetaldehyde (2) pre-treatment of the yeast, would also result in a comparable yield of *l*-PAC (3).

Baker's yeast, pH6 citrate buffer and petroleum spirit/acetaldehyde (2) mixture, was stirred at 20°C for 3h. Sodium pyruvate (10b) and ethanol were then added and the mixture was stirred at 5°C for 0.5h prior to the addition of benzaldehyde (1) in petroleum spirit. The reaction mixture was stirred at 5°C and sampled at 24h and 48h. GC analysis indicated 19% conversion to *l*-PAC (3) after 24h and 30% after 48h. Isolation of the product after 48h and purification by distillation gave 13% yield of *l*-PAC (3).

In an experiment conducted without ethanol, a mixture containing pH6 citrate buffer, petroleum spirit, baker's yeast and acetaldehyde (2) was stirred at 20°C for 3h prior to the addition of sodium pyruvate (10b) and benzaldehyde (1) in petroleum spirit. The reaction medium was stirred at 5°C for 48h and resulted in a 20% conversion to *l*-PAC (3) with no by-products in evidence. *l*-PAC (3) was isolated in a yield of 10% and with an optical rotation of $[\alpha]_D = -208.8°$ (c = 1.75, $CHCl_3$) (Lit.[109], $[\alpha]_D = -408.7°$, c = 1.1, $CHCl_3$) indicating that it was the *l*-enantiomer. Chiral GC of the trifluoroacetyl derivative of the *l*-PAC (3) indicated a high enantiomeric purity (82% ee).

For reactions involving acetaldehyde (2) pre-treatment, a temperature of 5°C led to a 20% conversion of benzaldehyde (1) to *l*-PAC (3) after 48h (10% isolated yield with 82% ee) whereas the higher temperature of 20°C resulted in a 30% conversion after only 24h.

The above two reaction systems at 5°C, which included an acetaldehyde (2) pre-treatment with and without ethanol resulted in isolated yields of 13% and 10% respectively. Overall, for the above two reaction systems very little difference was observed in the isolated yield of *l*-PAC (3). Thus in the system involving

acetaldehyde (2) pre-treatment of yeast at 5°C the inclusion of ethanol had very little effect on the yield of *l*-PAC (3).

When reactions were performed under the standard conditions at 5°C, the diol (5) and diketone (20) by-products were not observed in the first 24h but were observed after longer reaction times. Reactions incorporating an acetaldehyde (2) pre-treatment showed no evidence of the by-products, even after 48h. Standard reaction conditions at 5°C resulted in a higher isolated yield (24%) of *l*-PAC (3) than reactions involving acetaldehyde (2) pre-treatment (10% yield); in both reaction systems the *l*-isomer was formed at a level of enantiomeric purity of 86% and 82% ee respectively.

In conclusion, *l*-PAC (3) production was optimised at a reaction temperature of 5°C, which is similar to the result found by Shin and Rogers[23] who had found an optimum reaction temperature of 4°C in their aqueous system using partially purified PDC.

2.10 Maximisation of product yield

Previous studies[80,81] have shown that once reaction conditions have been optimised then additional quantities of yeast will increase the conversion to the desired product. It was therefore anticipated that an increase in the ratio of yeast to benzaldehyde (1) would increase the yield of *l*-PAC (3). The amount of yeast employed in each of the reaction systems so far described was 5g of yeast/mmol of benzaldehyde (1). A series of reactions was performed with amounts of yeast ranging from 5 to 10g/mmol benzaldehyde (1). The amount of ethanol added to each of these reactions was 0.2ml/g yeast.

Baker's yeast (5 -10g) was added to a mixture of pH6 citrate buffer (0.8ml/g yeast), ethanol and petroleum spirit, and then 2.5g sodium pyruvate (10b) and benzaldehyde (1) were added. The reaction mixture was stirred at 20°C for 24h and the formation of *l*-PAC (3) was monitored using GC; the results are presented in Figure 2.10.
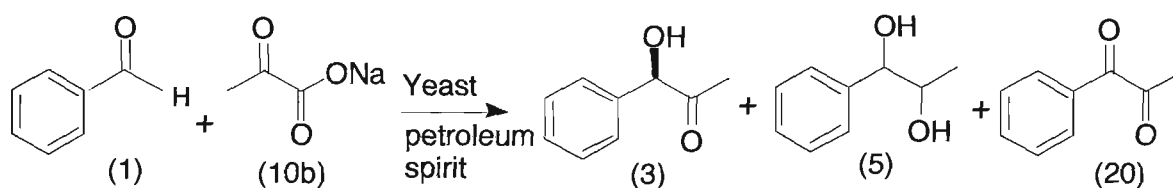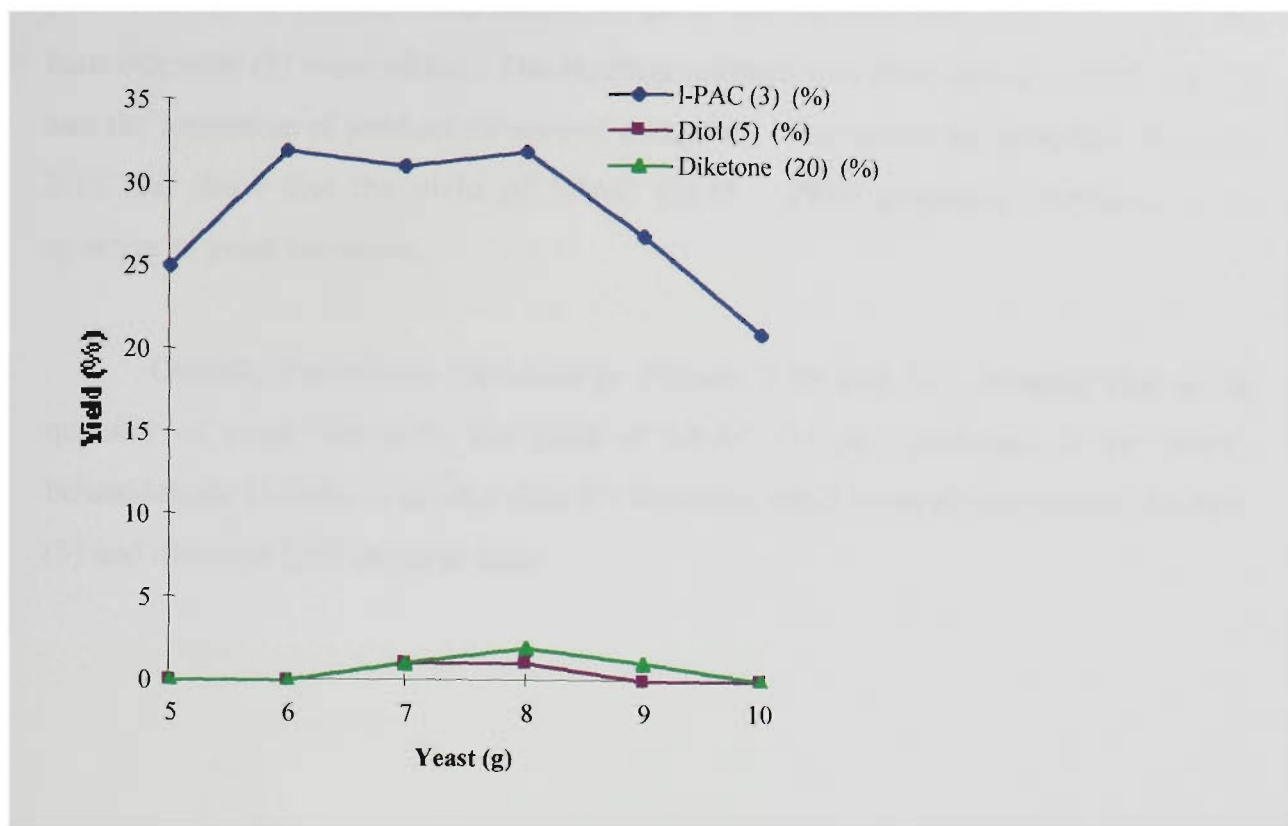
Figure 2.10 Variation in the quantity of yeast (5 - 10g) used for the acyloin condensation of benzaldehyde (1). All reactions contained 2.5g sodium pyruvate (10b), 0.8ml of pH6 citrate buffer, 0.2ml ethanol/g yeast and 1mmol of benzaldehyde (1) and were stirred for 24h at 20°C.

The results indicate that production of *l*-PAC (3) is optimal with 5-6g yeast/mmol benzaldehyde (1), however with amounts of yeast greater than 6g/mmol benzaldehyde (1), small amounts of the diol (5) and diketone (20) are formed.

In each of the above reactions reported in Figure 2.10, the amount of sodium pyruvate (10b) present was 2.5g, irrespective of the quantity of yeast present. To investigate whether the yeast/pyruvate ratio affected the production of *l*-PAC (3), the ratio of 0.5g sodium pyruvate/g yeast was used. This was the ratio (2.5g/5g yeast) which was found to be optimal in Section 2.4.

The pH6 citrate buffer, ethanol, sodium pyruvate (10b) (0.5 - 2.5g) and petroleum spirit (45ml) were mixed at 20°C for 1h and then yeast (1 - 5g) and benzaldehyde (1) were added. The reaction mixture was then stirred at 20°C for 24h and the formation of product monitored using GC. The results are reported in Figure 2.11 and show that the yield of *l*-PAC (3) (9 - 29%) gradually increases as the quantity of yeast increases.

Overall, the results presented in Figures 2.10 and 2.11 indicate that as the quantity of yeast increases, the yield of *l*-PAC (3) also increases; if the yeast : benzaldehyde (1) ratio is greater than 6:1 however, other by-products such as the diol (5) and diketone (20) begin to form.
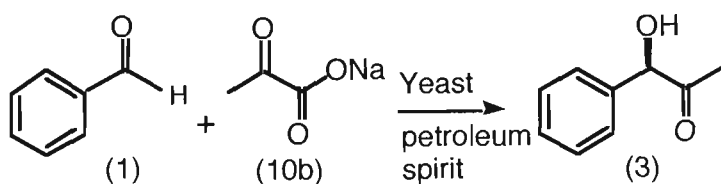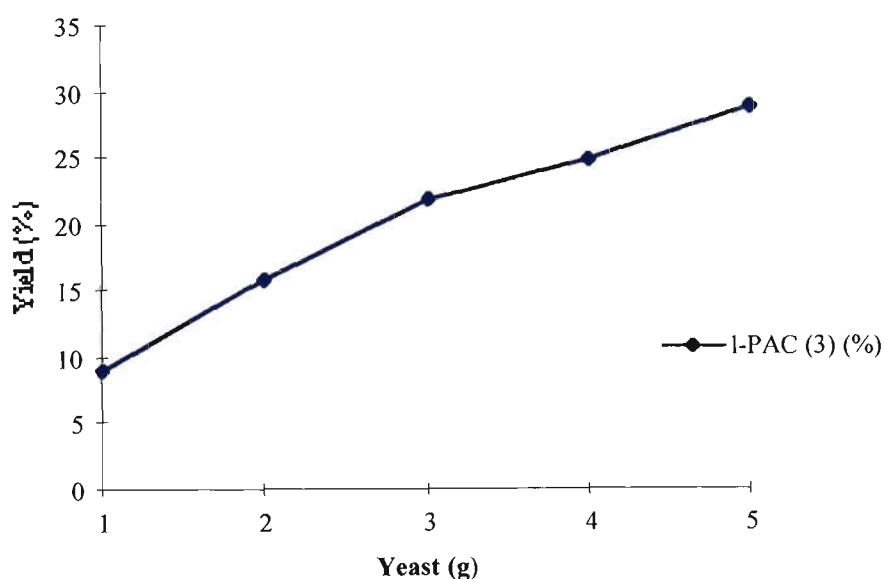
Figure 2.11 Variation in the quantity of yeast (1 - 5g) used for the acyloin condensation of benzaldehyde (1). All reactions contained 0.5g sodium pyruvate/g yeast. 1ml of pH6 citrate buffer, 0.2ml ethanol/g yeast, 9ml petroleum spirit/g yeast and 1mmol of benzaldehyde (1) and were stirred for 24h at 20°C.

Previous studies[21] have suggested that benzaldehyde (1) 'poisoned' the yeast. In an attempt to investigate whether poisoning was a factor in the present study, additional baker's yeast was added after 6.5h and the impact on *l*-PAC (3) production was studied.

Sodium pyruvate (10b) was dissolved in pH6 citrate buffer, added to ethanol and petroleum spirit and the mixture stirred at 20°C for 1h. Benzaldehyde (1) in petroleum spirit was then added and the mixture stirred at 20°C for 6.5h. GC analysis at this stage indicated 30% conversion to *l*-PAC (3). Additional yeast and citrate buffer were added and the reaction mixture was stirred at 20°C for a further 17.5h. GC analysis after the addition of the extra yeast showed that the remaining benzaldehyde (1) had been converted to benzyl alcohol (4) and that the amount of *l*-PAC (3) was not increased.

It was interesting to note that a comparison of the GC data in Figure 2.2 at 24h with the present result at 6.5h showed a similar conversion to *l*-PAC (3); at 6.5h, 30% *l*-PAC (3) was observed compared with 31% under similar conditions at 24h (Figure 2.2). This suggests that at 20°C maximal production of *l*-PAC (3) is achieved after 6.5h.

2.11 Conclusion

The results obtained from reactions performed under a variety of conditions showed that sodium pyruvate (10b) is an important ingredient in the biotransformation of benzaldehyde (1) to *l*-PAC (3). If less than 1 g of sodium pyruvate (10b) was used, either no reaction occurred or only benzyl alcohol (4) was produced. Overall, the results showed the optimal conditions for the biocatalytic conversion of benzaldehyde (1) to *l*-PAC (3) were 5g yeast/mmol benzaldehyde (1), 2.5g sodium pyruvate (10b), 0.5ml ethanol at a reaction temperature of 5°C over a 24h period. Although pretreating the yeast with acetaldehyde (2) for a maximum of 3h eliminated the production of benzyl alcohol (4) and unwanted by-products, the addition of small quantities of ethanol was just as effective and a good deal simpler. The order of addition of reagents also had an effect on the overall yield of *l*-PAC (3).

Better yields were obtained when the citrate buffer was saturated with the appropriate quantity of sodium pyruvate (10b) before activating the yeast.

The product was isolated in a 16% yield with 74% ee at 20°C and 24% yield with 86% ee at 5°C (enantiomeric purity was determined using chiral GC). Although Nikolova and Ward[53] did not specifically state a yield of *l*-PAC (3) calculations made from their tabulated and graphical data revealed that they had obtained a 4.5% yield of *l*-PAC (3) which was achieved using a moisture content of 10% in their reaction system. The yield obtained using the organic system in the present studies at 20°C was much higher and the method less cumbersome than that described by Nikolova and Ward.[89]

CHAPTER 3

REACTIONS USING PYRUVIC ACID

REACTIONS USING PYRUVIC ACID

3.1 Introduction

In a biocatalytic system which uses fermenting yeast, pyruvic acid (10) is converted to acetaldehyde (2) by pyruvic acid decarboxylase (PDC) (Step 1, Scheme 3.1). Acetaldehyde (2) condenses with benzaldehyde (1) *via* PDC to form *l*-PAC (3) (Step 2, Scheme 3.1). The pyruvate ion has been shown to play a significant role in both fermenting[21,23] and non-fermenting[32,33] biocatalytic systems (Section 2.4).
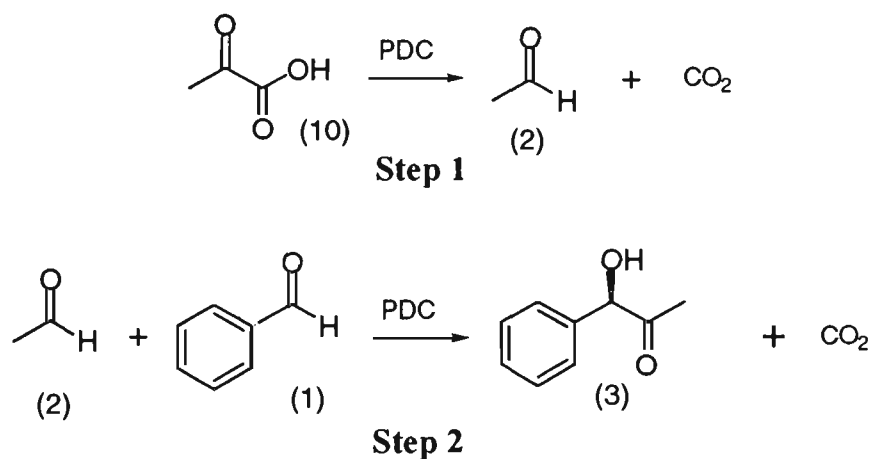
Scheme 3.1

It was thought that if pyruvic acid (10), which is cheaper than sodium pyruvate (10b), could be utilised in the biocatalytic conversion of benzaldehyde (1) to *l*-PAC (3) in an organic solvent system, then this would make the reaction more commercially viable.

3.2 Pyruvic acid

The standard reaction conditions including 5g yeast, 0.5ml ethanol and 1mmol benzaldehyde (1) at 20°C, which were established in Chapter 2, were employed for the yeast mediated acyloin condensation of benzaldehyde (1) to form *l*-PAC (3) using pyruvic acid (10) in place of sodium pyruvate (10b) (Scheme 3.2). Since pyruvic acid (10) is highly acidic (pH 2.2), it was added to 0.05M sodium citrate (pH8.3) instead of the pH6 citrate buffer used in Chapter 2. This was done so that less ammonium acetate was required to adjust the pH to the final value of 5.45.
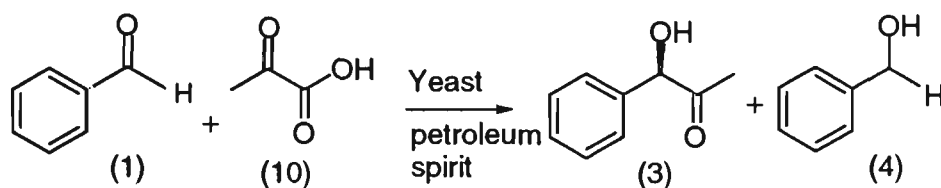
Scheme 3.2

Pyruvic acid (10) (0.3g) was added to 0.05M sodium citrate (5ml) and the pH adjusted to 5.45 using ammonium acetate. The pyruvic acid (10) solution and ethanol (0.5ml) in petroleum spirit (45ml) were added to baker's yeast (5g) and stirred. The benzaldehyde (1) was then added and the reaction mixture stirred at 20°C for 24h. GC analysis of the reaction mixture after 24h showed that benzaldehyde (1) had been converted to benzyl alcohol (4) (22%) and *l*-PAC (3) (12%). These results indicated that *l*-PAC (3) could be synthesised using pyruvic acid (10) in place of sodium pyruvate (10b) but that the system required modification in order to eliminate the benzyl alcohol (4).

