The Production of Acyloin Compounds

in *Saccharomyces cerevisiae* by Biotransformation

A thesis submitted for the Degree of

Doctor of Philosophy

by

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CONTENTS

LIST OF ABBREVIATIONS

A	Acrylamide
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
Al-DH	Aldehyde dehydrogenase
AP	Ammonium persulphate
ATP	Adenosine triphosphate
Bis	N,N'-methylenebisacrylamide
BSA	Bovine serum albumin
CoA	Coenzyme A
DCIP	2,6-dichlorophenol indophenol
DNA	Deoxyribonucleic acid
DNS	Dinistrosalicylic acid
EDTA	Ethylenediamine tetra-acetic acid
FAD	Flavin adenine dinucleotide (oxidised form)
$FADH_2$	Flavin adenine dinucleotide (reduced form)
GC	Gas chromatography
GSH	Glutathione
HETPP	2-hydroxyethyl thiamine pyrophosphate
'Hnmr	Nuclear magnetic resonance spectroscopy
HPLC	High performance liquid chromatography
MSG	Monosodium glutamate
NAD	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NBT	Nitro blue tetrazolium
NTG	N-methyl-N-nitro-N'-nitrosoguanidine
PAC	L-phenylacetyl carbinol
PDC	Pyruvate decarboxylase
Pi	Inorganic orthophosphate
PMS	Phenazine methosulphate
PS	Propioin synthase
TCA	Tricarboxylic acid
Temed	N, N, N', N' - tetramethylethylenediamine
TPP	Thiamine pyrophosphate

1. INTRODUCTION

1.1 EPHEDRINE (2-METHYLAMINO-1-PHENYLPROPAN-1-OL)

(-) - Ephedrine (1) and (+) - pseudoephedrine (2), the active principles of the chinese plant Ma Huang (Ephedra vulgaris), are classed among the alkaloids which have been known for about five thousand years. It was recorded as early as 1596 that an extract of this plant stimulated the blood circulation, and had diaphoretic, antipyretic, as well as cough relieving actions (Wooley and Wooley, 1972). The active principle was isolated in 1887 by the Japanese researcher Nagai in a pure state (Nagai, 1887). The name ephedrine was given to the substance as it occurs in Ephedra vulgaris. The structure exists in four isomeric forms, (-) - ephedrine and (+) - ephedrine and (+) - and (-) - pseudoephedrine, but only (-) - ephedrine and (+) - pseudoephedrine occur naturally.

Ephedrine is a sympathomimetic amine with direct and indirect effects on adrenoreceptors. It resembles adrenaline and amphetamine in its actions. It exists as colourless hexagonal prismatic crystals or a white crystalline powder with a bitter taste; odourless or with a slight aromatic odour. It is soluble at 20°C in 36 parts of water (The Pharmaceutical Codex, 1979).

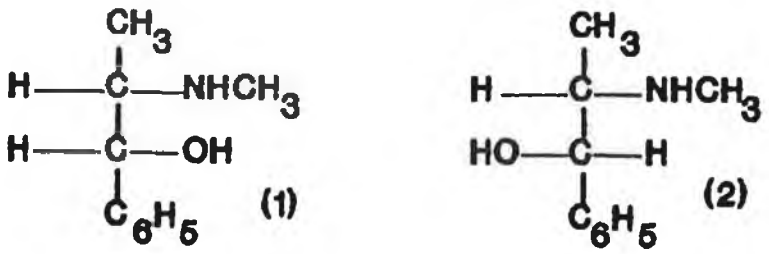


Figure 1.1 Structures of (-) - Ephedrine (1) and (+) - pseudoephedrine (2).

(-) - Ephedrine is pharmacologically more valuble than its diastereoisomer (+) - pseudoephedrine. To obtain (-) - ephedrine synthetically a stereoselective pathway giving (±) - ephedrine in preference to (±) - pseudoephedrine may be used, ((±) - ephedrine can subsequently be resolved by means of active mandelic acid). The precursor α-methylaminopropiophenone hydrochloride, when reduced with hydrogen over platinum gives almost exclusively (±) - ephedrine which may be readily isolated from the reduction products in over 90% yield (Hyde et al., 1928), (Figure 1.2(a)).

Another stereoselective synthesis of ephedrine is by the reductive amination of phenylacetyl carbinol (1-Hydroxy-1-phenyl-2-propanone) (4) with methylamine and hydrogen over platinum. By using (-) - phenylacetyl carbinol (PAC), the natural (-) - ephedrine may be obtained directly (Figure 1.2 (b)). PAC can be synthesised from (-) - mandelic acid (Freudenberg et al., 1932) or by yeast in the presence of benzaldehyde and a fermentable substrate such as sucrose (Neuberg and Hirsch, 1921).

(a) $C_6H_5COCH(NHCH_3)CH_3$ (3) $\xrightarrow{H_2/Pt/HCl}$ (±) Ephedrine

(b) $C_6H_5CHOHCOCH_3$ (4) $\xrightarrow{CH_3NH_2/H_2/Pt}$ (-) Ephedrine

Figure 1.2(a). Catalytic reduction of α-methylaminopropiophenone hydrochloride (3) to give (±) - ephedrine and (b) reductive amination of (-) - phenylacetyl carbinol (4) giving (-) - ephedrine.