The abovementioned system contained only 0.3g pyruvic acid (10), approximately one tenth the quantity of sodium pyruvate (10b) used for the yeast mediated acyloin condensation reactions in Chapter 2; clearly, the amount of this reagent required optimisation.

3.3 Optimisation of Pyruvic acid Concentration

Since a relatively high quantity of benzyl alcohol (4) was produced in the above reaction system the amount of ethanol was increased to 1ml to reduce the production of benzyl alcohol (4).

In order to optimise the quantity of pyruvic acid (10) added to the reaction system the quantity of this reagent was varied. Three different methods of adjusting the pH were investigated in order obtain the highest yield of *l*-PAC (3) and minimise unwanted by-products.

The first system was based on the conditions described above and involved dissolving pyruvic acid (10) (0.05g - 1.25g) in 0.05M sodium citrate and adjusting the pH to 5.45 using ammonium acetate. Baker's yeast (5g) was added to a mixture containing the pyruvic acid (10) solution (5ml) and ethanol (1ml) in petroleum spirit (45ml). Benzaldehyde (1) was then added and the reaction mixture stirred at 20°C for 24h. GC analysis of the reaction mixture after 24h gave the results presented in Figure 3.1.

Reactions containing 0.05g - 0.15g of pyruvic acid (10) resulted in low yields of *l*-PAC (3) (6 - 10%) and substantial amounts of benzyl alcohol (4) (20 - 24%). The yield of *l*-PAC (3) (15%) varied little using 0.2g - 0.5g of pyruvic acid (10). No benzyl alcohol (4) was produced when more than 0.3g of pyruvic acid (10) was used.
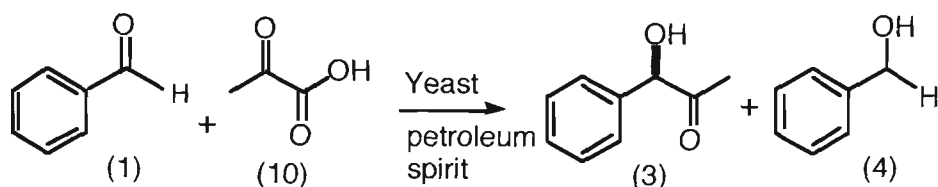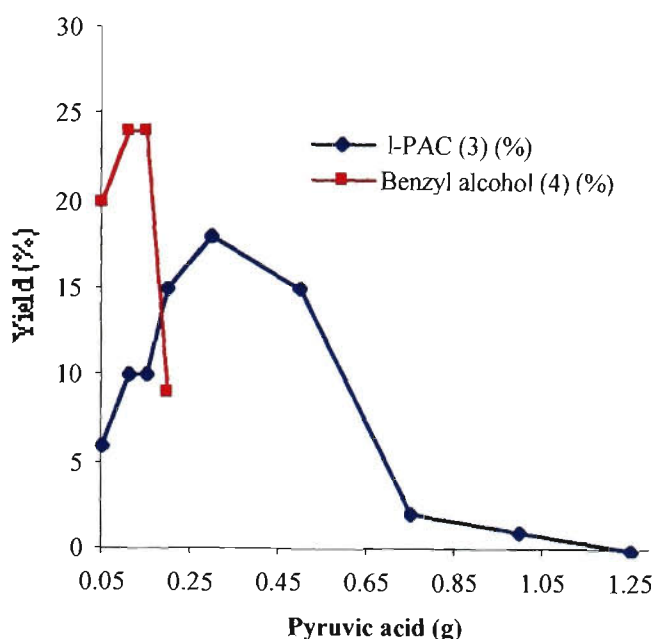
Figure 3.1 Variation in the amount of pyruvic acid (10) added to the reaction. All reactions contained 5g of yeast, 5ml 0.05M sodium citrate containing pyruvic acid (10) with the pH adjusted to 5.45. using ammonium acetate, 1ml ethanol,45ml petroleum spirit and 1.4mmol benzaldehyde (1) and were stirred for 24h at 20°C.

The reactions described in the previous chapter involved sodium pyruvate (10b) dissolved in a pH6 citrate buffer, without ammonium acetate. To remove any effect of ammonium acetate, a second system was investigated in which pyruvic acid (10) was dissolved in water and the pH adjusted using sodium citrate. The amount of pyruvic acid (10) added to the reaction was varied from 0.05g to 0.25g and the pH of the system was 5.45 in each case. The reaction mixture was stirred at 20°C for 24h and the results are reported in Figure 3.2.
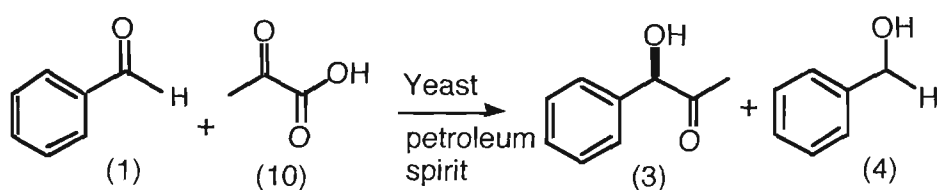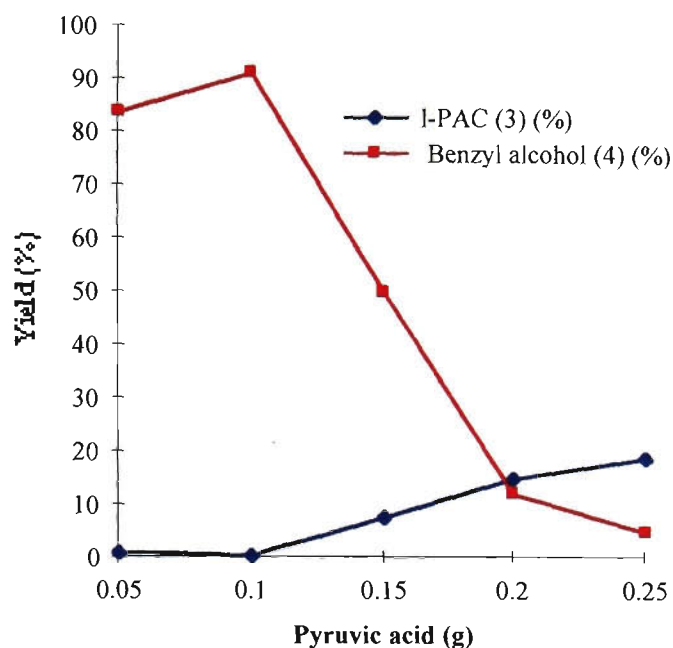
Figure 3.2 Variation in the amount of pyruvic acid (10) added to the reaction. All reactions contained 5g of yeast, 5ml water containing pyruvic acid (10) with the pH adjusted to 5.45. using sodium citrate, 1ml ethanol, 45ml petroleum spirit and 1.3mmol benzaldehyde (1) and were stirred for 24h at 20°C.

The yield of *l*-PAC (3) ranged from 1% to 19% with the lowest yield corresponding to 0.1g of pyruvic acid (10). As the amount of pyruvic acid (10) was increased the yield of *l*-PAC (3) increased to 19% whilst the yield of benzyl alcohol (4) decreased sharply from 91% to 5%.

The third system contained pyruvic acid (10) (0.05g -0.26g) which was dissolved in H_2O and the pH adjusted to 5.45 using ammonium acetate. To this solution (5ml), ethanol (1ml), petroleum spirit (45ml), and baker's yeast (5g) were added and the mixture was stirred at 20°C for 1.5h prior to the addition of benzaldehyde (1) (1mmol). The reaction was then stirred at 20°C for a further 24h. The reaction mixture was analysed using GC and results are shown in Figure 3.3.
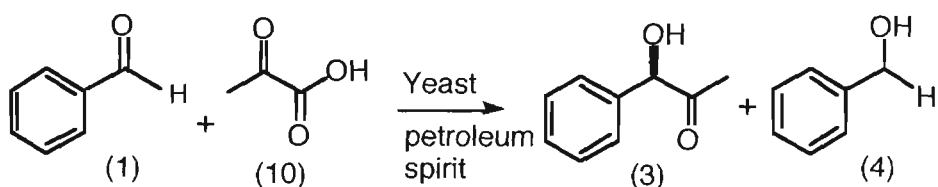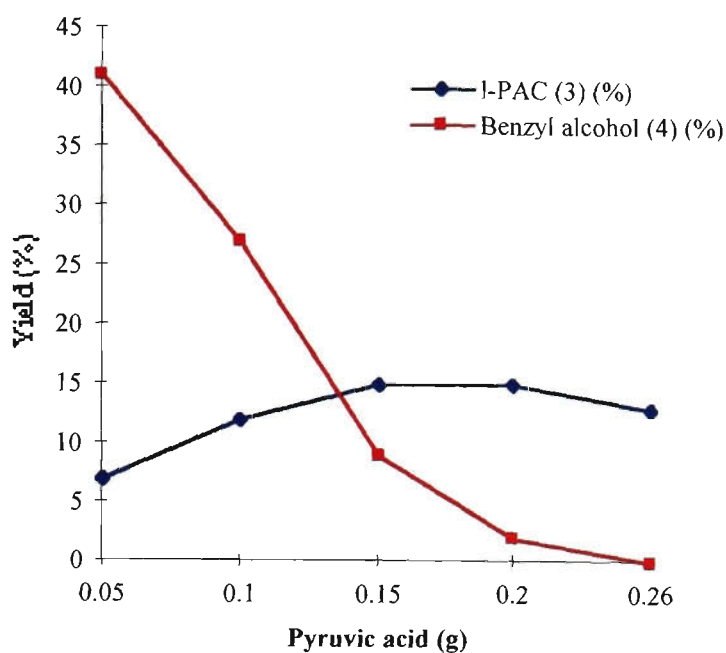
Figure 3.3 Variation in the amount of pyruvic acid (10) added to the reaction. All reactions contained 5g of yeast, 5ml water containing pyruvic acid (10) with the pH adjusted to 5.45 using ammonium acetate, 1ml ethanol, 45ml petroleum spirit and 1.3mmol benzaldehyde (1) and were stirred for 24h at 20°C.

The results indicate that as the amount of pyruvic acid (10) is increased from 0.05g to 0.26g, at a constant pH of 5.45, the yield of benzyl alcohol (4) decreases from 41 to 0% and the production of *l*-PAC (3) increases from 7 to 15%.

The results obtained from the three reaction systems indicate that the reagent used to adjust the pH of the system had little effect on the yield of *l*-PAC (3). Consequently, for all subsequent reactions, pyruvic acid (10) (0.3g) in 0.05M sodium citrate with the pH adjusted to 5.45 using ammonium acetate (Figure 3.1) was employed.

3.4 Reaction Time

Yeast reductase enzymes are known to deactivate after 24h at 20°C when using yeast, which is activated with a small quantity of water, in an organic solvent,[108] resulting in a maximum yield of product within this time. Results from the studies in Chapter 2 showed that the enzymes involved in the acyloin condensation were also deactivated after 24h. In order to discover if the yeast was deactivated with the inclusion of pyruvic acid (10), the yeast mediated acyloin condensation of benzaldehyde (1) using pyruvic acid (10) was studied over longer periods of time. If the yeast was not deactivated then the yield of *l*-PAC (3) should increase.

Pyruvic acid (10) (0.29g) in 0.05M sodium citrate (5ml, pH5.45), ethanol (0.5ml) and petroleum spirit (45ml) were added to baker's yeast (5g) and stirred. The benzaldehyde (1) (1mmol) was then added and the reaction mixture stirred at 20°C. Results after 24h showed benzyl alcohol (4), (22%) and *l*-PAC (3), (12%). A slight increase in the yield of benzyl alcohol (4), (25%) and a small quantity of the diol (5) (1%) and diketone (6) (1%) were observed after 72h, but the yield of *l*-PAC (3) remained constant.

The results suggest that the yeast is also largely deactivated after 24h in the presence of pyruvic acid (10) at 20°C. This result is consistent with those of other studies[108] and with the results obtained using sodium pyruvate (10b) whereby the extension of the reaction time beyond 24h only resulted in the formation of increased amounts of by-products.

3.5 Reactions at Low Temperature

3.5.1 *Reactions at 5°C with pyruvic acid (10)*

In Chapter 2, the reactions involving sodium pyruvate (10b) (Section 2.9), carried out at 5°C, showed an increase in the isolated yield of *l*-PAC (3) (16% at 20°C and 24% at 5°C) and a decrease in unwanted by-products. Reactions with pyruvic acid (10) were therefore investigated at 5°C in order to see if a similar trend would be observed. Although a reaction time beyond 24h did not result in a higher yield of *l*-PAC (3) when using sodium pyruvate (10b) the reactions at 5°C using pyruvic acid (10) were monitored over a longer period of time in order to compare the differences when using the two different reagents.

Pyruvic acid (10) (0.28g) was added to 0.05M sodium citrate (5ml) and the pH adjusted to 5.45 using ammonium acetate. The pyruvic acid (10)/ammonium acetate solution (5ml), ethanol (0.5ml) and petroleum spirit (45ml) were added to baker's yeast (5g) and stirred. The benzaldehyde (1) (1mmol) was then added and the reaction mixture stirred at 5°C for 72h. The reaction mixture was sampled every 24h and GC used to analyse the levels of *l*-PAC (3) and benzyl alcohol (4). The results showed that although the conversion to *l*-PAC (3) increased from 19% (24h) to 26% (72h), the conversion to benzyl alcohol (4) also increased from 2% (24h) to 10% (72h). There was therefore little gain in extending the reaction time beyond 24h. In practical terms, the outcome of the reaction is similar whether using sodium pyruvate (10b) or pyruvic acid (10).

The yeast was filtered from the reaction mixture after 24h at 5°C, extracted with diethyl ether and the combined filtrate and solvent extracts were distilled, product was obtained in an overall yield of 24%. The same isolated yield of product was obtained at 5°C using sodium pyruvate (10b) (Section 2.9).

The conversion to *l*-PAC (3) at 5°C (19% (24h)), and using 0.28g (3.2mmol) of pyruvic acid (10) and 0.5ml of ethanol was markedly greater than that at 20°C (6%). More benzyl alcohol (4) was formed at 20°C, (25%) than at 5°C (2%).

Although the production of the unwanted by-product, benzyl alcohol (4) was not completely eliminated at 5°C its production was considerably reduced.

A reaction with a larger amount of pyruvic acid (10) (0.5g/5g yeast) with the pH again adjusted to 5.45 using ammonium acetate, was performed in an attempt to eliminate the production of benzyl alcohol (4). The reaction was carried out over a 48h period and sampled every 24h. The conversion to *l*-PAC (3) was 30% after 24h and this result was unchanged after 48h. Benzyl alcohol (4) was absent from the reaction mixture both at 24h and 48h, however small amounts (2%) of by-products (5) and (12) were observed after 48h.

At a reaction temperature of 5°C, the greatest amount of *l*-PAC (3) (30%), was obtained when the reaction system contained 0.5ml of ethanol and 0.5g (6.4mmol) of pyruvic acid (10); benzyl alcohol (4) was absent from this system. Only 19% *l*-PAC (3) and a small quantity of benzyl alcohol (4) (4%), was obtained using 0.28g of pyruvic acid (10). For all further investigations 0.5g of pyruvic acid (10) and 0.5ml of ethanol were routinely used since this combination represented the optimal quantities of these reagents at 5°C.

At 5°C, the use of pyruvic acid (10) yielded a 30% conversion to *l*-PAC (3) whilst sodium pyruvate (10b) resulted in 24% *l*-PAC (3). The reaction systems contained 0.5g (6.4mmol) of pyruvic acid (10) or 2.5g (23mmol) of sodium pyruvate (10b). Only around one quarter of the cheaper reagent, pyruvic acid (10) is required to obtain a higher yield of *l*-PAC (3) which makes the use of this reagent commercially more viable than sodium pyruvate (10b).
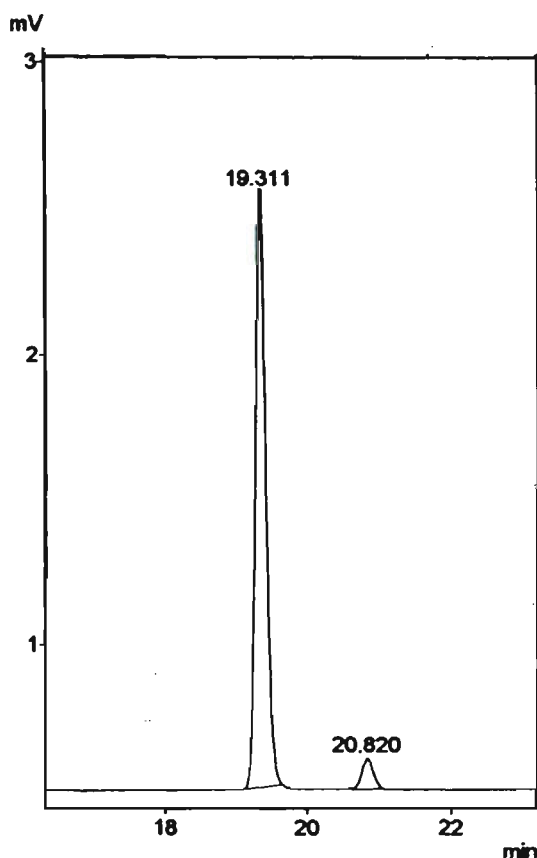
Figure 3.4 The chiral GC of the product derivatised using trifluoroacetic anhydride shows *l*-PAC (3) had been formed in an enantiomeric ratio of 95 : 5 (90% ee).

3.5.2 *Reactions at 5 °C without pyruvic acid*

It was of interest to investigate whether *l*-PAC (3) could be produced in the absence of pyruvic acid (10) but with yeast being activated by 0.05M sodium citrate and the pH adjusted to 5.5 using acetic acid.

Obviously, if acetic acid (or acetate) could be introduced as the acetyl donor, in place of pyruvic acid (10), into the yeast mediated acyloin condensation of benzaldehyde (1) to form *l*-PAC (3), then this would result in a cheaper means of production of *l*-PAC (3).

Sodium citrate (0.05M, 5ml), with pH adjusted to 5.5 using acetic acid, ethanol (0.5ml) and petroleum spirit (45ml) were added to yeast (5g) followed by the addition of benzaldehyde (1) (1mmol) and the mixture was stirred at 5°C for 24h. GC analysis showed complete conversion of benzaldehyde (1) to benzyl alcohol (4) with no *l*-PAC (3) formation at all. Clearly, acetic acid (acetate) is not a suitable acetyl donor for the yeast mediated production of *l*-PAC (3).