1.2 L-PHENYLACETYL CARBINOL PRODUCTION: HISTORY AND DEVELOPMENT

When studying phytochemical reductions which would give simple aldehydes, ketones and diketones, in the presence of a sugar such as sucrose or glucose, Neuberg and co-workers (Neuberg and Hirsch, 1921; Neuberg and Ohle, 1922) examined the phytochemical transformation of benzaldehyde to benzyl alcohol using both top and bottom fermenting yeast and sucrose or glucose. After 3-5 days no sugar or benzaldehyde remained but the amount of benzyl alcohol produced was not proportional to the amount of substrate used. The product (dissolved in the filtrate) exhibited L-rotation, precipitated from Fehlings solution in the cold, was strongly L-optically active and reduced alkaline copper solution in the cold. All of these properties indicated an optically active alpha beta ketone alcohol:- L-phenylacetyl carbinol (Neuberg and Hirsch, 1921; Neuberg and Liebermann, 1921). In preliminary experiments these authors were unable to combine benzaldehyde and acetaldehyde using fermenting yeast. However, this could be achieved if acetaldehyde was replaced by carboxy-acetaldehyde. They found that the fermentation of sugar (glucose or sucrose) by fresh yeast or cell-free extract in the presence of benzaldehyde, led to the formation of an unbranched carbon chain between benzaldehyde and acetaldehyde (PAC). Since this reaction could be carried out using other aldehydes such as cinnamaldehyde they believed a carboligase to be the active ingredient in the formation of these compounds.

Smith and Hendlin (1953) looked at PAC synthesis by an acetone powder of brewers' yeast. They proposed a mechanism by which yeast

acetylate benzaldehyde via a coenzyme A dependant system (Figure 1.3), the optimal pH of this acetylation reaction lying between 4.5 and 5.5

$$\text{(1)}\quad 2\ \text{pyruvic acid} + \text{CoA} \xrightarrow[\text{NAD/Mg}^{2+}]{\text{TPP}} \text{CO}_2 + \text{lactic acid} + \text{acetyl CoA}$$

$$\text{(2)}\quad \text{Acetyl CoA} + \text{benzaldehyde} \xrightarrow{2\text{H}^+} \text{CoA} + \text{phenylacetyl carbinol}$$

Figure 1.3 Synthesis of phenylacetyl carbinol by acetylation of benzaldehyde via a coenzyme A dependant system, proposed by Smith and Hendlin (1953).

Hanc and Kakac (1956) have shown that pyruvic acid, produced during the fermentation of glucose or sucrose by yeast cells, is decarboxylated to activated acetaldehyde, which is enzymatically transformed into PAC on the addition of benzaldehyde. This reaction requires thiamine pyrophosphate as a coenzyme and they suggest that the reaction occurs without the carboligase assumed by Neuberg and co-workers but is catalysed by pyruvate decarboxylase (Figure 1.4).

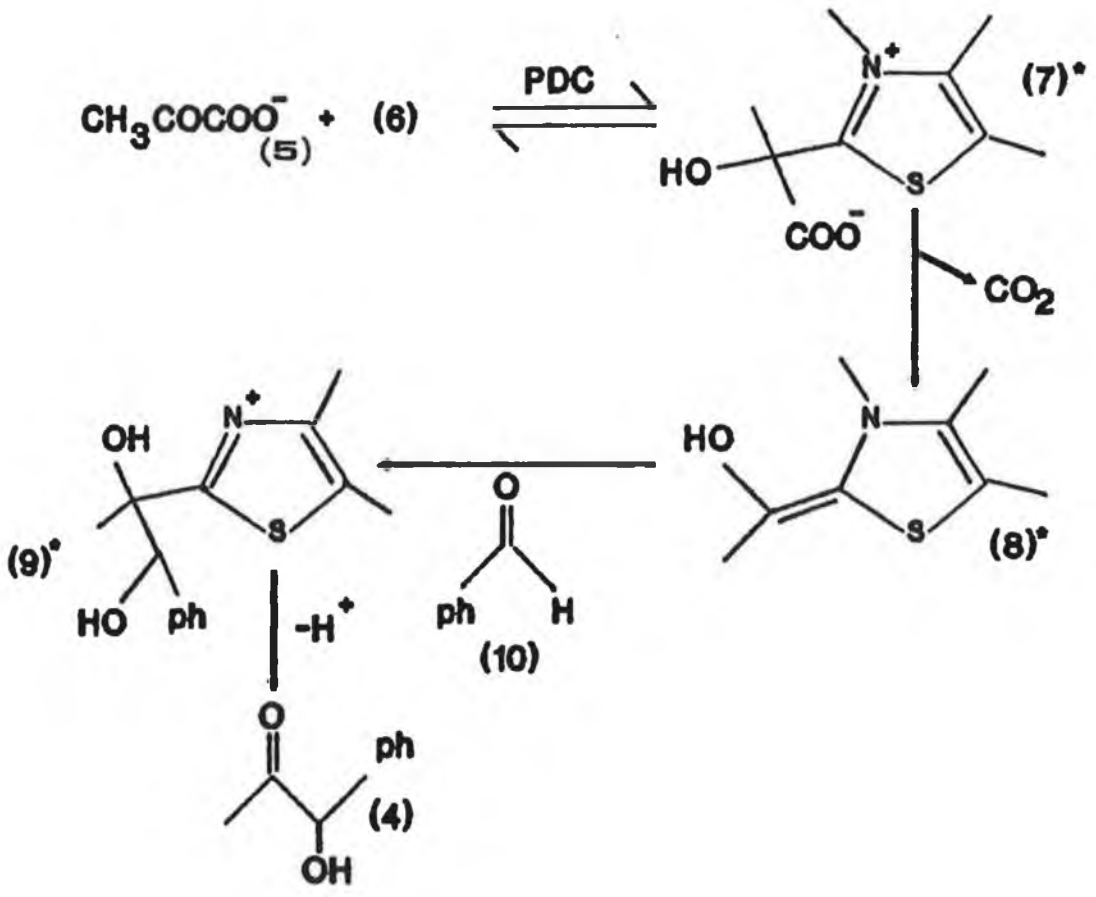
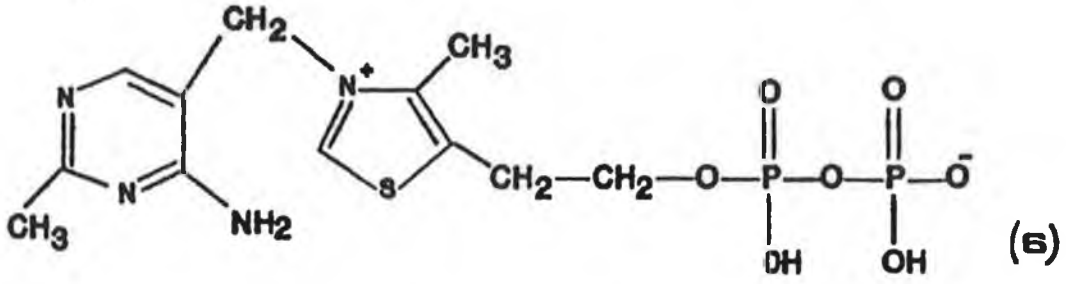


Figure 1.4 Scheme for the formation of PAC by decarboxylation of pyruvate (5) in the presence of benzaldehyde (10). (All asterisked species are enzyme bound), only the thiazole ring of TPP is shown, the entire structure is shown below (6). (6) is the cofactor, $(7)^*$ is the enzyme bound cofactor - pyruvate, $(8)^*$ is HETPP; $(9)^*$, condensation of benzaldehyde with 'active acetaldehyde' and (4), PAC.



NOTE: PDC = Pyruvate decarboxylase

PAC production is influenced by pyruvate decarboxylase activity and pyruvate concentration (Vojtisek and Netrval, 1982). They found that when PAC production levels off, the biotransformation can be restored with initial velocity by addition of pyruvate. Therefore, the intracellular pyruvate concentration is probably the rate limiting factor.

Under normal fermentation conditions, a quantitative conversion of benzaldehyde to PAC is never observed. About a half of the aldehyde is reduced by the yeast to benzyl alcohol (Neuberg and Ohle, 1922; Smith and Hendlin, 1953; Ose and Hironaka, 1957). Smith and Hendlin (1954) investigated the use of NAD analogues as a means of controlling the reaction. They examined the effects of some nicotinic acid derivatives on the rate of benzyl alcohol formation. In a cell-free system, these compounds inhibited formation of benzyl alcohol but had a negligible inhibitory effect in a whole cell fermentation system.

Ose and Hironaka (1957), extended the studies of Neuberg and Hirsch who had discovered the formation of PAC while studying the reduction of benzaldehyde by yeast. They analysed the extract obtained using the fermentation methods of the latter authors and measured the proportions of benzyl alcohol to PAC formed in the presence and absence of added acetaldehyde. In the absence of acetaldehyde, about 35% (w/w) of the benzaldehyde was converted to PAC with about 50% (w/w) being converted to benzyl alcohol. They used acetaldehyde as a hydrogen acceptor to inhibit conversion of benzaldehyde to benzyl alcohol. Small amounts of acetoin were formed (7% (w/w) based on added acetaldehyde). Using acetaldehyde,

they obtained a yield of 70% (w/w) PAC and 25% (w/w) benzyl alcohol, based on benzaldehyde added. Becvarova et al. (1963) also found that the addition of acetaldehyde reduced the amount of benzyl alcohol produced to 25% (w/w).

German patent 1543691 (1966) claimed production of PAC in yields of 55-75% (w/w) based on benzaldehyde to give 6-8 g/l of PAC after 6-10 h. The medium contained acetaldehyde.

Groger et al. (1966) described production of PAC from benzaldehyde plus a 50% (v/v) solution of acetaldehyde/water, added incrementally in the ratio 1:1.5 to a medium containing mineral salts and molasses. They obtained maximum titres of 7-8 g PAC/l.

Gupta et al. (1979) obtained a yield of 5.24 g/l PAC using Saccharomyces cerevisiae (CBS 1171) after an 8 h fermentation using 100 g/l yeast (wet weight, containing 70-75% moisture). Vojtisek and Netrval (1982) used Saccharomyces carlsbergensis (strain 'Budvar') to produce PAC in a medium containing sodium pyruvate. They obtained 10.4 g/l PAC after 8 h when sodium pyruvate was added to the medium hourly (The first and last two additions were of 1.5% (w/v), in the remaining cases 1% (w/v) was added).

Voets et al. (1973) carried out some studies on the effect of aeration on PAC production by S. cerevisiae. They suggest that low aeration results in a high production of benzyl alcohol. In general, yields of PAC produced in agitated but non-aerated batch cultures were approximately 40% lower than in aerated batches. Agarwal et al. (1987) investigated the effects of cell age, cell

concentration, dissolved oxygen and benzaldehyde concentration on PAC production. They suggest that benzaldehyde concentration should be maintained within the range 4 mM - 16 mM (0.4 - 1.7 g/l) and 15 - 18 h old cells should be used for maximum rates of conversion (i.e. cells withdrawn from a fermenter 15 - 18 h following inoculation with a 5% (v/v) seed culture showed highest rates of PAC formation). They observed that with high cell densities the rate (mmol/h) of PAC formation was higher than when lower cell densities were used, but specific rates (mmol/g dry cell/h) of PAC formation were higher when lower cell densities were used. They attribute this finding to oxygen limitation under high cell density conditions.