3.6 Acetaldehyde and Pyruvic acid.

In Section 2.6, pre-treatment of the yeast with acetaldehyde (2), in reaction systems containing sodium pyruvate (10b), was shown to eliminate the formation of benzyl alcohol (4). It was therefore of interest to examine whether pre-treating the yeast with acetaldehyde (2), prior to the addition of benzaldehyde (1), in reactions containing pyruvic acid (10), could also prevent the formation of benzyl alcohol (4) (Scheme 3.3).
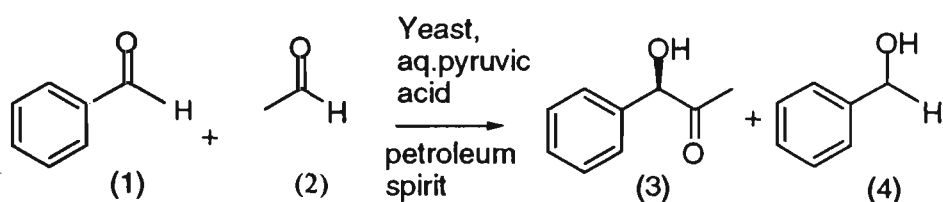
Scheme 3.3

Pyruvic acid (10) (0.5g) with pH adjusted to 5.45 using ammonium acetate solution (5ml), acetaldehyde (2) (0.56g) and petroleum spirit (45ml) were added to baker's yeast (5g) and the mixture was shaken at 20°C for 3h. Ethanol (0.5ml) and benzaldehyde (1) (1mmol) were then added and the reaction mixture was shaken at 20°C for a further 48h. GC analysis revealed only starting material.

The reaction was also carried out without adjusting the pH of the system and GC analysis after 24h again showed only starting material. A similar result was observed when the same reaction was conducted at 5°C.

It was interesting to note that although the pyruvic acid (10)/pH5.45 adjusted system (incorporating a 3h acetaldehyde (2) pre-treatment) failed to yield any *l*-PAC (3) at either 20°C or 5°C, the corresponding sodium pyruvate (10b) system resulted in 7% *l*-PAC (3).

It was observed that very little *l*-PAC (3) was formed when either sodium pyruvate (10b) or pyruvic acid (10) was added prior to acetaldehyde (2) pre-treatment.

3.7 Optimisation of Reaction Conditions.

Previous studies involving reduction reactions[44-49] have shown that once conditions have been optimised then additional quantities of yeast can be added in order to increase the conversion to product. This was not the case with the yeast mediated acyloin condensation of benzaldehyde (1) involving sodium pyruvate (10b) since very little increase in *l*-PAC (3) occurred as the amount of yeast was increased (Section 2.10).

In order to investigate the effect of increased quantities of yeast on the reaction system, a reaction utilising pyruvic acid (10) was carried out using 10g of yeast/mmol of benzaldehyde (1).

Pyruvic acid (10) (1.04g) was added to 0.05M sodium citrate (10ml) and the pH adjusted to 5.45 using ammonium acetate. Baker's yeast (10g) and benzaldehyde (1) (1mmol) were added to this solution (10ml), followed by ethanol (1ml) and petroleum spirit (80ml) and the reaction mixture was stirred at 5°C for 24h. GC analysis indicated 30% conversion to *l*-PAC (3). The corresponding result for a reaction system employing 5g yeast/mmol benzaldehyde (1), and an equivalent quantity of pyruvic acid (10) was also 30%.

The product of the reaction with 10g yeast was isolated and purified in a 20% yield. The isolated product following derivatisation with trifluoroacetic anhydride was analysed by chiral GC and exhibited a ratio of 95:5 (90% ee). The optical rotation of PAC (3) ($[\alpha]_D$ = -262.6°, (c = 0.745, CHCl$_3$), (Lit.,[109] $[\alpha]_D$ = -408.7°, c = 1.1, CHCl$_3$) showed that the *l*-enantiomer had been formed.

The results indicate that there is little difference between using 10g or 5g of yeast/mmol benzaldehyde (1) and consequently the optimal quantity of yeast /mmol of benzaldehyde (1) was set at 5g at a reaction temperature of 5°C.

3.8 Conclusion.

The results clearly show that pyruvic acid (10) is just as able to act as an acetyl donor as sodium pyruvate (10b) in the production of *l*-PAC (3). Under similar conditions, at a reaction temperature of 5°C, the yield of product, using pyruvic acid (10), was 20% (90% ee) compared with a yield of 24% (86% ee) when using sodium pyruvate (10b).

The ratio of the amount of sodium pyruvate (10b) and pyruvic acid (10) employed in these reactions is 23mmol : 6mmol so that only about one quarter of the quantity of pyruvic acid (10) is required for a similar yield of *l*-PAC (3). Thus the use of pyruvic acid (10), a significantly cheaper reagent, in place of sodium pyruvate (10b), would considerably reduce the production cost of *l*-PAC (3).

CHAPTER 4

NMR STUDIES

NMR STUDIES

4.1 The Biosynthesis of *l*-Ephedrine

The biosynthesis of *l*-ephedrine, in the plant, *Ephedra gerardiana*, has been studied by Grue-Sorensen and Spenser.[113,114] Their experiments, using both ^{13}C and ^{14}C labelled pyruvic acid, demonstrated that the C_2 of pyruvate is incorporated into PAC (3), which is then converted to ephedrine (7) (Scheme 4.1).
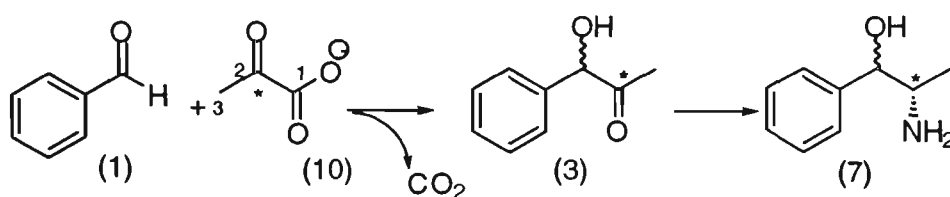
*Denotes ^{13}C or ^{14}C label

Scheme 4.1

The abovementioned studies were not entirely conclusive and in order to establish the origin of the C_6-C_1 subunit of *l*-ephedrine (7) Grue-Sorensen and Spenser[115] conducted further experiments using [*carbonyl*-^{13}C, ^{2}H]benzaldehyde and [*carbonyl*-^{13}C]benzoic acid. They showed by ^{13}C NMR spectroscopy that [*carbonyl*-^{13}C]benzoic acid supplies the benzylic moiety of the *Ephedra* alkaloids.

As a result of their experiments, Grue-Sorensen and Spenser[115] established the major steps (benzoic acid to ephedrine) leading to the *Ephedra* alkaloids (Scheme 4.2). Phenylalanine (26), from the plant, cleaves *via* interaction with ammonia lyase to cinnamic acid (27) which is then converted to either the benzoic acid (28) or SCoA moiety. Condensation of pyruvic acid (10) with benzoic acid (28) produces the dione (12). Transamination of (20) results in the cathinione (29) which is then reduced to produce *l*-ephedrine (7) and *d*-pseudoephedrine (8).
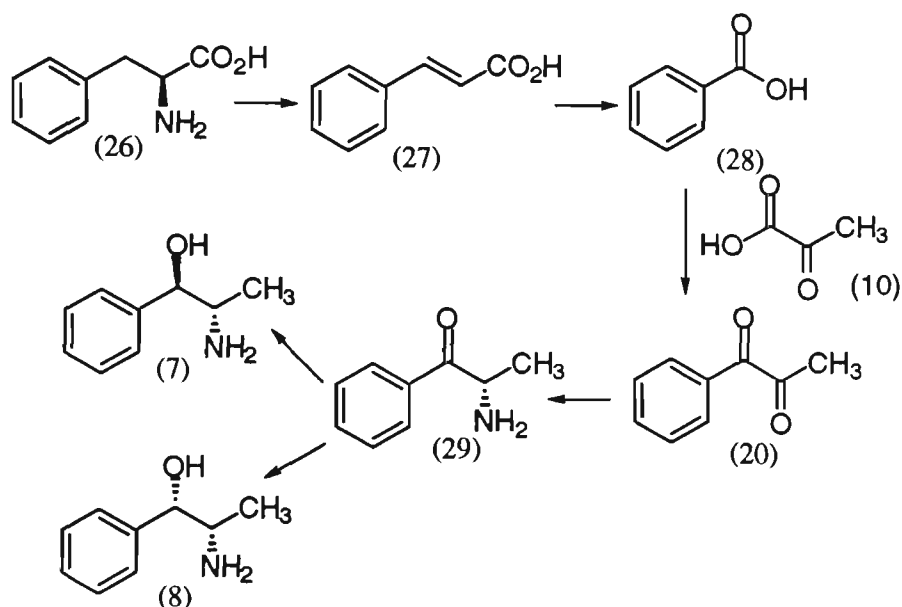
Scheme 4.2

4.2 The Biosynthesis of *l*-PAC (3) Using Fermenting Yeast.

The commercial production of *l*-PAC (3), the precursor of *l*-ephedrine (7), is conducted using fermenting yeast. Although fermenting yeast provides a cheap source of pyruvate decarboxylase (PDC), the enzyme responsible for catalysing the reaction leading to *l*-PAC (3), a number of other enzymes such as alcohol dehydrogenase (ADH) and oxidoreductases are also present and are associated with the production of the major by-product, benzyl alcohol (4).[10,22,30]

The mechanism of the biotransformation of benzaldehyde (1) to *l*-PAC (3) using fermenting yeast has been studied by a number of groups.[10,24,29,116] The process is initiated by glucose (9), a component of the fermentation broth, being converted *in situ* to pyruvic acid (10) and then to acetaldehyde (2) (Scheme 4.3).
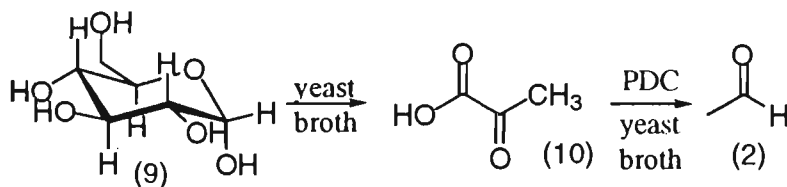
Scheme 4.3

The overall biotransformation process involves the condensation of acetaldehyde (2), which has been formed *in situ* from pyruvic acid (10), with benzaldehyde (1). This reaction is catalysed by PDC to give *l*-PAC (3) (Scheme 4.4). A second reaction which also occurs is the reduction of benzaldehyde (1) to benzyl alcohol (4) due to the activity of ADH and/or oxidoreductase enzymes.[10,22,30]
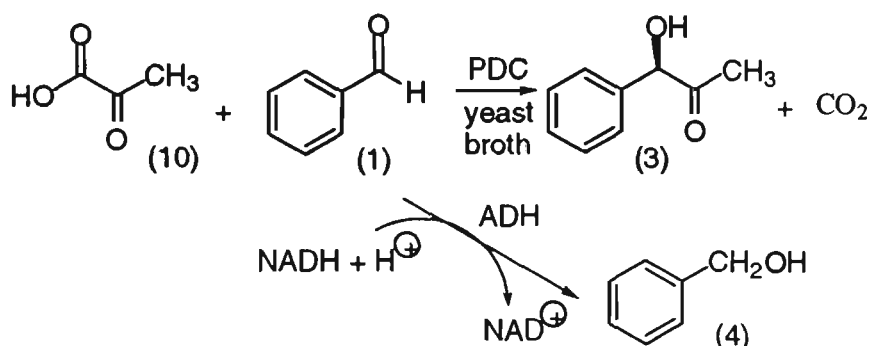
Scheme 4.4

4.3 The Biosynthesis of *l*-PAC (3) Using Yeast in an Organic Solvent.

In an organic system, glucose (9) is not added since fermentation does not occur; the addition of either sodium pyruvate (Section 2.4) or pyruvic acid (10) (Section 3.3) is therefore essential for the formation of *l*-PAC (3). The present study (Chapters 2 and 3) has shown that the addition of ethanol to the reaction medium increases the yield of *l*-PAC (3) and eliminates the production of benzyl alcohol (4). Pre-treatment of yeast with acetaldehyde (2) (Section 2.6) has also been shown to eliminate the production of benzyl alcohol (4).

$2\text{-}^{13}\text{C}$ sodium pyruvate and $1\text{-}^{13}\text{C}$ ethanol were independently used to study the yeast mediated acyloin condensation of benzaldehyde (1) to form *l*-PAC (3) (Scheme 4.5, (i) and (ii) respectively) in an organic solvent, in order to discover whether the origin of the acetyl group is the yeast, the pyruvate or the ethanol.
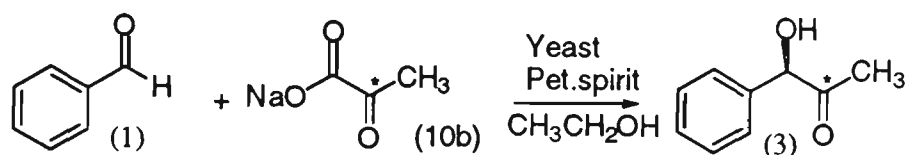
(i)
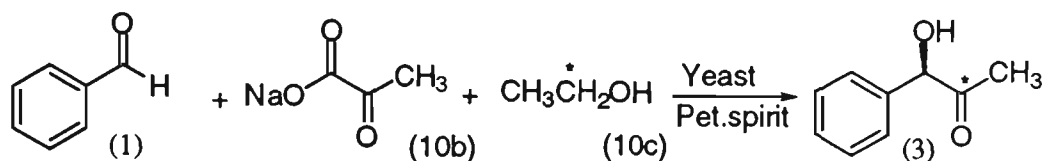
(ii)

*Denotes labelled carbon

Scheme 4.5

The reactions involving labelled materials were carried out on a small scale using 1g of yeast, compared with reactions described in Chapters 2 and 3 which employed 5g of yeast. All reagents were scaled down accordingly and the reactions were conducted at 5°C since the optimum yield of *l*-PAC (3) (26%) was obtained at this temperature using 5g of yeast.

4.3.1 *Reactions using 2-^{13}C sodium pyruvate*

2-^{13}C Sodium pyruvate was used in the yeast mediated condensation reaction in order to investigate the mechanism of the synthesis of *l*-PAC (3). If the acetyl group in *l*-PAC (3) was provided by the sodium pyruvate (10b), as was found in the fermenting system,[10,23] then ^{13}C NMR of the *l*-PAC (3) product would show a strong carbonyl peak. It was also anticipated that this study would reveal why such a high ratio of sodium pyruvate (10b):benzaldehyde (1) was required.

2-^{13}C sodium pyruvate, pH6 citrate buffer and ethanol in petroleum spirit were stirred at 20°C for 1h, baker's yeast and benzaldehyde (1) in petroleum spirit were added and the mixture was stirred at 5°C for a further 48h. GC analysis showed 37% conversion to *l*-PAC (3).

The ^{13}C NMR spectrum of the product mixture (Figure 4.1), after 48h, showed a strong C-OH peak (δ56.87ppm) for ethanol. A large excess of sodium pyruvate (10b) is used in the yeast mediated acyloin condensation reaction in an organic solvent and the presence of labelled ethanol indicates that excess pyruvate has been reduced to ethanol. This result suggests the alcohol dehydrogenase and/or oxidoreductase enzymes in the yeast, which convert benzaldehyde (1) to benzyl alcohol (4) in fermenting systems, may be involved in the conversion of pyruvate (10) to ethanol *via* acetaldehyde (2).
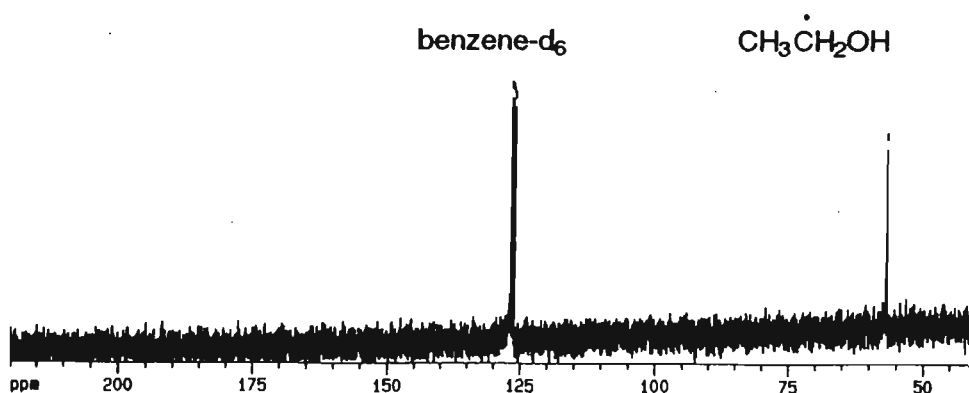
Figure 4.1 ^{13}C NMR spectrum of the product mixture after 48h when using 2-^{13}C sodium pyruvate in the yeast mediated acyloin condensation of benzaldehyde at a reaction temperature of 5°C. Results show the formation of 1-^{13}C ethanol from 2-^{13}C sodium pyruvate.

The reaction mixture was then filtered and excess solvent evaporated from the filtrate *in vacuo*. ^{13}C NMR of the concentrated sample showed a strong carbonyl peak at (δ) 205.4ppm (Figure 4.2), clear evidence that the C-2 from the pyruvate was incorporated into *l*-PAC (3). This carbonyl peak was not observed in the ^{13}C NMR of the crude sample (Figure 4.1) due to the low concentration of *l*-PAC (3).