Tripathi et al. (1988) evaluated PAC biotransformation efficiencies of harvested whole yeast cells grown continuously under glucose limited conditions at different dilution rates. They found that cells from increasing dilution showed increasing specific rates of product formation.

Becvarova and Hanc (1963) studied production of PAC by 6 yeast species. After a single addition of benzaldehyde (2 ml/l), the greatest amount of PAC was formed by Hansenula anomala, S. cerevisiae and S. carlsbergensis, yielding 46 - 51.5% (w/w) (calculated on added benzaldehyde). During fermentation with a higher benzaldehyde concentration (8ml/l), S. carlsbergensis utilized 70% aldehyde for the formation of PAC while the rest was mostly reduced to benzyl alcohol. Netrval and Vojtisek (1982) investigated the "initial rate" of PAC production (i.e. the PAC produced 30 min. after the addition of benzaldehyde) in 38 yeast

strains, mostly of the genera Saccharomyces and Candida. The amount of PAC produced varied from 0 to 1.24 g/l. The 15 strains which had the highest "initial rate" of PAC production were tested over a more prolonged period of time. The highest titre of PAC (6.3 g/l) was produced by the strain S. carlsbergensis "Budvar".

1.2.1 Physical and Chemical Properties of Biotransformation Substrate and Products

The physical and chemical properties of benzaldehyde, benzyl alcohol and PAC are summarised in Table 1.1

Table 1.1 Physical and chemical properties of biotransformation substrate, benzaldehyde and products, benzyl alcohol and PAC.

Property	Benzaldehyde	Benzyl alcohol	PAC
Physical Characteristics	Strongly refractive liquid	Liquid, aromatic odour	Yellowish Oil
Molecular formula	C_7H_6O	C_7H_8O	$C_9H_{10}O_2$
Molecular weight	106.12	108.13	150.0
Boiling Point	179°C	204.7°C	205-7°C
n^{20}_D	1.5456	1.54035	1.5205
$[\alpha]^{20}_D$	0.0	0.0	-157° in ethanol
Solubility	Miscible with alcohol, ether oils. Sol. in 350 parts H_2O	Miscible with abs. and 94% alcohol, ether, chloroform. Sol. in 25 parts H_2O	Miscible with most organic solvents
Other notable chemical properties and uses	Oxidises in air to benzoic acid. It reduces ammoniacal $AgNO_3$ but not Fehlings solution.	Used in the perfume industry and in embedding for electron microscopy	It reduces ammoniacal $AgNO_3$ & Fehlings Solution

1.2.2 Analysis of L-Phenylacetyl Carbinol

Neuberg and Ohle (1922) attempted to determine PAC using the Fehlings method, exploiting the considerable reducing power of PAC. When this method was applied to PAC producing fermentation cultures, it was necessary to ensure that all reducing sugar present in the fermentation had been used up in glycolysis. Depending on the speed of titration, divergent values were obtained using this method so it ultimately proved unsatisfactory (Groger and Erge, 1965). Another method involving reducing substance determination was used by Smith and Hendlin (1953) involving use of iodine and hydroxylamine-HCl. This method again was considered to be unsuitable for the determination of crude PAC preparations because of the presence of other reducing substances in fermentation broths.

A number of authors described polarographic methods for PAC determination (Hanc and Kakac, 1956; Fedoronko, 1958; Becvarova et al., 1963). Paper chromatographic procedures were described by some authors. Hanc and Kakac (1956) used Whatman number 1 paper impregnated with formamide and developed with benzol. PAC was detected as its 2,4-dinitrophenylhydrazone derivative using 2,4-dinitrophenylhydrazine. Macek et al. (1964) used a similar method. Bauerova and Bauer (1959) described a paper chromatographic method for the determination of PAC in crude ketol preparations. Isoamyl alcohol-water with ascending chromatography was used. Blue tetrazolium was used to detect PAC spots. (PAC has considerable reducing power and blue tetrazolium is used in estimation and detection of reducing compounds). Bauerova and Bauer (1959) also described a colorimetric method for determination of PAC based on

the reaction of PAC with blue tetrazolium. They indicated that the measurement was not influenced by the presence of benzaldehyde. Becvarova et al. (1963) and Macek et al. (1964) described the use of gas chromatography for the analysis of PAC and other compounds using a silicone elastomer as a stationary phase and helium as the carrier gas. It was demonstrated that PAC, acetyl benzoyl, benzyl alcohol and benzaldehyde could be separated using this GC method. Groger and Erge (1965) described a colorimetric method for determination of PAC. They applied the colorimetric method of Westerfeld (1945) for determination of acetoin and diacetyl, based on the Voges-Proskauer reaction to determine PAC. Groger and Erge (1965) also described a thin layer chromatography procedure for separation of PAC from other fermentation components. Stationary phase consisted of silica-gel and chloroform was used as solvent. Dinitrophenylhydrazine-HCl or iodine vapour were used to detect the separated components. Fey et al. (1971) described 2 methods for determination of PAC, a spectrophotometric and a volumetric method, each based on the selective oxidation of the α-Ketol group by periodic acid. The volumetric method is based on the titration of free iodine with sodium arsenite following addition of potassium iodide. The spectrophotometric method involves the calculation of PAC concentration from the absorbance (at 748 - 759 nm depending on concentration) of the excess periodic acid as compared to its absorbance at its initial concentration.