The reaction mixture was then filtered and excess solvent evaporated from the filtrate *in vacuo*. ^{13}C NMR of the concentrated sample showed a strong carbonyl peak at (δ) 205.4ppm (Figure 4.2), clear evidence that the C-2 from the pyruvate was incorporated into *l*-PAC (3). This carbonyl peak was not observed in the ^{13}C NMR of the crude sample (Figure 4.1) due to the low concentration of *l*-PAC (3).
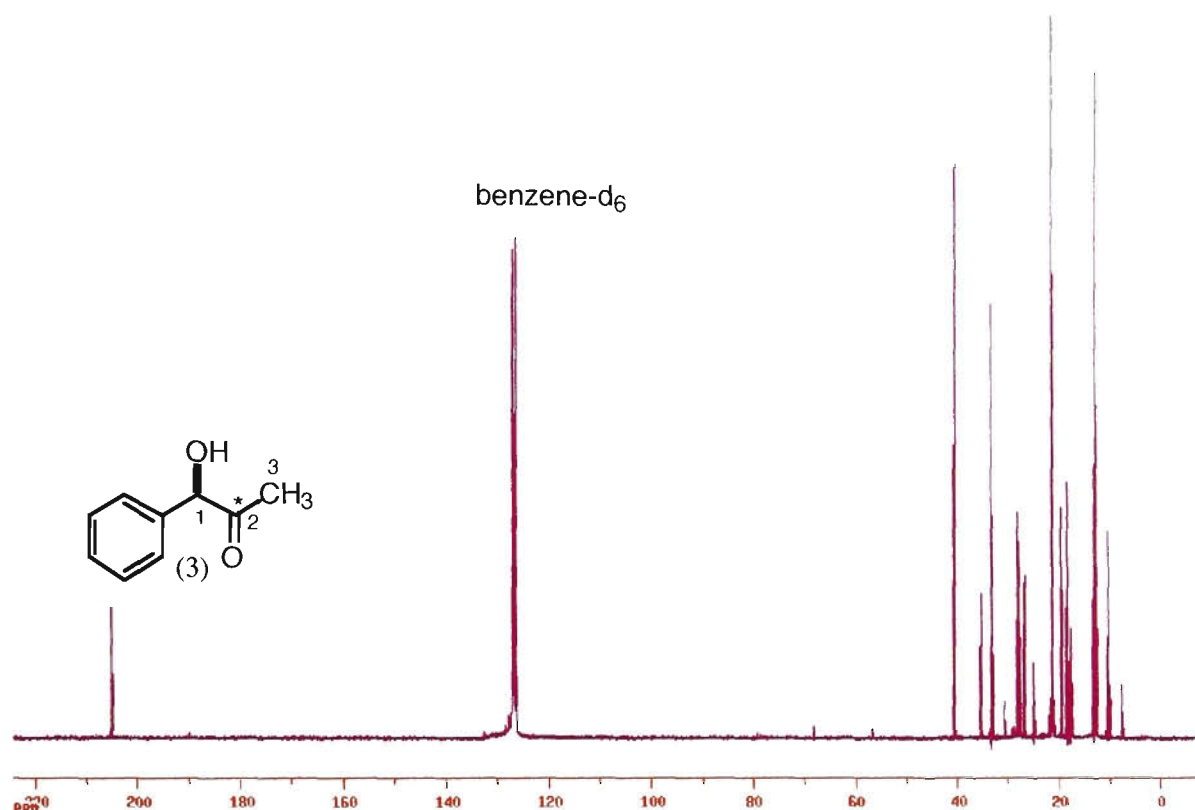
Figure 4.2 ^{13}C NMR spectrum of *l*-PAC (3) following filtration and evaporation of the reaction mixture after 48h when using 2-^{13}C sodium pyruvate (10) in the yeast mediated acyloin condensation of benzaldehyde (1) at a reaction temperature of 5°C. Results show the strong carbonyl peak at δ205.4ppm.

The product was dissolved in CDCl$_3$ and analysed using ^{1}H NMR (Figure 4.3). The ^{1}H NMR of the product showed a distinct doublet for the CH at δ5.1ppm, (J$_{C-H}$ = 3Hz) due to coupling to the ^{13}C carbonyl. A singlet for CH is not observed in the ^{1}H NMR indicating that the carbon of the carbonyl is exclusively ^{13}C in the *l*-PAC (3) formed in this reaction. This result indicates that the C-2 from the sodium pyruvate (10b) is incorporated into the *l*-PAC (3) and that there is no contribution to the acetyl group from the yeast.
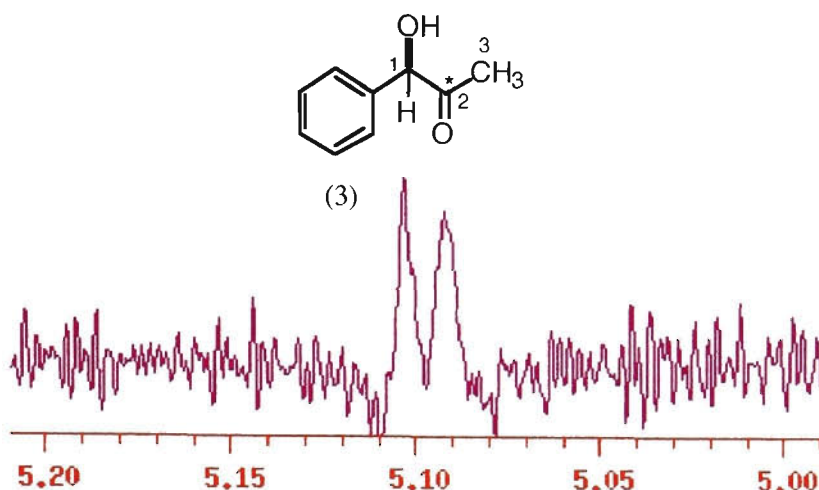
Figure 4.3   ¹H NMR spectrum of *l*-PAC (3) following filtration and evaporation of the reaction mixture after 48h when using 2-¹³C sodium pyruvate (10b) in the yeast mediated acyloin condensation of benzaldehyde (1) at a reaction temperature of 5°C. Results show a distinct doublet at $\delta 5.1$ppm ($J_{C-H} = 3$Hz) for the H at C-1.

4.3.2 *Reactions using 1-^{13}C ethanol*

In the above reaction system in which 2-^{13}C sodium pyruvate was used, ^{13}C NMR data indicated that the sodium pyruvate (10b) was converted to 1-^{13}C ethanol. This result poses a number of questions regarding the reaction pathway followed in an organic solvent. The possibilities include: (i) pyruvate (10) is directly involved in the condensation reaction whilst some pyruvate (10) is converted to ethanol (10c) *via* acetaldehyde (2), (ii) pyruvate (10) is converted to acetaldehyde (2) which provides the acetyl group in the condensation whilst some acetaldehyde (2) is converted to ethanol (10c), (iii) pyruvate (10) is converted to ethanol, which is then converted to *l*-PAC (3) (Scheme 4.6).
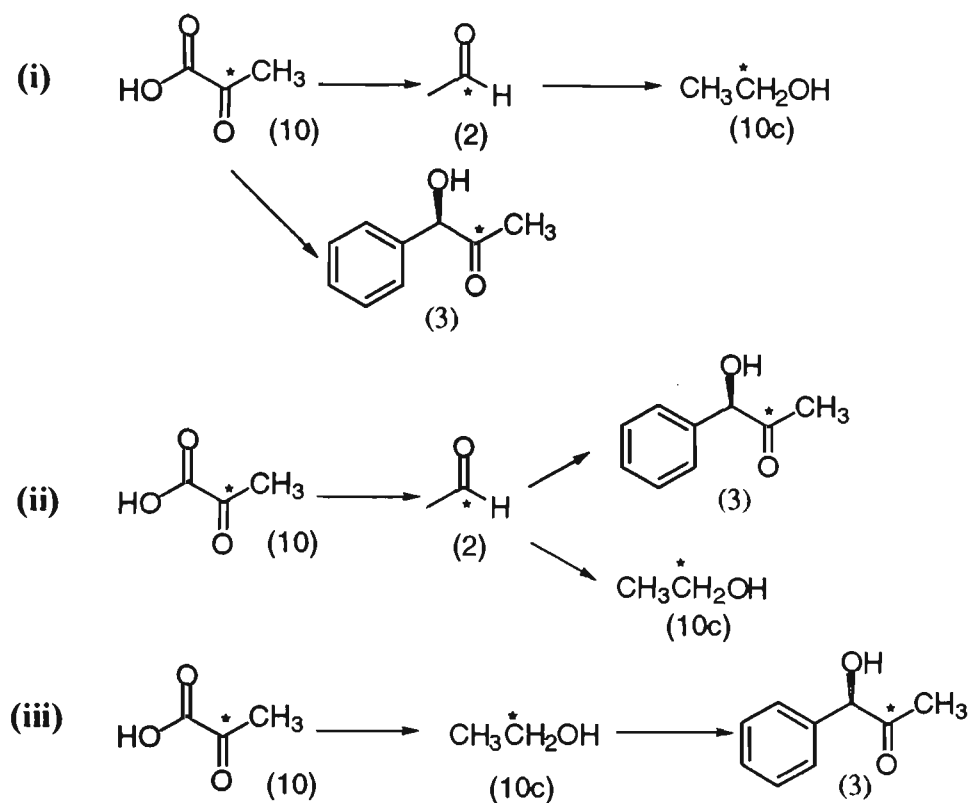
Scheme 4.6

In order to examine the three possibilities, 1-^{13}C ethanol was added to the reaction medium. If ethanol (10c) was the source of the acetyl group, the ^{13}C NMR of the product mixture would show the presence of ^{13}C in the carbonyl signal of *l*-PAC (3).

A mixture of sodium pyruvate, pH6 citrate buffer and 1-^{13}C ethanol in petroleum spirit was stirred at 20°C and then yeast and benzaldehyde (1) added. The mixture was then stirred at 5°C for 24h, filtered and analysed using GC, which showed 44% conversion to *l*-PAC (3).

^{13}C NMR analysis (Figure 4.4) of the reaction mixture showed the presence of only 1-^{13}C ethanol (C-OH, 56.86ppm). Although the results of studies described in Section 2.5 indicated that the addition of ethanol enhanced the production of *l*-PAC (3), the present results indicate that the ethanol is not incorporated into *l*-PAC (3), thereby eliminating pathway (iii) (Scheme 4.6).
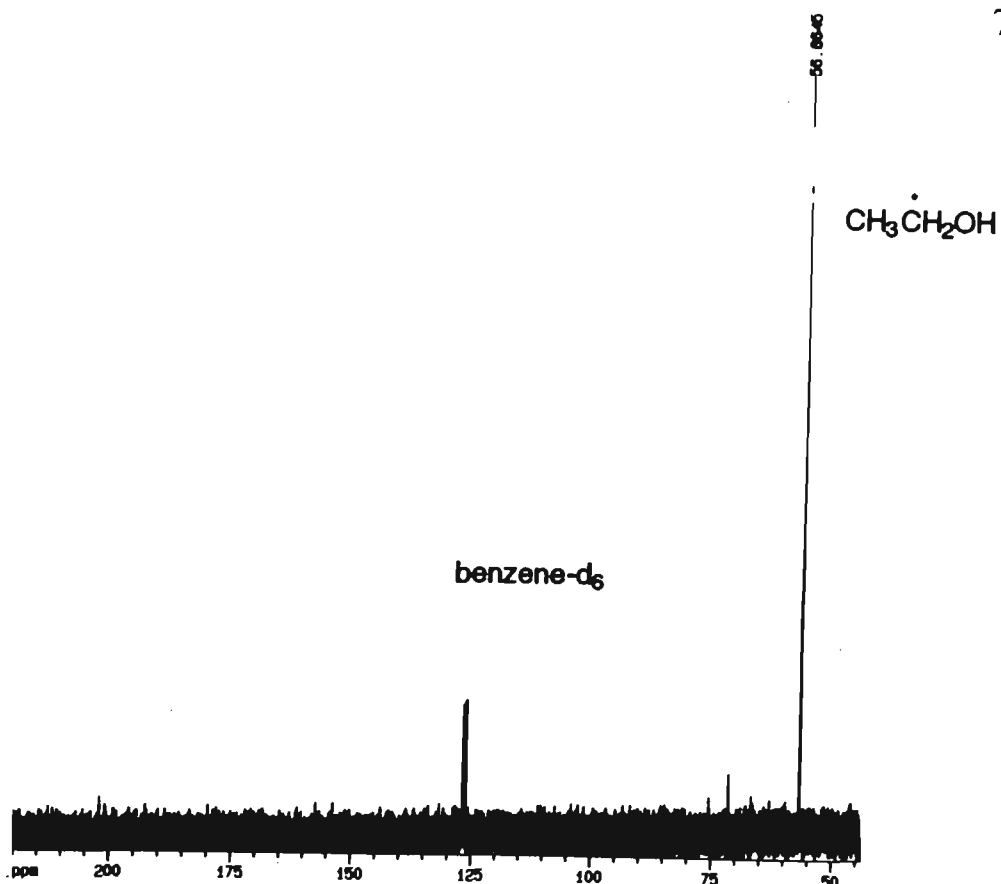
Figure 4.4 ¹³C NMR of the reaction mixture following the yeast mediated acyloin condensation of benzaldehyde using 1-¹³C ethanol at a reaction temperature of 5°C. Results show C-1 at δ56.86ppm.

Results reported in Section 4.3.1 indicates that the pyruvate (10) used in the synthesis of *l*-PAC (3) also results in the formation of ethanol. This can occur as a result of either of the pathways labelled as (i) or (ii) in Scheme 4.6. It is also noteworthy that labelled acetaldehyde (δ196.7ppm, CHO) was not observed in the ¹³C NMR spectrum of the reaction mixture. This is also consistent with either (i) or (ii) in Scheme 4.6.

¹³C labelled acetaldehyde (2) ($2000/g) was not employed to investigate the feasibility of path (ii) but this could certainly be the subject of further studies in order to clarify whether acetaldehyde (2) is involved in the reaction.

4.4 ^{13}C NMR Studies of the Kinetics of the Reaction

The kinetics of the biotransformation of benzaldehyde (1) to *l*-PAC (3) was studied using ^{13}C NMR. The rate of reaction was studied at reaction temperatures of 5°C, 10°C and 20°C.

The yeast mediated acyloin condensation of [*carbonyl*-^{13}C]benzaldehyde in an NMR tube was monitored over a 68h period using a 500MHz NMR spectrometer. The reaction was carried out at 5°C, 10°C and 20°C to:

> (a) find the optimum reaction temperature,
>
> (b) to observe the formation of product and
>
> (c) study the rate of reaction.

It is known that reductase activity of yeast is deactivated in petroleum spirit after 24h at 20°C[59] and it was of considerable interest to discover if acyloin deactivation occurred at temperatures below 20°C.

Pyruvic acid/buffer mixture (pH5.45), petroleum spirit, benzene-d$_6$ (internal standard), ethanol and [*carbonyl*-^{13}C]benzaldehyde were added to a 10mm NMR tube containing baker's yeast. The tube was placed in a 500MHz NMR instrument set at the desired reaction temperature (5°C, 10°C or 20°C) and a ^{13}C NMR spectrum recorded hourly for 68h. The supernatant was then decanted from the NMR tube and analysed using GC. The results are given in Table 4.1 and show that as the temperature decreased, the conversion to *l*-PAC (3) increased. The yield of *l*-PAC (3) at 5°C, using a yeast : benzaldehyde (1) ratio of 0.1g : 0.02mmol, was the same as that obtained using 5g of yeast and 1mmol of benzaldehyde (1), indicating the NMR system is a viable model for studying the larger scale reactions.

Table 4.1 GC results of the conversion from benzaldehyde (1) to *l*-PAC (3) following the yeast mediated acyloin condensation of benzaldehyde (1) at reaction temperatures of 5°C, 10°C and 20°C.

Reaction Temp. (°C)	*l-* PAC (3), (%)	BenzylAlcohol (4), (%)	By-products (5), (6)
5	26 (26*)	0	0
1 0	1 7	0	0
2 0	1 2	6	5

*Yield using 5g of yeast.

4.4.1 *The effect of temperature on the l-PAC (3) reaction*

A time lapse sequence of ^{13}C NMR spectra of the yeast mediated acyloin condensation of benzaldehyde (1) at 5°C was recorded. Each individual spectrum was the result of 128 scans collected over approximately 7 minutes and spectra were recorded at hourly intervals for 68h. The first spectrum was taken 30 - 45 minutes after the reaction was initiated. Figure 4.5 displays the full time lapse sequence of signals obtained at 185ppm due to the carbonyl ^{13}C of benzaldehyde (1) and 80ppm due to the carbinol ^{13}C of *l*-PAC (3). The intensity of each peak in the sequence of the 80ppm signal was measured relative to the benzene-d_6 signal for each spectrum and the values plotted using Microsoft Excel® (Figure 4.6). Similar spectra were recorded at 10°C and 20°C and the time lapse data obtained from the *l*-PAC (3) carbinol ^{13}C signal for each of these reactions is also plotted in Figure 4.6
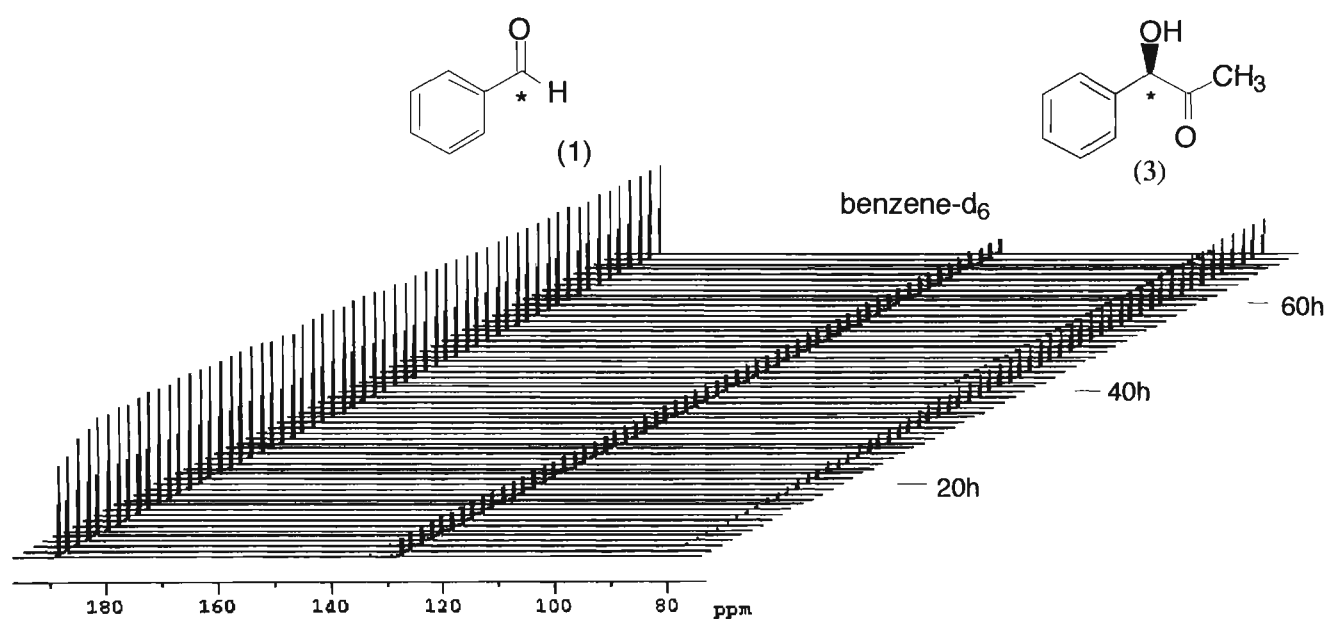
Figure 4.5 Time lapse sequence of ¹³C NMR spectra of the yeast mediated acyloin condensation of benzaldehyde (1) at 5°C. *Denotes the labelled carbonyl of benzaldehyde (1) at 185ppm and the labelled carbinol of *l*-PAC (3) at 80ppm.
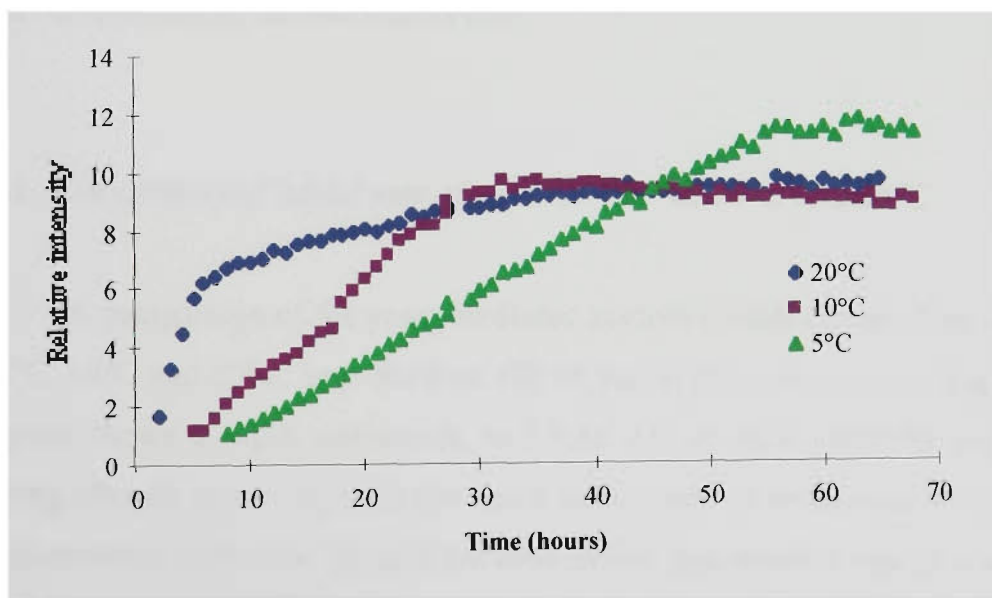
Figure 4.6 Plot of the time lapse NMR data recorded hourly during the yeast mediated acyloin condensation of benzaldehyde (1) at reaction temperatures of 5°C, 10° and 20°C.

The progress of the yeast mediated acyloin condensation of benzaldehyde (1) to form *l*-PAC (3), at 5°C, is shown in Figure 4.6. The reaction began slowly but

reduction in the rate of reaction. These results indicate that the yeast was not deactivated at 5°C until after about 55h when the graph of the relative intensity of the product peak plateaus indicating the production of *l*-PAC (3) has ceased.

The relative intensity of the product peak (80ppm) at 10°C is plotted in Figure 4.6 and shows a reasonably constant rate of production of *l*-PAC (3) from 5 - 30h. Between 30 and 68h the graph of relative intensity of the product peak has formed a plateau indicating that the production of *l*-PAC (3) has ceased. This result indicates that the yeast was deactivated after about 30h at a reaction temperature of 10°C.

The relative intensity of the product peak (80ppm) at 20°C is plotted in Figure 4.6 and shows an almost immediate initiation of *l*-PAC (3) production followed by a constant production rate between about 2 and 6h; after this time the reaction slows dramatically and by 25h has all but ceased. This is a similar result to that reported in Section 2.7 in which a 14% yield of *l*-PAC (3) was observed after 6h with little increase at later times. These two results indicate that at a reaction temperature of 20°C the production of *l*-PAC (3) has almost ceased after about 6h. This indicates that the yeast enzymes responsible for the production of *l*-PAC (3) have little activity after 6h exposure to the reaction system.

4.4.2 *Comparison of initial rate of reaction at 5°C, 10°C and 20°C*

A comparison of the yeast mediated acyloin condensation of benzaldehyde (1) at 5°C, 10°C and 20°C, over the first 10h of the reaction, is given in Figure 4.7. This diagram shows a rapid conversion to *l*-PAC (3) at 20°C with the reaction almost ceasing after 6h compared with the much slower rate of reaction at 10°C, which does not commence until after 5h, and the even slower but steadier rate of reaction at 5°C, which does not commence before 8h. The slopes of the three relative intensity plots provide a relative measure of the initial reaction rates at 20°C, 10°C and 5°C and have values of 0.83 : 0.21 : 0.14. Thus the initial rate of reaction at 20°C is about 6 times faster than that at 5°C but at 10°C it is only 1.5 times faster than at 5°C.
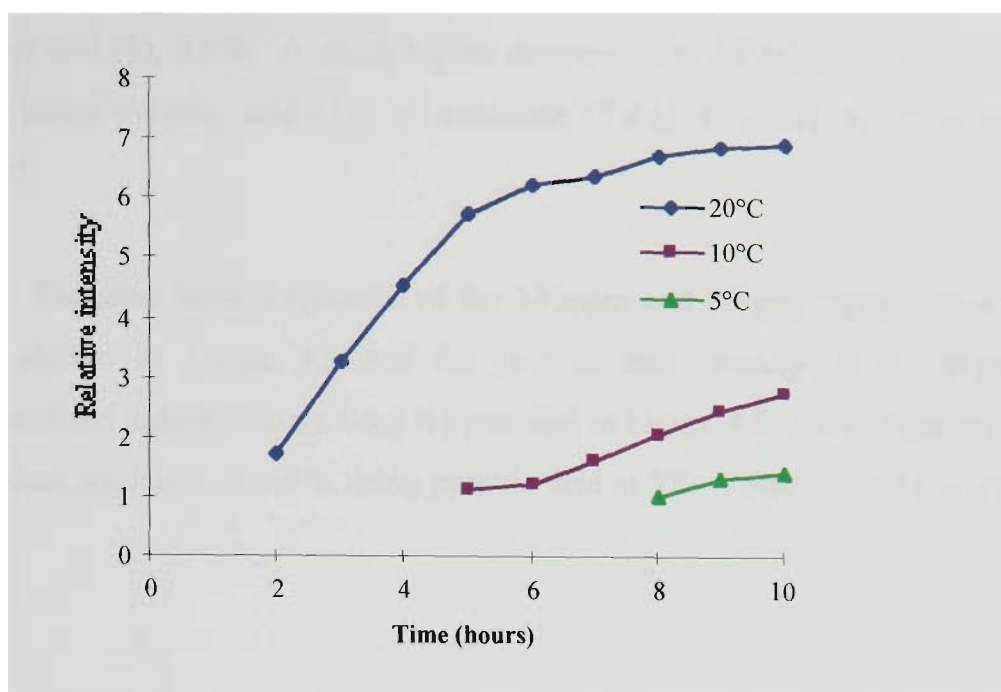
Figure 4.7 Plot of the time lapse NMR data over the first 10h during the yeast mediated acyloin condensation of benzaldehyde (1) at reaction temperatures of 5°C, 10°C and 20°C.

The above results indicate that deactivation of the yeast can be delayed by carrying out the reaction at 5°C. The initial rate of reaction may be much slower than at 20°C, but the overall production of *l*-PAC (3) is increased due to the extended activity of the enzymes involved in the acyloin condensation of benzaldehyde (1).

4.4.3 Reaction at 5°C using sodium pyruvate

As already noted, the temperature study was carried out using pyruvic acid (10) as the acetyl group source. In order to permit a comparison of the reaction rates of pyruvic acid (10) and sodium pyruvate (10b), a similar reaction was carried out at 5°C employing sodium pyruvate (10b) as the acetyl source.

Sodium pyruvate (10b) was dissolved in pH6 citrate and added to a 10mm NMR tube containing baker's yeast, petroleum spirit, ethanol, benzene-d_6, (internal standard) and [*carbonyl*-^{13}C]benzaldehyde. The NMR tube was placed in the 500MHz NMR which had been set to 5°C and a spectrum recorded every hour for 68h. The supernatant was decanted and analysed using GC and showed a 13%

conversion to *l*-PAC (3) plus a small amount of the by-products, benzyl alcohol (4), 1% and diol (6), 0.5%. A much higher conversion to *l*-PAC (3), 26%, was obtained when using pyruvic acid (10) as substrate (Table 4.1) and no by-products were formed.

The time lapse sequences of the 185ppm and 80ppm signals were similar to those shown in Figure 4.5 and the plot of the intensity of the 80ppm signal (standardised against benzene-d_6) is presented in Figure 4.8. For comparison, the time lapse data sequence obtained using pyruvic acid at 5°C is also plotted in Figure 4.8.
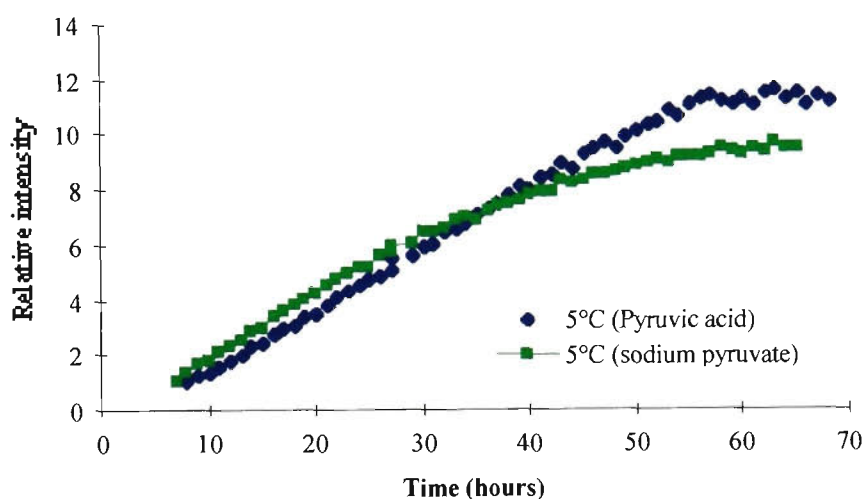
Figure 4.8 Plot of the time lapse NMR data showing *l*-PAC formation during the yeast mediated acyloin condensation of benzaldehyde (1) using (i) pyruvic acid (10) and (ii) sodium pyruvate (10b) at 5°C.

^{13}C NMR studies at 5°C of the yeast mediated acyloin condensation of benzaldehyde (1) using pyruvic acid (10) as the substrate, and with the pH adjusted to 5.45, showed a higher conversion to *l*-PAC (3) and a longer period of yeast activity than was the case when sodium pyruvate (10b) was used as the substrate. Since pyruvic acid (10) is a significantly cheaper reagent than sodium pyruvate (10b), pyruvic acid (10) is the preferred substrate for the yeast mediated acyloin condensation of benzaldehyde (1) to give *l*-PAC (3).

The ^{13}C NMR studies clearly show the most productive and efficient results are obtained at a reaction temperature of 5°C using pyruvic acid as the substrate.

4.5 Conclusion

Studies which were reported in Chapter 2 showed that some important requirements of the yeast mediated acyloin condensation of benzaldehyde (1) in an organic solvent were needed in order to obtain optimal formation of *l*-PAC (3). These requirements included; sodium pyruvate (10b) (23mmol) : benzaldehyde (1) (1mmol), pH5.45, addition of ethanol (0.5ml) to prevent the formation of benzyl alcohol (4) and 5°C. In order to find a more economical means of production of *l*-PAC (3) the cheaper reagent, pyruvic acid (10) was employed. The information regarding reaction conditions and requirements as found in Chapter 2 were utilised and the reaction fully explored using pyruvic acid (10). The studies which were reported in Chapter 3 revealed that the cheaper reagent could be successfully used in the reaction and that a lower ratio of pyruvic acid (10) (6mmol) : benzaldehyde (1) (1mmol) was required. The exploration of the use of pyruvic acid (10) in place of sodium pyruvate (10b) led to a patent application for this process (see Appendix).

^{13}C NMR was employed to reveal details of; (i) the reaction pathway, (ii) the role of reagents in the reaction and (iii) how temperature effected the rate of reaction and the deactivation of the yeast enzymes. ^{13}C NMR studies revealed that pyruvate (10) was responsible for the carbonyl of *l*-PAC (3) and although the inclusion of ethanol was found to enhance the production of *l*-PAC (3) and decrease/eliminate the production of benzyl alcohol (4), ethanol was not directly involved in the condensation of benzaldehyde (1) to form *l*-PAC (3). Temperature studies revealed that the initial reaction rate was higher at 20°C, but that the yeast enzymes were deactivated after a short period (6h) at a temperature of 5°C the enzymes were not deactivated for at least 55h.

CHAPTER 5

EXPERIMENTAL

EXPERIMENTAL

5.1 General

Proton (^{1}H) NMR spectra were recorded at 300MHz and carbon (^{13}C) at 75.43MHz with a Bruker DPX300 spectrometer. The spectrometer was used in conjunction with a Silicon Graphics Indy work station. The ^{1}H and ^{13}C NMR spectra refer to deuterochloroform solutions with tetramethylsilane (TMS) as the external reference ($\delta 0.00$ ppm) unless otherwise stated. Resonance data are reported according to the following: chemical shift measured in parts per million (ppm) downfield from TMS, multiplicity, number of hydrogens, observed coupling constant (J, Hz) and assignment. Multiplicities are denoted as singlet (s), doublet (d), triplet (t), quartet (q) and multiplet (m). The temperature controlled ^{13}C NMR experiments were recorded at 125.72MHz on a Bruker DRX500 at CSIRO, Division of Molecular Science, Clayton, Victoria.

Infra-red (IR) spectra were obtained using a BioRad Digilab FTIR spectrophotometer (cm^{-1} scale) in conjunction with the Win-IR data program. Spectra refer to thin liquid films. The intensity of each frequency of absorption (υ_{max}) is reported as strong (s), medium (m) or weak (w).

Optical rotations were measured with an Optical Activity PolAAr 2000 AA series polarimeter.

Gas chromatographic (GC) analysis of products were performed on a Shimadzu GC-17A using a BP1 column, 0.25mm ID, 15m in length and phase thickness, 0.25μm. Chiral gas chromatography was conducted using a Chiraldex G-

TA (30m x 0.25mm) column and phase thickness, 0.125µm. Operating conditions are shown in Table 5.1.

Table 5.1 GC operating conditions used for the analysis of samples following
(i) the yeast mediated acyloin condensation of benzaldehyde and,
(ii) the *l*-PAC product which had been derivatised using trifluoroacetic anhydride.

	BP1 column (i)	Chiraldex G-TA (ii)
Carrier gas	He, 12psi, 0.65ml/min	He, 12psi, 0.65ml/min
Split ratio	70:1	70:1
Temperatures: oven	40°C for 3min., then 8°C/min. to 150°C	95°C
injector	200°C	200°C
detector	250°C	250°C

Radial chromatography was carried out using a Chromatotron model 7924T on glass plates coated with Merck Kieselgel 60PF$_{254}$ silica gel 2mm thick. The elution solvent was ether/petroleum spirit (60:40, v/v). The components were visualised by inspection under ultraviolet (UV) light.

The pH was measured using an Orion Research Model 701 digital pH meter and a combination pH electrode.

5.2 Materials

The baker's yeast used in the reactions was that which is readily available in the supermarkets and sold under the Mauripan label and was supplied by Mauri Integrated Ingredients, Footscray. BDH AnalaR petroleum spirit (40 - 60°C) was used without purification. ^{13}C labelled reagents were purchased from Cambridge Isotope Laboratories, Massachusetts. All other reagents were purchased from Sigma-Aldrich.

5.2.1 *Solvent purification*

(a) Ethanol: Anhydrous ethanol was prepared by the method of Vogel[117] and stored over 5A molecular sieves.

(b) Dichloromethane: Dichloromethane (200ml) was shaken with portions of concentrated H_2SO_4 until the acid layer remained colourless, then washed with water, 5% $NaHCO_3$ (3 x 100ml) and water (2 x100ml), pre-dried over $CaCl_2$ and distilled from $CaSO_4$. The distillate was stored over $CaCl_2$.[118]

(c) Tetrahydrofuran (THF): Tetrahydrofuran containing a small amount of benzophenone was dried over sodium wire until a blue colour was evident. The solvent was stored over sodium wire and distilled prior to use.[119]

5.2.2 *Purification of reagents*

(a) Benzaldehyde: Benzaldehyde was washed with 10% Na_2CO_3 until the evolution of CO_2 ceased, then with saturated Na_2SO_4 and H_2O and dried over $CaCl_2$. The benzaldehyde was distilled under nitrogen at reduced pressure.[120]

(b) Trifluoroacetic anhydride: This solvent was purified by Mr. Abilio Ten using a standard proceedure.[121]

5.3 Buffer Solutions

5.3.1 *pH 6 citrate buffer*

0.1M Citric acid (9.5ml) and 0.1M tri-sodium citrate (40.5ml) were diluted to 100ml to give a pH6 buffer solution.[122]

5.3.2 *pH 4 and 5 buffer solutions*

These solutions were prepared according to the method described by Perrin and Dempsey.[122]

5.3.3 *pH8.3 solution*

The pH8.3 solution was 0.05M sodium citrate.

5.4 Synthesis of Phenylacetylcarbinol (PAC (3))[91]

5.4.1 *Preparation of 2-methyl-1,3-dithiane (25b)*

(25b)
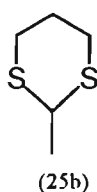
A stirred solution of acetaldehyde (3.2ml, 60mmol) and 1,3-propanedithiol (5.0ml, 48mmol) in dichloromethane (30ml) was treated with a moderate stream of HCl gas (generated from NaCl and concentrated H_2SO_4) for 1.5h. The organic phase was washed with 2M KOH (20ml), brine (20ml) and then dried over $MgSO_4$. The solvent was evaporated *in vacuo* to give a colourless liquid (2.06g,

32% yield). ^{1}H NMR (δ), 1.45, d, 3H, (J = 6.9Hz), CH$_3$; 1.97, m, 2H, CH$_2$; 2.85, m, 4H, 2xCH$_2$; 4.11, q, 1H, (J = 6.9Hz), CH. The ^{1}H NMR data is identical with the published spectrum.[123] ^{13}C NMR (δ), 20.6, CH$_3$; 24.6, CH$_2$; 30.1, 2 x SCH$_2$; 41.4, CH.