1.3 BAKERS' YEAST

Many authors have studied PAC production in bakers' yeast (*Saccharomyces cervisiae*), (Neuberg and Hirsch, 1921; Neuberg and Liebermann, 1921; Hanc and Kakac, 1956; Groger *et al.*, 1966; Voets *et al.*, 1973; Vojtisek and Netrval, 1982). Fungi and yeasts are eukaryotic organisms distinct from more primitive prokaryotic organisms (bacteria and blue-green algae). They contain a higher degree of organisation with a distinct nucleus bounded by a membrane and subcellular organelles such as mitochondria and endoplasmic reticulum. The yeast plasma membrane serves as a physical barrier for the cellular protoplast, as an osmotic barrier controlling the entry and exit of solutes into and out of the protoplast, and possibly as a template for cell wall synthesis (Soumalainen and Nurminen, 1976). The cell membrane is particularly important in biotransformations using whole cells where there may be a high concentration of substrate and/or products which could be toxic to intracellular components. The maintenance of membrane integrity depends on the stability of its components in the presence of these compounds. The structure of yeast membranes is typically eukaryotic. It incorporates sterols into a lipid bilayer composed of phosphotidylserine (PS), phosphotidylethanolamine (PE), phosphotidylcholine (PC), phosphotidylinositol (PI), cardiolipin and sphingo-lipids. The predominant fatty acids associated with these lipids are the C-16 and C-18 acids, palmitic (16:0), palmitoleic (16:1), stearic (18:0), and oleic (18:1). Yeast synthesise only monoenoic fatty acids

(Henry, 1982). About 80% of the total cellular phospholipid and sterol are found in the plasma membrane. The predominant membrane phospholipid is PC.

1.3.1 Carbohydrate Metabolism

The initial reaction in sucrose utilisation is the extracellular cleavage of the sugar to its monosaccharide constituents by invertase. The same gene codes for both an internal non-glycosylated invertase enzyme and a glycosylated, external enzyme which is secreted into the periplasmic space between the cell wall and the cytoplasmic membrane (Carlson and Botstein, 1982). The products of invertase action, glucose and fructose, are transported into the cell by facilitated diffusion (carrier-protein mediated diffusion into the cell, down a concentration gradient) (Frankel, 1982). Once a sugar has entered the cell it enters the glycolytic pathway. PAC is produced by fermenting yeast in the presence of glucose or sucrose and a benzaldehyde co-substrate. Glucose and fructose are phosphorylated by glucokinase and hexokinase. The importance of the glycolytic pathway in yeast is evident by the proportion of total cellular protein present as glycolytic enzymes. Estimates range from 30 - 65% (Hess et al., 1969; Bannelos and Gancedo, 1978; Frankel, 1982).

Levels of these enzymes seem to depend on substrate as their levels have been reported to be low in acetate grown cells. Upon the addition of glucose or galactose the rates of individual enzyme synthesis increase 3 - 100 fold (Maitra and Lobo, 1971). In flask

cultures of S. cerevisiae the levels of phosphofructokinase respond to levels of both glucose and ethanol in the medium (Foy and Bhattacharjee, 1978).

Carbohydrate metabolism in yeast involves complex regulatory mechanisms (Entian et al., 1984). In the presence of hexoses, fermentation of these sugars has priority over respiratory degradation, and gluconeogenesis is prevented. Carbon catabolite repression and carbon catabolite inactivation are the two main regulatory systems responsible for these effects. Carbon catabolite inactivation is the rapid degradation of some enzymes, mostly gluconeogenic in the presence of glucose. The mechanism of catabolite inactivation is not yet known (Trivedi and Tesch, 1986) but it is thought to procede via an initial protein modification, which inactivates the enzyme. This is followed by protein specific protease action which degrades the modified enzyme (Holzer, 1976; Holzer, 1978).

Glucose starvation has a major effect on glycolytic enzymes. In the absence of glucose, phosphofructokinase decays the fastest with diphasic kinetics (Maitra and Lobo, 1971), pyruvate decarboxylase also decays rapidly in the absence of glucose (Polakis and Bartley, 1965). Other glycolytic enzymes such as triose phosphate isomerase, phosphoglycerate kinase and pyruvate kinase decay only after a lag period.

Glucose fermentation by yeast is an anaerobic process, but yeast are aerobes. Under anaerobic conditions yeasts ferment very actively but hardly grow. On aeration, fermentation decreases in

favour of respiration. In some yeasts, fermentation can be completely suppressed by vigorous aeration (Pasteur effect). Pasteur discovered this effect more than 100 years ago during investigations on fermentation processes in wine making. The Pasteur effect involves several regulatory mechanisms working together. One of these applies at the level of phosphorylation and can be explained by competition for ADP and Pi. The dehydrogenation step in the catabolism of glyceraldehyde phosphate requires phosphate and ADP. Degradation of glucose via glycolysis is thus dependent on ADP and Pi. Without these, dehydrogenation of glyceraldehyde - 3 - phosphate cannot take place. Under aerobic conditions the ADP and phosphate are competed for by respiratory chain phosphorylations. A second regulatory mechanism is also responsible for the Pasteur effect: the enzyme phosphofructokinase is allosterically inhibited by ATP. The enzyme is also inhibited by citrate.