5.4.2 Preparation of 2-methyl-α--phenyl-1,3-dithiane-2-methanol (25c).[91]
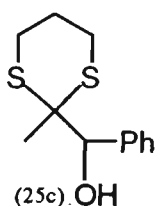
Butyl lithium (1.6M, 5ml, 8mmol) was added to the 1,3-dithiane (11b) (1.02g, 7mmol) in dry THF (10ml) at -78°C. The reaction was stirred for 2h then warmed to 20°C and stirred for a further 25h. Benzaldehyde (1ml, 9.8mmol) was added and the mixture stirred at 20°C for 45h. The reaction mixture was washed with 1M NH$_4$Cl (2 x 25ml) and saturated NaCl (2 x 25ml). The solvent was evaporated *in vacuo* to give the pure product (0.5g, 28% yield). ^{1}H NMR (δ), 1.58, s, 3H, CH$_3$; 2.00, m, 2H, CH$_2$; 3.17, m, 4H, 2xCH$_2$; 5.12, s, 1H, CH; 7.5, m, 5H, Ph.

5.4.3 Preparation of PAC (3)
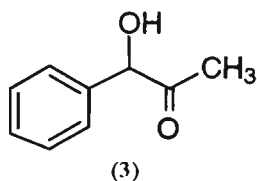
The dithiane (11c) (0.21g, 0.87 mmol) was added at 20°C to a suspension of HgCl$_2$ (0.92g, 3.4mmol) in ethanol/water (25:30, v/v) which had been adjusted to pH 7 using K$_2$CO$_3$ and the mixture heated under reflux for 5.5h. The "off-white" suspension was filtered and the collected solid thoroughly washed

with 1:1 hexane/dichloromethane. The filtrate layers were separated and the organic phase washed with 5M ammonium acetate (20ml), water (20ml) and brine (20ml). The aqueous phase was then extracted with diethyl ether (2x20ml) and the organic phases combined and dried over $MgSO_4$. The solvent was then evaporated *in vacuo* to give the product (0.08g, 64% yield). ^1H NMR (δ) (lit.[109]), 2.09 (2.06), s, 3H, CH_3; 5.11 (5.08), s, 1H, CH; 7.37 (7.33), m, 5H, Ph. ^{13}C NMR (δ),23.5, CH_3; 79.5, CH; 126.7, 128.1, 128.4, 136, Ph; 206.0, CO;.

5.4.4 *Preparation of 1-phenylpropan-1,2-diol (5)*[109]

(5)
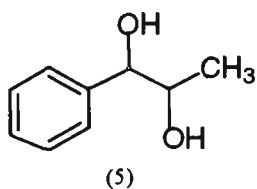
PAC (3) (0.5g, 3.3mmol) was dissolved in dry ethanol (5ml), $NaBH_4$ (0.3g, 7.9mmol) added and the mixture stirred at 20°C for 1h. The excess $NaBH_4$ was filtered off and the solution acidified with 2M HCl and then neutralised using $NaHCO_3$. The reaction mixture was extracted with dichloromethane and the solvent evaporated *in vacuo*. ^1H NMR (δ), 1.05, d, 3H, (J = 6.4Hz), CH_3; 2.62, 2.78, each m 2H, 2xOH; 3.84, dq, 1H, (J = 6.4, 7.3Hz) $CHCH_3$; 4.36, d, 1H, (J = 7.3Hz), CHPh; 7.33, 5H, Ph. The ^1H NMR data is identical with that reported in the literature.[109]

5.5 Yeast Mediated Acyloin Condensation of Benzaldehyde Using Sodium Pyruvate.

5.5.1 *Yeast mediated acyloin condensation of benzaldehyde at 20 °C.*

Sodium pyruvate (1g, 9mmol), pH6 citrate buffer (5ml) and petroleum spirit (45ml) were added to dried baker's yeast (5g) followed by benzaldehyde (0.12g, 1mmol). The reaction mixture was stirred at 20°C for 24h. GC analysis showed an 8% conversion to *l*-PAC (3) and a 21% conversion to benzyl alcohol.

5.5.2 *Reaction using sodium pyruvate and ethanol at 20 °C.*

Sodium pyruvate (2.5g, 23mmol), pH6 citrate buffer (5ml) and ethanol/petroleum spirit (0.5ml/40ml) were mixed and shaken at 20°C for 1h. Baker's yeast (5g) and benzaldehyde (0.12g, 1mmol) in petroleum spirit (5ml) were added and the reaction mixture shaken at 20°C for 24h. GC analysis indicated a 30% conversion to *l*-PAC (3). The yeast was filtered off, washed with diethyl ether and the product purified *via* flash distillation (200°C/1mm) to give *l*-PAC (3) (0.024g, 16% yield). $[\alpha]_D = -239.2°$ (c = 0.64, CHCl$_3$), (lit.[109], $[\alpha]_D = -408.7°$, c = 1.1, CHCl$_3$). IR (υ_{max} cm^{-1}), (lit.[116]) 3300-3600 (3480), bs, 3063, w, 3000, w, 1713 (1720), s, 1600, w, 1556, w, 1494, m, 1454, m, 1359, s, 1230, m, 1177, m, 1091, m, 1068, m, 1027, w, 970, w, 754, m, 701, s. ^{1}H and ^{13}C NMR were identical to the previously prepared product. *l*-PAC (3) was derivatised *via* the following method to determine the enantiomeric excess (ee). Dichloromethane (0.5ml) and freshly distilled trifluoroacetic anhydride (0.5ml) were added to a GC vial containing about 5 mg of *l*-PAC (3). The vial was capped and left at room temperature for 30min. Excess reagent was evaporated using an hotplate and dry dichloromethane added to the vial. The derivatised product was analysed using chiral GC and showed an enantiomeric ratio of 93:7 (86% ee).

5.5.3 *Reaction using sodium pyruvate and ethanol at 5°C.*

Sodium pyruvate (2.5g, 23mmol), pH6 citrate buffer (5ml), ethanol/petroleum spirit (0.5ml/40ml) were mixed and shaken at 20°C for 1h. Baker's yeast (5g) and benzaldehyde (0.13g, 1.2mmol) in petroleum spirit (5ml) were added and the reaction stirred at 5°C for 48h. GC analysis indicated a 24% conversion to *l*-PAC (3). The yeast was filtered off, washed with diethyl ether and the product purified *via* flash distillation (200°C/1mm) to give *l*-PAC (3) (0.044g, 24% yield). $[\alpha]_D = -375.8°$ (c = 1.6, CHCl$_3$) (lit.[109], $[\alpha]_D = -408.7°$, c = 1.1, CHCl$_3$). The derivatised product was analysed using chiral GC and showed an enantiomeric ratio of 95:5 (90% ee).

5.5.4 *Optimisation of reaction conditions.*

In the following, one or more of the reaction conditions employed using sodium pyruvate at 20°C was varied.

5.5.4.1 *Effect of sodium pyruvate*

(a) **With pH 6 sodium citrate buffer.** The quantity of sodium pyruvate was varied from 0.5 - 3.0g. The general reaction conditions were as follows petroleum spirit (45ml), pH6 sodium citrate buffer (5ml), ethanol (0.5ml), sodium pyruvate and benzaldehyde (1) (0.12g, 1mmol) were added to baker's yeast (5g) and the mixture was stirred at 20°C for 24h. The GC results are given in Table 5.2.

Table 5.2 The effect of sodium pyruvate (10b) on the production of *l*-PAC (3) and various other by-products from the yeast mediated acyloin condensation of benzaldehyde (1). All reactions contained 5g of yeast, 5ml buffer, 45ml petroleum spirit, 0.5ml of ethanol, 1mmol of benzaldehyde and were stirred for 24h at 20°C.

Sodium pyruvate (g)	*l*- PAC (3) (%)	Benzyl alcohol (4)
0	0	0
0.5	0	7
1	12	0
1.5	14	0
2	15	0
2.5	31	0
3	28	1

(b) **With pH 6 sodium citrate buffer and adjusting the pH to 5.45 with ammonium acetate.** In the following reactions, sodium pyruvate (1 - 2.5g) was added to pH6 sodium citrate buffer and the pH adjusted to 5.45 using ammonium acetate. Reactions were carried out with and without ethanol. General reaction conditions were as follows: sodium pyruvate was dissolved in pH6 citrate buffer (5ml) and the pH adjusted to 5.45 by adding ammonium acetate; baker's yeast (5g), petroleum spirit (45 ml) and benzaldehyde (0.12g, 1mmol) were added and the mixture stirred at 20°C for 24h. The GC results are given in Table 5.3.

Table 5.3 Effect of sodium pyruvate (1 - 2.5g/5g yeast) on the production of *l*-PAC (3) with pH6 citrate buffer and adjusting the pH to 5.45 with ammonium acetate with ethanol (0.5ml) and without ethanol at 20°C. GC results of product formation.

Sodium pyruvate (g)	Ethanol (ml)	*l*- PAC (3) (%)	Benzyl alcohol (4) (%)	Diol (5) (%)	Diketone (12) (%)
1	0.5	14	4	1	2
2	0.5	27	1	1	3
2.5	0.5	31	0	0	0
1	0	8	21	0	2
2	0	15	5	0	3
2.5	0	8	0	0	0

5.5.4.2 *Effect of ethanol used.*

(a)   **With 2.5g sodium pyruvate/g yeast.**   Sodium pyruvate (2.5g, 22.7mmol), pH6 citrate buffer (5ml) and ethanol/petroleum spirit (0.5 - 2ml/40ml) were stirred at 20°C for 1h . To this mixture was added baker's yeast (5g) and benzaldehyde (0.13g, 1.2 mmol) in petroleum spirit (5ml) and stirred at 20°C for 24h. The GC results are given in Table 5.4.

Table 5.4 The effect of ethanol (0.5 – 2ml) on the production of *l*-PAC (3) and various other by-products from the yeast mediated acyloin condensation of benzaldehyde (1). All reactions contained 5g of yeast, 2.5g sodium pyruvate, 5ml buffer, 45ml petroleum spirit, 1.2mmol of benzaldehyde and were stirred for 24h at 20°C.

Ethanol (ml)	*l*-PAC (3) (%)	Diol (5) (%)	Diketone (12) (%)
0.5	31	0	0
0.6	34	2	2
0.7	32	1	2
0.8	30	1	2
0.9	29	1	1
1	21	0	1
1.5	0	0	0
2	0	0	0

(b)   **With 2.0g sodium pyruvate/g yeast.**   Sodium pyruvate (2g, 18.2mmol), pH6 citrate buffer (5ml) and ethanol/petroleum spirit (0.5 – 0.9ml/40ml) were stirred at 20°C for 1h . To this mixture was added baker's yeast (5g) and benzaldehyde (0.13g, 1.2 mmol) in petroleum spirit (5ml) and stirred at 20°C for 24h. The GC results are given in Table 5.5.

Table 5.5 The effect of ethanol (0.5 – 0.9ml) on the production of *l*-PAC (3) and various other by-products from the yeast mediated acyloin condensation of benzaldehyde (1). All reactions contained 5g of yeast, 2g sodium pyruvate, 5ml buffer, 45ml petroleum spirit, 1.2mmol of benzaldehyde and were stirred for 24h at 20°C.

Ethanol (ml)	*l*- PAC (3) (%)	Benzyl alcohol (4) (%)
0.5	15	0
0.6	18	0
0.7	14	1
0.8	14	2
0.9	13	3

5.5.4.3 *Effect of pH of buffer solution*

In the following reactions, the pH of the buffer used to activate the yeast was varied from 4 - 8.3.

Sodium pyruvate (2.5g, 23mmol), pH (4 - 8.3) citrate buffer (5ml) and ethanol/petroleum spirit (0.5ml/40ml) were mixed and stirred at 20°C. Baker's yeast (5g) and benzaldehyde (1) (0.12g, 1mmol) in petroleum spirit (5ml) were added and the reaction mixture stirred at 20°C for 24h. The conversion to *l*-PAC (3) was determined by GC and the results are given in Table 5.6.

Table 5.6 Variation in the pH (4 - 8.3) of the buffer solution. All reactions contained 5 g of yeast, 5ml buffer, 45ml petroleum spirit, 2.5 g of sodium pyruvate (10b), 0.5ml of ethanol and 1mmol of benzaldehyde (1) and were stirred for 24h at 20°C.

pH	*l* -PAC (3) (%)
4	16
5	15
6	31
8.3	9

5.5.4.4 *Effect of baker's yeast*

(a) **With 0.8ml pH6 citrate buffer/g yeast in 45ml petroleum spirit and a constant quantity of sodium pyruvate (2.5g) and ethanol (1ml).** Sodium pyruvate (2.5g, 22.7mmol), ethanol (1ml) and petroleum spirit (45ml) were added to pH6 citrate buffer (0.8ml/g yeast) and the mixture was stirred at 20°C for 1h. Baker's yeast (5 - 10g) and benzaldehyde (0.15g, 1.4mmol) in petroleum spirit (5ml) were then added and the reaction mixture stirred at 20°C for a further 24h. The conversion to *l*-PAC (3) was determined by GC and the results are given in Table 5.7

Table 5.7 Variation in the quantity of yeast (5 - 10g) used for the acyloin condensation of benzaldehyde (1). All reactions contained 2.5g sodium pyruvate (10b), 0.8ml of pH6 citrate buffer, 0.2ml ethanol/g yeast and 1mmol of benzaldehyde (1) and were stirred for 24h at 20°C.

Yeast (g)	*l*-PAC (3) (%)	Diol (5) (%)	Diketone (12) (%)
5	25	0	0
6	32	0	0
7	31	1	1
8	32	1	2
9	27	0	1
10	21	0	0

(b) **With 1ml pH6 citrate buffer/g yeast, 0.5g sodium pyruvate/g yeast and 9ml petroleum spirit/g yeast.**

(i) **1g of baker's yeast:** Sodium pyruvate (0.5g, 4.5mmol), ethanol (0.2ml) and petroleum spirit (9ml) were added to pH6 citrate buffer (1ml) and the mixture was stirred at 20°C for 1h. Baker's yeast (1g) and benzaldehyde (0.16g, 1.5mmol) were then added and the reaction mixture stirred at 20°C for a further 24h. GC analysis showed benzaldehyde (91%) and *l*-PAC (3) (9%).

(ii)   **2g of baker's yeast:** Sodium pyruvate (1.0g, 9mmol), ethanol (0.4ml) and petroleum spirit (18ml) were added to pH6 citrate buffer (2ml) and the mixture was stirred at 20°C for 1h. Baker's yeast (2g) and benzaldehyde (0.14g, 1.3mmol) were then added and the reaction mixture stirred at 20°C for a further 24h. GC analysis showed benzaldehyde (84%) and *l*-PAC (3) (16%).

(iii)   **3g of baker's yeast:** Sodium pyruvate (1.5g, 13.6mmol), ethanol (0.6ml) and petroleum spirit (27ml) were added to pH6 citrate buffer (3ml) and the mixture was stirred at 20°C for 1h. Baker's yeast (3g) and benzaldehyde (0.14g, 1.3mmol) were then added and the reaction mixture stirred at 20°C for a further 24h. GC analysis showed benzaldehyde (78%) and *l*-PAC (3) (22%).

(iv)   **4g of baker's yeast:** Sodium pyruvate (2.0g, 18.2mmol), ethanol (0.8ml) and petroleum spirit (36ml) were added to pH6 citrate buffer (4ml) and the mixture was stirred at 20°C for 1h. Baker's yeast (4g) and benzaldehyde (0.14g, 1.3mmol) were then added and the reaction mixture stirred at 20°C for a further 24h. GC analysis showed benzaldehyde (75%) and *l*-PAC (3) (25%).

(v)   **5g of baker's yeast:** Sodium pyruvate (2.5g, 22.7mmol), ethanol (1.0ml) and petroleum spirit (45ml) were added to pH6 citrate buffer (5ml) and the mixture was stirred at 20°C for 1h. Baker's yeast (5g) and benzaldehyde (0.16g, 1.5mmol) were then added and the reaction mixture stirred at 20°C for a further 24h. GC analysis showed benzaldehyde (70%) and *l*-PAC (3) (30%).

5.5.5 *Acetaldehyde pre-treatment*

5.5.5.1 *Effect of acetaldehyde pre-treatment time*

For the following reactions, the yeast was pre-treated with acetaldehyde for various times (1 - 24h) before the addition of benzaldehyde. The results are given in Table 5.8.

Sodium pyruvate (2.5g, 23mmol), 0.05M sodium citrate (5ml) and petroleum spirit (45ml) were added to baker's yeast (5g) and then acetaldehyde (0.5ml, 9mmol) was added and the mixture stirred at 20°C (1 - 24h) prior to the addition of benzaldehyde (0.12g, 1mmol). The mixture was then stirred for 24h and the results of GC analysis are given in Table 5.8.

Table 5.8 Pre-treatment of the yeast with acetaldehyde (2) for between 1 - 24h prior to the addition of benzaldehyde (1). All reactions contained 5 g of yeast, 5ml buffer, 45ml petroleum spirit, 2.5 g of sodium pyruvate (10b), which was added prior to acetaldehyde (2) pre-treatment, and 1mmol benzaldehyde (1) and were stirred for 24h at 20°C.

pretreatment time (h)	*l* -PAC (3) (%)	Benzyl alcohol (4) (%)
1	4	0
2	4	0
3	7	0
4	7	1
5	9	1
6	8	0
12	6	0
24	4	2

5.5.5.2 *Acetaldehyde pre-treatment prior to sodium pyruvate addition*

Baker's yeast (5g), pH6 citrate buffer (5ml) and acetaldehyde/petroleum spirit mixture (0.5g, 10.8mmol/40ml) were stirred at 20°C for 24h. Sodium pyruvate (2.5g, 23mmol) was then added and the mixture was shaken at 20°C for 1h. Benzaldehyde (0.12g, 1mmol) in petroleum spirit (5ml) was then added and the mixture was stirred at 20°C a further 24h. GC analysis showed a 30% conversion to *l*-PAC (3).

5.5.5.3 *Acetaldehyde pre-treatment with a reaction temperature of 5°C*

Baker's yeast (5g), pH6 citrate buffer (5ml) and acetaldehyde/petroleum spirit mixture (0.5g, 10.8mmol/40ml) were stirred at 20°C for 3h. Sodium pyruvate (2.5g, 23mmol) was then added and the mixture was shaken at 20°C for 1h. Benzaldehyde (0.12g, 1mmol) in petroleum spirit (5ml) was then added and the mixture was stirred at 5°C for 48h. GC analysis showed a 20% conversion to *l*-PAC (3). The baker's yeast was filtered, washed with diethyl ether and the product purified by flash distillation (200°C/1mm) to give *l*-PAC (3) (0.018g, 10% yield). $[\alpha]_D$ = -208.8° (c = 1.75, $CHCl_3$) (lit.[109], $[\alpha]_D$ = -408.7°, c = 1.1, $CHCl_3$). The derivatised product was analysed using chiral GC and showed an enantiomeric ratio of 91:9 (82% ee).

5.5.6 *Slow addition of benzaldehyde*

Sodium pyruvate (2.54g, 23mmol), pH6 citrate buffer (5ml) and ethanol/petroleum spirit (0.5ml/40ml) were stirred at 20°C for 1.75h and baker's yeast (5g) added. Benzaldehyde (1mmol)/petroleum spirit (10ml) mixture was slowly added (0.025ml/min) to the reaction mixture over a period of 7h at 20°C. The reaction was stirred at 20°C for a further 17h. GC analysis showed benzaldehyde (1) (93%) and *l*-PAC (3) (7%).

5.5.7 *Addition of benzaldehyde in batches*

Sodium pyruvate (2.54g, 23mmol), pH6 citrate buffer (5ml) and ethanol/petroleum spirit (0.5ml/40ml) were stirred at 20°C for 1.75h and baker's yeast (5g) added. Benzaldehyde (0.05g, 0.5mmol) in 2.5ml petroleum spirit was then added and the reaction mixture stirred at 20°C for 6h. An equivalent amount of benzaldehyde (0.05g, 0.5mmol) in 2.5ml petroleum spirit was further added and the reaction mixture stirred at 20°C for a total of 24h. GC analysis showed benzaldehyde

(1) (86%) and *l*-PAC (3) (14%) after 6h and no further increase in *l*-PAC (3) after 24h.

5.6 Yeast Mediated Acyloin Condensation of Benzaldehyde Using Pyruvic Acid

5.6.1 *Reaction time*

Pyruvic acid (0.23g, 3.4mmol) was added to 0.05M sodium citrate (5ml) and the pH adjusted to 5.45 with ammonium acetate. This solution and ethanol (0.5ml) in petroleum spirit (40ml) were added to baker's yeast (5g). Benzaldehyde (0.13g, 1.2mmol) in petroleum spirit (5ml) was then added and the reaction mixture stirred at 20°C for 72h. The reaction mixture was analysed by GC, after 24h (*l*-PAC (3), 12%; benzyl alcohol (4), 22%) and after 72h *l*-(PAC (3), 12%; benzyl alcohol (4), 25%; diol (5), 1%; diketone (20), 1%).

5.6.2 *Effect of pyruvic acid*

The concentration of pyruvic acid was varied and the pH adjusted to 5.45 using the following methods.

5.6.2.1 *0.05M Sodium citrate with the pH adjusted using ammonium acetate*

Pyruvic acid (0.05g - 1.25g, 0.6 - 14.2mmol) was dissolved in 0.05M sodium citrate (5ml) and the pH adjusted to 5.45 using ammonium acetate. This solution, (5ml) along with ethanol (1ml) in petroleum spirit (40ml), were added to baker's yeast. Benzaldehyde (0.15g, 1.4mmol) in petroleum spirit (5ml) was then added and

the reaction mixture stirred at 20°C for 24h. The results of GC analysis are given in Table 5.9.

Table 5.9 Variation in the amount of pyruvic acid (10) added to the reaction. All reactions contained 5g of yeast, 5ml 0.05M sodium citrate containing pyruvic acid (10) with the pH adjusted to 5.45. using ammonium acetate, 1ml ethanol,45ml petroleum spirit and 1.4mmol benzaldehyde (1) and were stirred for 24h at 20°C.

Pyruvic acid (g)	l -PAC (3) (%)	Benzyl alcohol (4) (%)
0.05	6	20
0.11	10	24
0.15	10	24
0.2	15	9
0.3	18	0
0.5	15	0
0.75	2	0
1	1	0
1.25	0	0

5.6.2.2 *Water with the pH adjusted to 5.45 using sodium citrate*

Pyruvic acid (0.05g - 0.25g, 0.6 - 2.8mmol) was dissolved in water (5ml) and the pH adjusted to 5.45 using sodium citrate. This solution, (5ml) along with ethanol (1ml) in petroleum spirit (40ml), were added to baker's yeast. Benzaldehyde (0.14g, 1.3mmol) in petroleum spirit (5ml) was then added and the reaction mixture and stirred at 20°C for 24h. The results of GC analysis are given in Table 5.10.

Table 5.10 Variation in the amount of pyruvic acid (10) added to the reaction. All reactions contained 5g of yeast, 5ml water containing pyruvic acid (10) with the pH adjusted to 5.45. using sodium citrate, 1ml ethanol, 45ml petroleum spirit and 1.3mmol benzaldehyde (1) and were stirred for 24h at 20°C.

Pyruvic acid (g)	l -PAC (3) (%)	Benzyl alcohol (4) (%)
0.05	1	84
0.1	0.5	91
0.15	8	50
0.2	15	12
0.25	19	5

5.6.2.3 *Water with the pH adjusted to 5.45 using ammonium acetate*

Pyruvic acid (0.05g - 0.26g, 0.6 - 3mmol) was dissolved in water (5ml) and the pH adjusted to 5.45 using ammonium acetate. This solution (5ml) along with ethanol (1ml) in petroleum spirit (40ml), was added to baker's yeast. Benzaldehyde (0.14g, 1.3mmol) in petroleum spirit (5ml) was then added and the reaction mixture stirred at 20°C for 24h. The results of GC analysis are given in Table 5.11.

Table 5.11 Variation in the amount of pyruvic acid (10) added to the reaction. All reactions contained 5g of yeast, 5ml water containing pyruvic acid (10) with the pH adjusted to 5.45 using ammonium acetate, 1ml ethanol, 45ml petroleum spirit and 1.3mmol benzaldehyde (1) and were stirred for 24h at 20°C.

Pyruvic acid (g)	*l* -PAC (3) (%)	Benzyl alcohol (4) (%)
0.05	7	41
0.1	12	27
0.15	15	9
0.2	15	2
0.26	13	0

5.6.3 Reactions at 5°C

5.6.3.1 *Reaction with pyruvic acid*

Pyruvic acid (0.5g, 6mmol) was dissolved in 0.05M sodium citrate (5ml) and the pH adjusted to 5.45 with ammonium acetate. This solution was then added to ethanol/petroleum spirit (0.5ml/40ml), baker's yeast (5g) and benzaldehyde (0.12g, 1mmol) in petroleum spirit (5ml) and the mixture was stirred at 5°C for 48h. The mixture was sampled after 24h and 48h. GC analysis showed a 30% conversion of benzaldehyde (1) to *l*-PAC (3) after both 24h and 48h and the inclusion of diol (5) (1%) and diketone (20) (1%) after 48h.

5.6.3.2 *Effect of yeast with pyruvic acid*

Pyruvic acid (1g, 12mmol) was dissolved in 0.05M sodium citrate (10ml) and the pH adjusted to 5.45 with ammonium acetate. This solution was then added to ethanol/petroleum spirit (1.0ml/80ml), baker's yeast (10g) and benzaldehyde (0.12g, 1mmol) and the mixture was stirred at 5°C for 24h. GC analysis showed a 30% conversion of benzaldehyde (1) to *l*-PAC (3). The mixture was then filtered and the baker's yeast washed with diethyl ether. The combined organic layers were washed with 10% sodium carbonate, the aqueous layer was extracted with ether, the combined organic layers dried over sodium sulphate and the solvent evaporated *in vacuo*. The product was separated (petroleum spirit 40-60/diethyl ether, 50:50 mixture) using radial chromatography and purified by flash distillation (200°C/1mm) to give *l*-PAC (3) (0.015g, 20% yield). $[\alpha]_D$ = -262.6°, (c = 0.745, CHCl$_3$), (lit.[109], $[\alpha]_D$ = -408.7°, c = 1.1, CHCl$_3$). The derivatised product was analysed by chiral GC and showed an enantiomeric ratio of 95:5 (90% ee). ^{1}H and ^{13}C NMR spectra were identical to those previously recorded.

5.6.3.3 *Reaction without pyruvic acid*

To the sodium citrate/acetic acid mixture (5ml), at pH5.5, ethanol/petroleum spirit (0.5/40ml), baker's yeast (5g) and benzaldehyde/petroleum spirit (1mmol/5ml) were added and the mixture was stirred at 5°C for 24h. GC revealed only benzyl alcohol (4).

5.6.4 *Acetaldehyde pre-treatment*

5.6.4.1 *Reaction at 20°C*

(a) **With pH adjustment.** Pyruvic acid (0.6g, 6mmol) was mixed in 0.05M sodium citrate (5ml) and the pH adjusted 5.45 with ammonium acetate. Baker's yeast (5g) and acetaldehyde (0.56g, 13mmol) in petroleum spirit (40ml) were added and the mixture shaken at 20°C for 3h. Ethanol (0.5ml) and benzaldehyde (0.13g, 1.2mmol) in petroleum spirit (5ml) were then added and the mixture was then shaken at 20°C for 24h. GC analysis of the reaction mixture showed only benzaldehyde (1).

(b) **Without pH adjustment.** Water (4ml) and petroleum spirit (45ml) were added to baker's yeast (5g) and the mixture stirred at 20°C. Pyruvic acid (2.0ml, 28.8mmol), acetaldehyde (0.29g, 6.6mmol) and benzaldehyde (0.17g, 1.6mmol) were then added and the mixture stirred at 20°C for 24h. GC analysis after this time showed only benzaldehyde (1).

5.6.4.2 *Acetaldehyde pre-treatment at a reaction temperature of 5°C*

Pyruvic acid (0.52g, 6mmol) was dissolved in 5ml of 0.05M sodium citrate. Baker's yeast (5g) and acetaldehyde (0.56g, 13mmol) in petroleum spirit (40ml) were added and the mixture was shaken at 20°C for 3h. Ethanol (0.5ml) and benzaldehyde (0.13g, 1.2mmol) in petroleum spirit (5ml) were added and the reaction mixture was then stirred at 5°C for 24h. GC analysis of the reaction mixture revealed only benzaldehyde (1).

5.7 ^{13}C NMR Studies

5.7.1 *2-^{13}C sodium pyruvate*

2-^{13}C Sodium pyruvate (0.52g, 5mmol), pH6 citrate buffer (1ml) and ethanol/petroleum spirit (0.1ml/8ml) were stirred together at 20°C for 1h. Baker's yeast (1g) and benzaldehyde (0.3mmol) were added and the reaction mixture was stirred at 5°C for 48h. GC analysis of the reaction mixture showed *l*-PAC (3) (37%). The yeast was removed and excess solvent evaporated. ^{13}C NMR of the residue demonstrated incorporation of ^{13}C into the *l*-PAC (3). ^{13}C NMR (δ), 206.41, CO. ^{1}H NMR (δ), 2.2, s, 3H, CH$_3$; 5.11, d, (J$_{C-H}$ = 3Hz) 1H, CH; 7.37, m, 5H, Ph.

5.7.2 *1-^{13}C ethanol*

Sodium pyruvate (2.5g, 23mmol), pH6 citrate buffer (5ml) and 1-^{13}C ethanol/petroleum spirit (0.5ml/40ml) were stirred together at 20°C for 0.5h. To this solution was added baker's yeast (5g) and benzaldehyde (0.18g, 1.7mmol) and the reaction mixture stirred at 5°C for 24h. GC analysis of the reaction mixture showed *l*-PAC (3) (44%). Following evaporation of the solvent, ^{13}C NMR of the residue failed to demonstrate any incorporation of ^{13}C into the *l*-PAC (3).

5.7.3 *Reactions with [carbonyl-^{13}C] benzaldehyde*

For all of the following reactions a 10mm NMR tube containing the reaction mixture was fitted with a vortex plug and placed in a Bruker 500MHz NMR spectrometer operating at 125.72 MHz for ^{13}C at temperatures of 5°C, 10°C or 20°C. The tube was spun at 20Hz 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 recorded at hourly intervals over a period of 68h in order to observe the conversion of substrate to product. Each accumulated data set was Fourier transformed after application of line broadening of 1Hz.

The intensity of the product peak (80ppm) was measured relative to the benzene-d_6 and the values were plotted using Microsoft Excel $^{®}$.

5.7.3.1 *Reaction at 5°C*

The following materials were added to a 10mm NMR tube: baker's yeast (0.1g), a pH5.45 solution of pyruvic acid (0.1mmol) in citrate buffer (0.1ml), petroleum spirit (2.5ml), ethanol (10µl) and benzene-d_6 (120µl) as internal standard and as a lock substance. The [*carbonyl*-^{13} C] benzaldehyde (3.0µl) was then added and the reaction mixture was maintained at 5°C for 68h. ^{13}C NMR showed a gradual decrease of the benzaldehyde carbonyl peak and a corresponding gradual increase of the C1 peak of the *l*-PAC (3) at 80ppm. The supernatant was then decanted and analysed using GC to show a 26% conversion of benzaldehyde (1) to *l*-PAC (3).

5.7.3.2 *Reaction at 10°C*

The following materials were added to a 10mm NMR tube: baker's yeast (0.1g), a pH5.45 solution of pyruvic acid (0.1mmol) in citrate buffer (0.1ml), petroleum spirit (2.5ml), ethanol (10µl) and benzene-d_6 (120µl) as internal standard and as a lock substance. The [*carbonyl*-^{13} C] benzaldehyde (3.0µl) was then added and the reaction mixture was maintained at 10°C for 68h. ^{13}C NMR showed a gradual decrease of the benzaldehyde carbonyl peak and a corresponding gradual increase of

the C1 peak of the *l*-PAC (3) at 80ppm. The supernatant was then decanted and analysed using GC to show a 17% conversion of benzaldehyde (1) to *l*-PAC (3).

5.7.3.3 *Reaction at 20°C*

The following materials were added to a 10mm NMR tube: baker's yeast (0.1g), a pH5.45 solution of pyruvic acid (0.1mmol) in citrate buffer (0.1ml), petroleum spirit (2.5ml), ethanol (10μl) and benzene-d_6 (120μl) as internal standard and as a lock substance. The [*carbonyl*-^{13}C]benzaldehyde (3.0μl) was then added and the reaction mixture was maintained at 5°C for 68h. ^{13}C NMR showed a gradual decrease of the benzaldehyde carbonyl peak and a corresponding gradual increase of the C1 peak of the *l*-PAC (3) at 80ppm. The supernatant was then decanted and analysed using GC to showing 12% *l*-PAC (3), 6% benzyl alcohol (4) and 5% by-products ((5) and (12)).

5.7.3.4 *Reaction at 5°C with sodium pyruvate*

Sonication was used to dissolve sodium pyruvate (50mg) in pH6 citrate buffer (0.1ml) in a 10mm NMR tube. Baker's yeast (0.1g), petroleum spirit (2.5ml), ethanol (10μl) and benzene-d_6 (120μl), as internal standard and as a lock substance were each added to the tube. The [*carbonyl*-^{13}C] benzaldehyde (3.0μl), was then added and the reaction mixture was held at 5°C for 68h. ^{13}C NMR showed a gradual decrease of the benzaldehyde carbonyl peak and a corresponding gradual increase of the C1 peak of the *l*-PAC (3) product at 80ppm. The supernatant was then decanted and analysed using GC to show both *l*-PAC (3) (13%) benzyl alcohol (4) (1%) and diol (5) (0.5%).

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APPENDIX

YEAST-BASED PROCESS FOR PRODUCTION OF L-PAC

This invention relates to organic compounds
useful as precursors for the synthesis of a variety of
products, particularly for synthesis of compounds useful as
pharmaceutical agents. The method of the invention
utilises yeast-mediated catalysis in organic solvents, and
in particular the yeast-mediated condensation between
pyruvate and a substituted aromatic aldehyde to yield the
corresponding acyloin (hydroxy ketone) compound. In a
preferred embodiment, the reaction is that between pyruvate
and benzaldehyde to yield phenylacetylcarbinol, the
precursor to ephedrine, in high enantiomeric purity.

BACKGROUND OF THE INVENTION

Physicochemical methods for production of
enantiomerically pure compounds usually involve multi-step
synthesis incorporating one or more steps which are
asymmetric, and laborious purification procedures. Such
methods are not only tedious, but frequently provide
relatively poor yields. Alternatively enantiomerically-
pure starting materials can be used, together with
enantioselective reaction steps; however, such pure
starting materials are available only for a very limited
number of desired compounds.

In an attempt to overcome the difficulties of
using traditional organic chemical methods, biological
systems have been intensively investigated. Such systems
show a very high degree of stereoselectivity in their
reactions, and therefore microbiological, enzymatic or
chemoenzymatic reactions for achieving specific reaction
steps with a variety of reagents have been attempted. For
example, microorganisms of a number of genera have been
proposed for synthesis of optically active α-substituted
derivatives of 3-hydroxypropionic acid for use as
intermediates in the synthesis of compounds such as
α-tocopherol, muscones and pharmaceutical, insecticidal and

agricultural chemical agents (U.S. Patent No. 4734367 by Hoffman-La Roche, Inc.). Most such procedures use whole-cell fermentation systems in aqueous media, or isolated enzymes with a specific desired activity. However, fermentation systems present the disadvantage that purification of the desired product can be difficult, and yields tend to be low; while the yield and convenience of the reaction can be improved by utilising immobilised cells, or cells which have been selected or genetically modified, this adds significantly to the cost of the process. The use of purified enzymes is normally prohibitively expensive, and again without the use of immobilised enzyme the yield tends to be low and purification difficult.

In recent years, intense efforts have been directed towards development of methods which are highly selective, provide a good rate of transformation, and enable easy, non-chromatographic separation and purification of the product. It would be particularly desirable if reactions could be carried out in organic solvents, since these are particularly convenient for large scale reactions and purifications.

It has been shown that dry baker's yeast is able to effect non-fermentative reduction of α-keto esters in organic solvents such as hexane or benzene, to produce the corresponding α-hydroxy esters with good yield and selectivity (Nakamura et al, 1988; Nakamura et al, 1990; Nakamura et al, 1991; Nakamura et al, 1993); reduction of β-keto esters in petroleum ether, diethyl ether, toluene, carbon tetrachloride and petrol has also been demonstrated (Jayasinghe et al, 1993; Jayasinghe et al, 1994; North, 1996). Although initially it was thought that immobilisation of yeast, for example in polyurethane, was essential in order to maintain stability of cell membrane-bound coenzymes for the dehydrogenases and reductases which catalyse the reaction (Nakamura et al, 1988; Nakamura et al, 1990), it was subsequently found that the addition of a

very small proportion of water to the organic system would avoid the need for immobilisation (Nakamura et al, 1991).

Ephedrine (α-[1-(methylamino)ethyl]benzene-methanol), originally isolated from plants of the genus *Ephedra*, occurs as the naturally-occurring isomers *l*-ephedrine and *d*-pseudoephedrine, and other pharmacologically active isomers include *d*-ephedrine and *l*-pseudoephedrine. These compounds are adrenergic sympathomimetic agents and have antihistamine activity; *l*-ephedrine is widely used as a bronchodilator, while *d*-pseudoephedrine is widely used as a decongestant. Compounds of these groups are present in a very wide range of prescription and over-the-counter pharmaceutical formulations.