Carbon from the glycolytic pathway is fed directly into the TCA cycle by the conversion of pyruvate to acetyl-CoA and CO_2 by pyruvate dehydrogenase. Pyruvate dehydrogenase is subject to catabolite repression, and under conditions favouring fermentation, pyruvate is decarboxylated to acetaldehyde by pyruvate decarboxylase (Polakis and Bartley, 1965). At this point PAC is formed in the presence of benzaldehyde. Alcohol dehydrogenase catalyses the conversion of acetaldehyde to ethanol, using NAD as coenzyme. The enzyme also converts a proportion of benzaldehyde (when present) to benzyl alcohol. The reduction of NAD to NADH in the alcohol dehydrogenase reaction regenerates the NADH required for the glyceraldehyde - 3 - phosphate dehydrogenase reaction and

thus maintains glycolysis. Therefore, alcohol dehydrogenase is involved in the general coenzyme equilibration mechanism of the cell (Wiesenfeld et al., 1975). Using strains of S. cerevisiae which had altered levels of alcohol dehydrogenase and pyruvate decarboxylase, Sharma and Tauro (1986) showed that these enzymes had a major role in determining the rate of ethanol production. For fast ethanol formation the level of both pyruvate decarboxylase and alcohol dehydrogenase should be high. Accumulated ethanol can be metabolised by yeast under aerobic conditions. Ethanol enters the TCA cycle via the action of another alcohol dehydrogenase, acetaldehyde dehydrogenase (ADH II) and acetyl CoA synthetase (Frankel, 1982).

1.4 YEAST PYRUVATE DECARBOXYLASE

Pyruvate decarboxylase (2-oxo-acid-carboxy-lyase) catalyses the decarboxylation of 2-oxo acids to the corresponding aldehydes. The enzyme contains thiamine pyrophosphate (TPP) and Mg^{2+} as cofactors. It shows a requirement for these cofactors only after dialysis for 18 h at 2°C against 0.01 M potassium phosphate pH 6.2 (Hussain Quadri and Hoare, 1967). Ullrich et al. (1966) determined the coenzyme content of the enzyme by fluorescence assay of thiochrome. They found three thiamine pyrophosphates per enzyme molecule. When the holoenzyme was saturated with thiamine pyrophosphate and Mg^{2+} an increase in activity of about 30% was observed, indicating that the protein molecule probably has a fourth binding site for the coenzyme.

The molecular weight, determined by density gradient centrifugation was 175,000 (Ullrich et al., 1966). Several authors have determined the molecular weight of the holoenzyme to be 250,000 (Kuo et al., 1986).

Pyruvate decarboxylase is composed of two subunits of two monomers each, as shown by ultracentrifugation and gel chromatography (Hopmann, 1980). The enzyme exists as two distinct isozymes, one consisting of identical, the other of dissimilar, subunits. Otherwise, kinetically and mechanistically, the two isozymes appear to be virtually indistinguishable (Kuo et al., 1986).

The shape of the v/s curve for pyruvate decarboxylase is sigmoidal. The enzyme is activated by its substrate, pyruvate, but is practically inactive when the substrate concentration approaches zero (Hubner and Schellenberger, 1986). Acetaldehyde inhibits the decarboxylation of pyruvate but does not inactivate the enzyme. Activity may be restored by removal of the acetaldehyde. The inhibition of decarboxylation by acetaldehyde is not competitive with pyruvate and was shown by Juni (1961) to be substrate-independent. The inhibitory effect of acetaldehyde on decarboxylation decreases with increasing chain length of the substrate. Other aldehydes also inhibit the decarboxylation of pyruvate. Juni (1961) investigated the effect of a range of aldehydes on pyruvate decarboxylase activity. Benzaldehyde inhibited the enzyme by 30% as compared to a 65% inhibition by acetaldehyde.

1.4.1 Mechanism of Pyruvate Decarboxylase

TPP is the pyrophosphoric acid ester of thiamine, or vitamin B_1,

a necessary growth factor for many micro-organisms and most vertebrate species. TPP is ubiquitous in nature as the coenzyme for a number of enzymes catalyzing the decarboxylation of 2-oxo acids and the transfer of C_2 moieties. During the decarboxylation of pyruvate the 2-hydroxyethyl derivative of TPP (HETPP) is formed, which may be viewed as an activated, or coenzyme-bound, form of acetaldehyde. Free acetaldehyde is formed from HETPP by pyruvate decarboxylase. Interaction by a hydrogen bond of the apoenzyme with HETPP increases the basicity of the amino group at position 4 of the pyrimidine ring. The increased basicity permits the hydroxyl proton of the α-hydroxyethyl group of HETPP to react with the basic amino group, thus labilizing the carbon-carbon bond of the hydroxyethyl group at the C_2 position of TPP to form acetaldehyde. A scheme for the decarboxylation of pyruvate is illustrated in Figure 1.5.

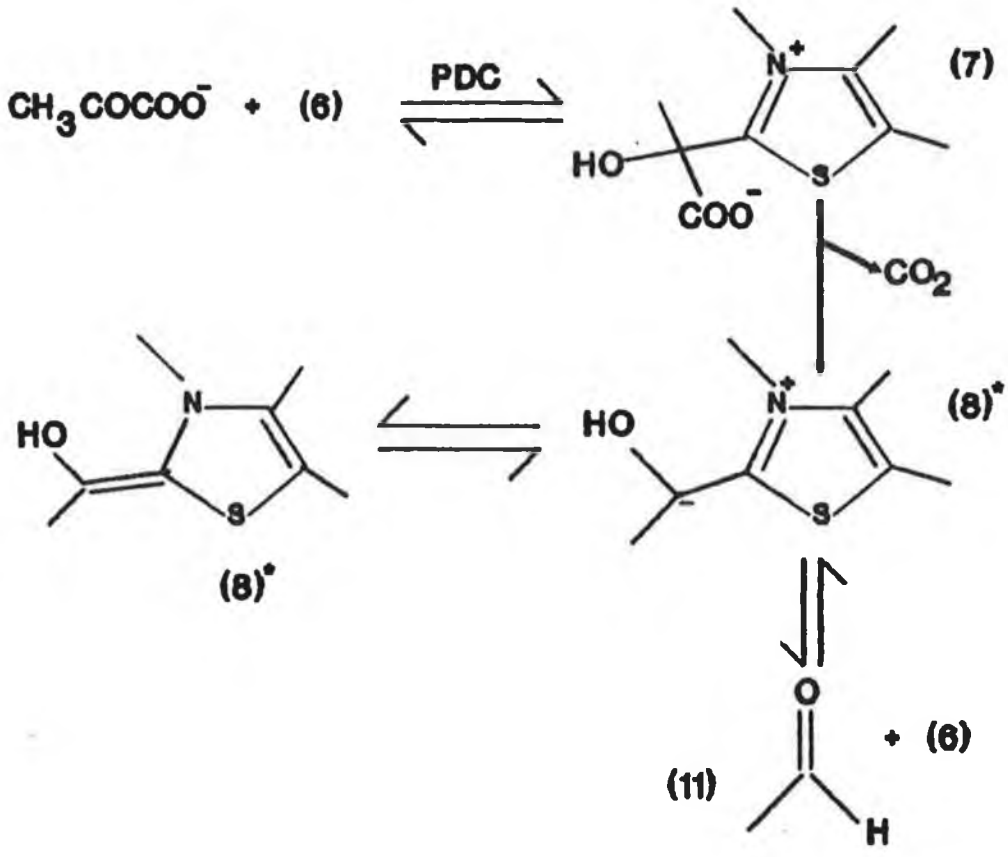


Figure 1.5 Scheme for the decarboxylation of pyruvate (all asterisked species are enzyme bound). Only the thiazole ring of TPP is shown, the entire structure is shown in Figure 1.4. (6) is the cofactor, $(7)^*$ is the enzyme bound cofactor - pyruvate, $(8)^*$ are the resonance forms of HETPP and (11) is acetaldehyde.

NOTE: PDC = pyruvate decarboxylase

Juni (1961) postulated a two-site mechanism for the decarboxylation of pyruvate. A simplified outline for this theory is presented in Figure 1.6. The attachment of pyruvate to site 1 of the enzyme is shown in step A. Bonding with the α-carbon of pyruvate takes place at position 2 of the thiazole ring of TPP. This is followed by decarboxylation which leaves an aldehyde unit still firmly bound to the enzyme surface (step B), as the HETPP complex. This is also called 'active acetaldehyde'. 'Active acetaldehyde' is then irreversibly transferred to site 2 as indicated in step C. It is postulated that once the aldehyde unit becomes attached to the second site it can reversibly dissociate from the enzyme to yield free acetaldehyde.

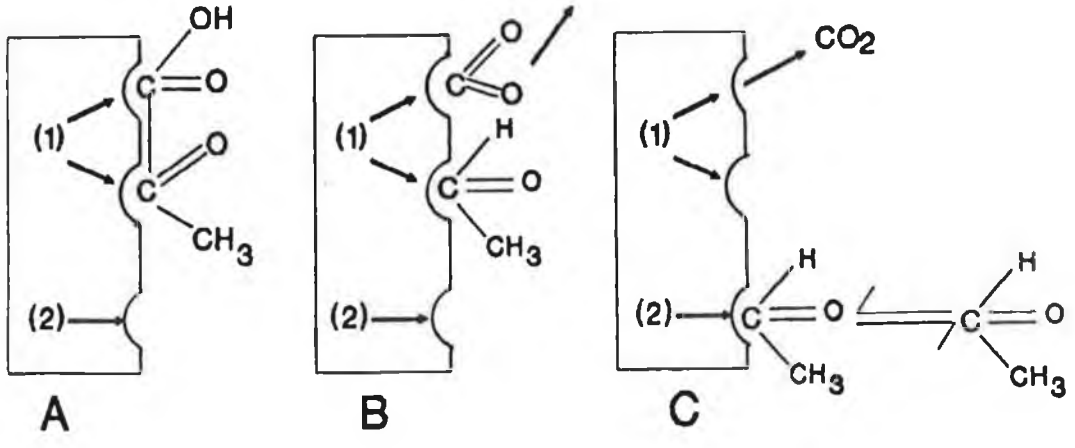


Figure 1.6 Two-site mechanism for pyruvate decarboxylase.

The non-enzymatic decarboxylation of pyruvate catalysed by thiamine does not result in the formation of free acetaldehyde. The sole product of the decarboxylation is α-acetolactate. Results of studies of the non-enzymatic decarboxylation of a series of α-keto acids, as catalysed by thiamine in alkaline solution indicate that aldehyde - TPP complexes of aldehydes with chain lengths greater than acetaldehyde are relatively unstable and decompose spontaneously to free aldehydes, thus avoiding the requirement for the second enzymatic site.

In the presence of hydrogen acceptors such as 2,6-dichlorophenolindophenol (DCIP) pyruvate decarboxylase catalyses the oxidative decarboxylation of 2-oxo acids to the corresponding carboxylic acids (Hubner et al. 1986). This reaction (unphysiological with respect to the enzyme) can be compared with the first step of the oxidative decarboxylation of 2-oxo acids by the pyruvate dehydrogenase complex.

1.4.2 Other TPP requiring Decarboxylases

Yeast pyruvate decarboxylase is by far the best studied of the non-oxidative α-keto acid decarboxylases that require TPP for activity. Phenylglyoxylate decarboxylase (benzoylformate decarboxylase) and phenylpyruvate decarboxylase, both involved in the catabolism of aromatic compounds, belong to this class of enzymes (Barrowman and Fewson, 1985). The occurrence of phenylglyoxylate decarboxylase has been reported in different strains of Acineobacter calcoaceticus and in various Pseudomonas species (Barrowman et al., 1986). Phenylpyruvate decarboxylase has also been found in A. calcoaceticus and in Achromobacter eurydice.