The production of *l*-phenylacetylcarbinol, a precursor of *l*-ephedrine, by catalysis using whole baker's yeast cells in aqueous medium was one of the first microbial biotransformation processes to be used commercially (Neuberg and Hirsch, 1921; see also Hildebrandt and Klavehn, 1934). This reaction involves the yeast-induced condensation of benzaldehyde with acetyl-coenzyme A. The reaction has been widely investigated, and has been shown to be mediated by the enzyme pyruvate decarboxylase (Groger, Schmander and Mothes, 1966). It has also been shown that the reaction has a relatively broad specificity for the substrate, enabling a variety of substituted aromatic aldehydes to be converted to the corresponding substituted optically-active phenylacetylcarbinols (Long, James and Ward, 1989).

Although this yeast-catalysed system has been widely exploited, this has normally utilised aqueous systems, which are inconvenient for large-scale extraction and purification, which require organic solvents. Additionally, fermentation systems present the disadvantage that purification of the desired product can be difficult, and yields tend to be low; while the yield and convenience of the reaction can be improved by utilising immobilised

cells, or cells which have been selected or genetically modified, this adds significantly to the cost of the process. The use of purified enzymes is normally prohibitively expensive, and again without the use of immobilised enzyme the yield tends to be low and purification difficult.

We have now surprisingly found that yeast-mediated acyloin condensation of benzaldehyde can be achieved in an organic solvent using non-fermenting yeast, and that addition of a small proportion of ethanol to the reaction mixture suppresses formation of undesired side-products. Even more surprisingly, by performing the reaction at reduced temperature, an even greater reduction of side-reactions can be achieved, without loss of catalytic activity. The effect of reduction in temperature appears to be generally applicable to both aqueous and non-aqueous systems utilising a non-fermenting yeast.

Although Ward and co-workers have carried out investigations using whole cell yeast biotransformation in two-phase organic systems with a water content of at least 10% (Nikolova and Ward, 1991; 1992a; 1992b; Ward, 1995), the yields of phenylacetylcarbinol were low, and the levels of side-products were unacceptably high.

The first description of the synthesis of 1-ephedrine was contained in a patent by Hildebrandt and Klavehn (1934) and made use of the discovery by Neuburg and Hirsch (1921) that fermenting strains of *Saccharomyces cerevisae* in aqueous systems would convert benzaldehyde to phenylacetylcarbinol. The yield of the carbinol was typically about 18%, and significant amounts of both benzyl alcohol and benzoic acid were obtained as side-products.

In a preferred embodiment, yields of around 24% with the almost total absence of side-products were obtained using the method of the invention.

SUMMARY OF THE INVENTION

In a first aspect, the invention provides a method of synthesis of a substituted carbinol compound, comprising the step of subjecting the corresponding substituted aromatic aldehyde to acyloin condensation mediated by a yeast in an organic solvent under non-fermenting conditions, in the presence of an aliphatic alcohol or aliphatic aldehyde.

Any yeast capable of effecting reduction may be used. It is economically advantageous to use the cheapest yeast available, and ordinary baker's yeast, *Saccharomyces cerevisiae*, is preferred. Strains of yeast adapted to other purposes, including brewing yeast and wine or sherry yeasts could also be employed. Strains specifically adapted to an organic solvent environment or for enhanced reduction efficiency may be used; such strains include conventionally-selected and genetically modified strains. For maximum efficiency of reaction, it is advisable to present the maximum surface area of yeast for contact with the reactants. This can be effected by using "active" dried yeast, which is readily commercially available as "instant dry yeast", and may be stored at room temperature. Alternatively, well-pulverised dry baker's yeast may be used. Other yeasts, such as those described in U.S. Patent No. 4734367, or fungi such as those disclosed in Chênevert *et al* (1992) may also be used. The person skilled in the art will readily be able to test whether any specific organism will function for the purposes of the invention, using the methods described herein.

Preferably the aliphatic alcohol or aliphatic aldehyde is ethanol or acetaldehyde, suitably 0.1 ml per g yeast. This results in a significant increase in the yield of carbinol, and reduces the amount of aromatic alcohols produced as a side-reaction. Ethanol is preferred, since this results in superior conversion of the aromatic aldehydes to the desired carbinol, and lower yield of undesired reduction product. Without wishing to be

bound by any particular theory, it is believed that the
ethanol or acetaldehyde provides an alternative substrate
for the reductase enzymes thus inhibiting the formation of
side products such as benzyl alcohol. Therefore, it is
5   predicted that other aliphatic alcohols or aliphatic
aldehydes could perform the same function.

Although the reaction can be performed at ambient
temperature, suitably 16-24°C, preferably 20°C, we have
surprisingly found that significantly better results are
10   obtained at lower temperatures, in the range 0-5°C. The
reason for the improved performance and further reduction
of side-reactions which is observed is not presently
understood; however, we have observed that the activity of
the yeast at these reduced temperatures is comparable to
15   that at ambient temperature. This result is particularly
surprising, because it would normally be expected that a
yeast-mediated reaction would demonstrate a temperature
optimum at ambient or slightly elevated temperature,
although Shiu and Rogers (1996), have shown that isolated
20   pyruvate decarboxylase, the enzyme involved in the acyloin
condensation reaction, exhibits increased activity at 4°C.

The solvent may be any suitable organic solvent
of low or moderate polarity, such as petroleum ether,
carbon tetrachloride, hexane and other hydrocarbons,
25   diethyl ether, toluene, or benzene. We have shown that
reduction of ethyl acetoacetate (another yeast-mediated
reaction) can be achieved in good yields and with high
enantioselectivity in a range of mixed organic solvent
systems, including 2-ethoxyethanol/diethyl ether,
30   pyridine/carbon tetrachloride, chloroform/petroleum ether
and ethyl acetate/toluene. We have found that petroleum
ether is especially suitable, and has the advantage of low
cost. We have found that in the yeast-mediated reduction
of ethyl acetoacetate, baker's yeast retains its reducing
35   activity when 1 to 30% v/v of a polar solvent is added to a
non-polar organic solvent, and this is also expected to be
the case in the current invention, because the crucial

factor in the choice of the solvent is whether the yeast remains active which is not dependent on the particular reaction concerned. Preferably 1 to 5% of polar solvent is added. The polar solvent is preferably chloroform, dichloromethane, methyl ethyl ketone, or methyl isobutyl ketone; the best results for mixed solvents were obtained with chloroform or dichloromethane in petroleum ether. In general, a single solvent is preferred.

It is known that in order to preserve functioning of an enzyme in an organic solvent environment it is necessary for the enzyme to be fully hydrated by being surrounded by a few layers of water molecules. This requirement is satisfied by providing a ratio of 0.6 to 1.2 ml water/g of yeast, preferably 1.0 ml water/g of yeast. This results in a single phase organic system as all of the water is absorbed into the yeast. A two-phase system reduces the yield of the product and makes isolation of the product considerably more difficult.

Once the yeast-mediated reaction has been completed, the yeast can readily be separated from the reaction mixture by filtration and washing. The reaction mixture, comprising product, unreacted starting material, solvent and minor impurities, is subjected to conventional purification, for example by flash distillation, to yield the purified product. Optionally the yeast can be extracted with an organic solvent such as ethyl acetate to yield a marginal amount of further product.

In a preferred embodiment, the invention provides a method for yeast-mediated conversion of benzaldehyde to phenylacetylcarbinol, according to the following reaction:
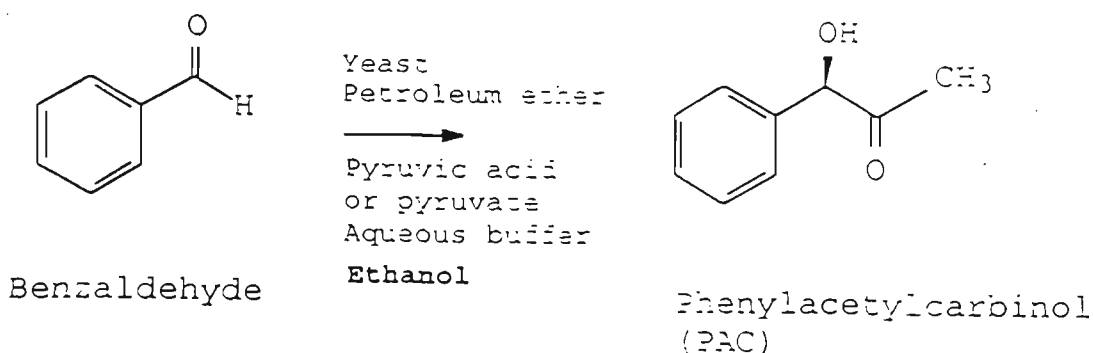
It will be clearly understood that the benzaldehyde, the pyruvic acid, or both may optionally be substituted, and that pyruvate, for example sodium pyruvate, may be used as an alternative to pyruvic acid. However, preferably pyruvic acid is used, since the sodium pyruvate is more expensive, and we have found that one quarter as much pyruvic acid is required compared to pyruvate. Aromatic aldehydes substituted with alkyl, aryl, halo, nitro, hydroxy, alkoxy, amino, carbonyl, thioxy or thioalkoxy groups or composites of these groups may also be used instead of benzaldehyde.

For either sodium pyruvate or pyruvic acid, the pH of the pyruvate/citrate buffer solution is preferably between 5 and 6, more preferably pH 5.45. Between 0.6 and 1.2 ml buffer/g of yeast should preferably be used for optimal results.

While the ratio of yeast to substrate will vary depending on the individual system, and is readily determined experimentally using routine trial and error methods, we have found that for the conversion of benzaldehyde to phenylacetylcarbinol the optimum ratio is 5 g yeast/mmol benzaldehyde; increasing the amount of yeast results in only a small increase in conversion, and lower amounts of yeast provide lower conversion.

Similarly, the optimum reaction time may readily be determined, and for the benzaldehyde-phenylacetyl-carbinol system we have investigated reaction times from 12 to 72 hours, and have found that when the reaction is continued for longer than 24 hours there is very little

improvement in conversion, and that there is an increase in production of by-products.

In a particularly preferred embodiment, production of undesired side-products is reduced by performing the catalysis reaction at below ambient temperature. Preferably the temperature is 0-5°C.

In a second aspect, the invention provides a method of synthesis of a substituted carbinol compound comprising the step of subjecting the corresponding substituted aromatic aldehyde to acyloin condensation mediated by a yeast in an organic solvent under non-fermenting conditions, in which the reaction mixture comprises water sufficient to activate the yeast but not to form a two phase system.

In a third aspect, the invention provides a method of synthesis of a substituted carbinol compound comprising the step of subjecting the corresponding substituted aromatic aldehyde to acyloin condensation mediated by a yeast in an organic solvent under non-fermenting conditions, in which the reaction is performed at 0-10°C.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of reference only to the following non-limiting examples.

Example 1   Yeast-Mediated Acyloin Condensation of
                   Benzaldehyde Using Pyruvic Acid

Pyruvic acid buffer was prepared by dissolving pyruvic acid (10.44 g, 119 mmol) in 100 ml of 0.05 M sodium citrate. Ammonium acetate was added to give a pH of 5.45. Benzaldehyde (106 mg, 1 mmol), petroleum ether (80 ml), ethanol (0.5 ml), baker's yeast (5 g) and the pyruvic acid buffer (5 ml) were stirred at 5°C for 24 h. The mixture was then filtered and the yeast washed with diethyl ether. The combined organic layers were then washed with 10%

sodium carbonate. After removal of the solvent in vacuo
the product was purified by flash distillation (200°C/1 mm)
to give phenylacetylcarbinol (30 mg, 20% yield). Gas
chromatography (GC) of the product showed pure PAC
(11.88 min.). Chiral GC showed a ratio of 95:5, 90% ee.

$[\alpha]_D$ = -262.6 (c = 0.745, CHCl$_3$). ^{1}H NMR (CDCl$_3$)
δ 7.33-7.52, M, Ph; δ5.12, s, CH; δ 2.06, s, CH$_3$.

10   Example 2   Yeast-Mediated Acyloin Condensation of
                     Benzaldehyde Using Sodium Pyruvate
        9.5 ml of 0.1 M citric acid and 40.5 ml of 0.1 M
tri-sodium citrate were diluted to 100 ml to give a pH 6
citrate buffer solution. (Ref.: Buffers for pH and Metal
15 Ion Control, D.D. Perrin and B. Dempsey, Publ. John Wiley
and Sons, pg 103). Petroleum spirit (40 ml), ethanol
(0.5 ml), the pH 6 citrate buffer (5 ml) and sodium
pyruvate (2.5 g, 23 mmol) were stirred at room temperature
for 1 h. Benzaldehyde (127 mg, 1.2 mmol) and baker's yeast
20 (5 g) were then added and the reaction stirred at 5°C for
24 h. The mixture was then filtered and yeast washed with
diethyl ether. After removal of the solvent in vacuo the
product was purified by flash distillation (200°C/1 mm) to
give phenylacetylcarbinol (44 mg, 24%). The GC of the
25 product showed pure PAC (11.88 min.). Chiral GC showed a
ratio of 97.5:2.5, 95% ee.

$[\alpha]_D$ = -375.8 (c = 1.6, CHCl$_3$). ^{1}H NMR (CDCl$_3$)
δ 7.33-7.52, M, Ph; δ5.12, s, CH; δ 2.06, s, CH$_3$.

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        It will be apparent to the person skilled in the
art that while the invention has been described in some
detail for the purposes of clarity and understanding,
various modifications and alterations to the embodiments
35 and methods described herein may be made without departing
from the scope of the inventive concept disclosed in this
specification.

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following pages, and are incorporated herein by this
reference.

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CLAIMS

1.   A method of synthesis of a substituted carbinol compound, comprising the step of subjecting the corresponding substituted aromatic aldehyde to acyloin condensation mediated by a yeast in an organic solvent under non-fermenting conditions, in the presence of an aliphatic alcohol or aliphatic aldehyde.

2.   A method according to Claim 1, in which the yeast is *Saccharomyces cerevisiae*.

3.   A method according to Claim 1 or Claim 2, in which the yeast is of a strain specifically adapted to an organic solvent environment or for enhanced reduction efficiency.

4.   A method according to any one of Claims 1 to 3, in which the yeast is active dried yeast.

5.   A method according to any one of Claims 1 to 4, in which the aliphatic alcohol or aliphatic aldehyde is ethanol or acetaldehyde respectively.

6.   A method according to claim 5, in which the proportion of ethanol or acetaldehyde is 0.1 ml/g yeast.

7.   A method according to Claim 5 or Claim 6, in which the aliphatic alcohol or aliphatic aldehyde is ethanol.

8.   A method according to any one of Claims 1 to 7, in which the reaction is performed at ambient temperature.

9.   A method according to any one of Claims 1 to 8, in which the reaction is performed at 16-24°C.

10.   A method according to Claim 9, in which the reaction is performed at 20°C.

11.   A method according to any one of Claims 1 to 7, in which the reaction is performed at 0-10°C.

12.   A method according to any one of Claims 1 to 11, in which the reaction is performed in an organic solvent selected from the group consisting of petroleum ether, carbon tetrachloride, hexane and other hydrocarbons, diethyl ether, toluene, and benzene.

13.   A method according to any one of Claims 1 to 12, in which the reaction is performed in petroleum ether.

14.   A method according to any one of Claims 1 to 11, in which the reaction is performed in a mixed organic solvent system.

15.   A method according to Claim 14, in which the mixed organic solvent system is selected from the group consisting of 2-ethoxyethanol/diethyl ether, pyridine/carbon tetrachloride, chloroform/petroleum ether and ethyl acetate/toluene.

16.   A method according to any one of Claims 1 to 15, in which 1 to 30% v/v of a polar solvent is added to a non-polar organic solvent.

17.   A method according to any one of Claims 1 to 16, in which 1 to 5% of polar solvent is added.

18.   A method according to Claim 17, in which the polar solvent is selected from the group consisting of chloroform, dichloromethane, methyl ethyl ketone, and methyl isobutyl ketone.

19.   A method according to Claim 18, in which the reaction is performed in chloroform or dichloromethane in petroleum ether.

20.   A method according to any one of Claims 1 to 19, in which the reaction mixture comprises water sufficient to activate the yeast but not to form a two phase system.

21.   A method according to Claim 20, in which the reaction mixture comprises water at a ratio of 0.6 to 1.2 ml water/g of yeast.

22.   A method according to Claim 1, in which the reaction is conversion of an optionally-substituted aromatic aldehyde to the corresponding acyloin compound by condensation with an optionally-substituted pyruvate compound.

23.   A method according to Claim 22, in which the aromatic aldehyde substituted with one or more groups selected from the group consisting of alkyl, aryl, halo,

nitro, hydroxy, alkoxy, amino, carbonyl, thioxy or thioalkoxy substituted with alkyl, aryl, halo, nitro, hydroxy, alkoxy, amino, carbonyl, thioxy or thioalkoxy alkyl, aryl, halo, nitro, hydroxy, alkoxy, amino, carbonyl,
5 thioxy and thioalkoxy.

24. A method according to Claim 22 or Claim 23, in which the reaction is conversion of benzaldehyde to phenylacetylcarbinol.

25. A method according to any one of Claims 22 to 24,
10 in which the pyruvate compound is pyruvic acid.

26. A method according to Claim 25, in which the reaction is performed at a pH of between 5 and 6.

27. A method according to any one of Claims 22 to 24, in which the pyruvate compound is sodium pyruvate.

15 28. A method according to Claim 27, in which the reaction is performed at a pH of between 5 and 8.

29. A method according to any one of Claims 1 to 31, in which the reaction time is 12 to 24 hours.

30. A method of synthesis of a substitute carbinol
20 compound comprising the step of subjecting the corresponding substituted aromatic aldehyde to acyloin condensation mediated by a yeast in an organic solvent under non-fermenting conditions, in which the reaction mixture comprises water sufficient to activate the yeast
25 but not to form a two phase system.

31. A method of synthesis of a substitute carbinol compound comprising the step of subjecting the corresponding substituted aromatic aldehyde to acyloin condensation mediated by a yeast in an organic solvent
30 under non-fermenting conditions, in which the reaction is performed at 0-10°C.

ABSTRACT

This invention relates to yeast-mediated catalysis in organic solvents, and in particular the yeast-mediated condensation between pyruvate and a substituted aromatic aldehyde to yield the corresponding acyloin (hydroxy ketone) compound.

The invention provides a method of synthesis of a substituted carbinol compound, comprising the step of subjecting the corresponding substituted aromatic aldehyde to acyloin condensation mediated by a yeast in an organic solvent under non-fermenting conditions. Preferably the yeast is *Saccharomyces cerevisiae*.

In a preferred embodiment, the reaction is that between pyruvate and benzaldehyde to yield phenylacetylcarbinol, the precursor to ephedrine, to yield a product of high enantiomeric purity.