The native molecular weight values for phenylglyoxylate decarboxylase and phenylpyruvate decarboxylase are estimated to be 232,000 ± 4,000 and 235,000 ± 3,000 respectively. They both decarboxylate aromatic but not aliphatic substrates. Each enzyme appears to be substrate specific. Neither decarboxylates pyruvate nor α-ketoadipate at more than about 2% of the rate of its own substrate (Barrowman and Fewson, 1985). The pH optima of phenylglyoxylate decarboxylase and phenylpyruvate decarboxylase are 5.9 and 7.0 respectively.

Barrowman et al. (1986) investigated possible immunological relationships among phenylglyoxylate decarboxylase and phenylpyruvate decarboxylase of A. calcoaceticus and pyruvate decarboxylase of brewers' yeast. Although the three enzymes are at least superficially similar, being tetramers of similar subunit molecular weight. In no case did an antiserum cross-react with a decarboxylase of a different substrate specificity from that against which it had been raised. Structural homology amongst the three enzymes cannot be ruled out however, as regions of amino acid homology might be buried within the native protein.

No studies have been published on the ability of these enzymes to form acyloin compounds as formed by pyruvate decarboxylase. Preliminary investigations in this area have been initiated in our laboratory (Bourgault, 1989).

1.4.3 Propioin Synthase

Morimoto et al. (1983) have isolated and characterised an enzyme from bakers' yeast which catalyses the formation of acyloin

compounds from the corresponding aldehydes without ketoacid. The enzyme, which they tentatively name propioin synthase, has been shown to catalyse the formation of propioin and acetoin (reactions illustrated in Figure 1.7) in addition to furoin, methylfuroin, isobutyroin and valeroin from the corresponding aldehydes.

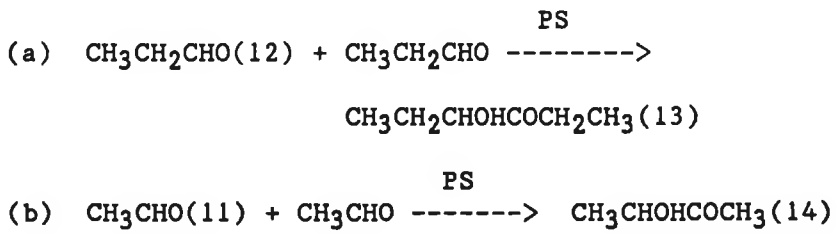


Figure 1.7 (a) Conversion of 2 molecules of propionaldehyde (12) to Propioin (13) and (b) 2 molecules of acetaldehyde (11) to acetoin (14) by the enzyme propioin synthase (PS).

Morimoto et al.(1988) purified the enzyme to homogeneity (on disc gel electrophoresis). It is most active at pH 6.8 - 7.0 and 37°C. Its activity is enhanced by $FeSO_4.7H_20$, $MnSO_4$, TPP, β-mercaptoethanol, $MgSO_4$, $CaCO_3$ and NaCl and inhibited by $AgNO_3$, $HgCl_2$, $CuSO_4$, $ZnSO_4$, iodoacetic acid and $(NH_4)_2SO_4$. Its molecular weight was 96,000 by sedimentation equilibrium and 100,000 by Sephadex G-200 column chromatography.

1.4.4 Formation of Acyloin Compounds

Acyloins are α-hydroxy ketones of the general formula RCHOHCOR, in which R represents an aliphatic residue. The term 'acyloin' is commonly used as a class name for the symmetrical keto alcohols, and the name of the individual compound is derived by adding the suffix oin to the stem of the acid to which the acyloin corresponds. A mixed acyloin in which the two alkyl groups are different is named as a derivative of carbinol (McElvain, 1948).

PAC is frequently referred to as an acyloin compound, even though, under the above definition this is not strictly correct. Methods of preparation that have been used for the synthesis of certain acyloins include the reaction of sodium with esters of aliphatic acids in inert solvents and photochemical and biological syntheses.

Acetoin is an acyloin compound commonly found in biological systems. There are at least three known mechanisms for acetoin formation, depending on the source of enzyme (Hussain Quadri and Hoare, 1973). Neuberg and Simon (1933) reported that in yeast one molecule of acetaldehyde, formed by decarboxylation of pyruvic acid, condenses with another molecule of pyruvic acid to form acetoin. The system present in various animal tissues has been studied in pig heart extracts (Green et al., 1942; Juni, 1952a). One molecule of pyruvate and one of acetaldehyde or two molecules of acetaldehyde can act as substrates for acetoin formation. Alkonyi et al. (1976) studied the formation of acetoin by the head-to-head combination of two acetaldehyde molecules as catalysed by the pyruvate dehydrogenase complex isolated from pigeon breast muscle. TPP and magnesium ions were required for the reaction. Bacteria do not use acetaldehyde for the formation of acetoin. Two molecules of pyruvate act as substrate for the formation of α-acetolactate which is decarboxylated to form acetoin. This system has been studied in cell free extracts of Aerobacter aerogenes (Silverman and Werkman, 1941; Juni, 1950; Dirscherl and Hofermann, 1951; Juni, 1952b) and in Streptococcus faecalis (Dolin and Gunsalus, 1951). Chuang and Collins (1968) studied acetoin

production in a range of other bacterial species and in strains of S. cerevisiae. The different pathways to acetoin formation in yeast, bacteria and mammalian species are summarised in Figure 1.8.

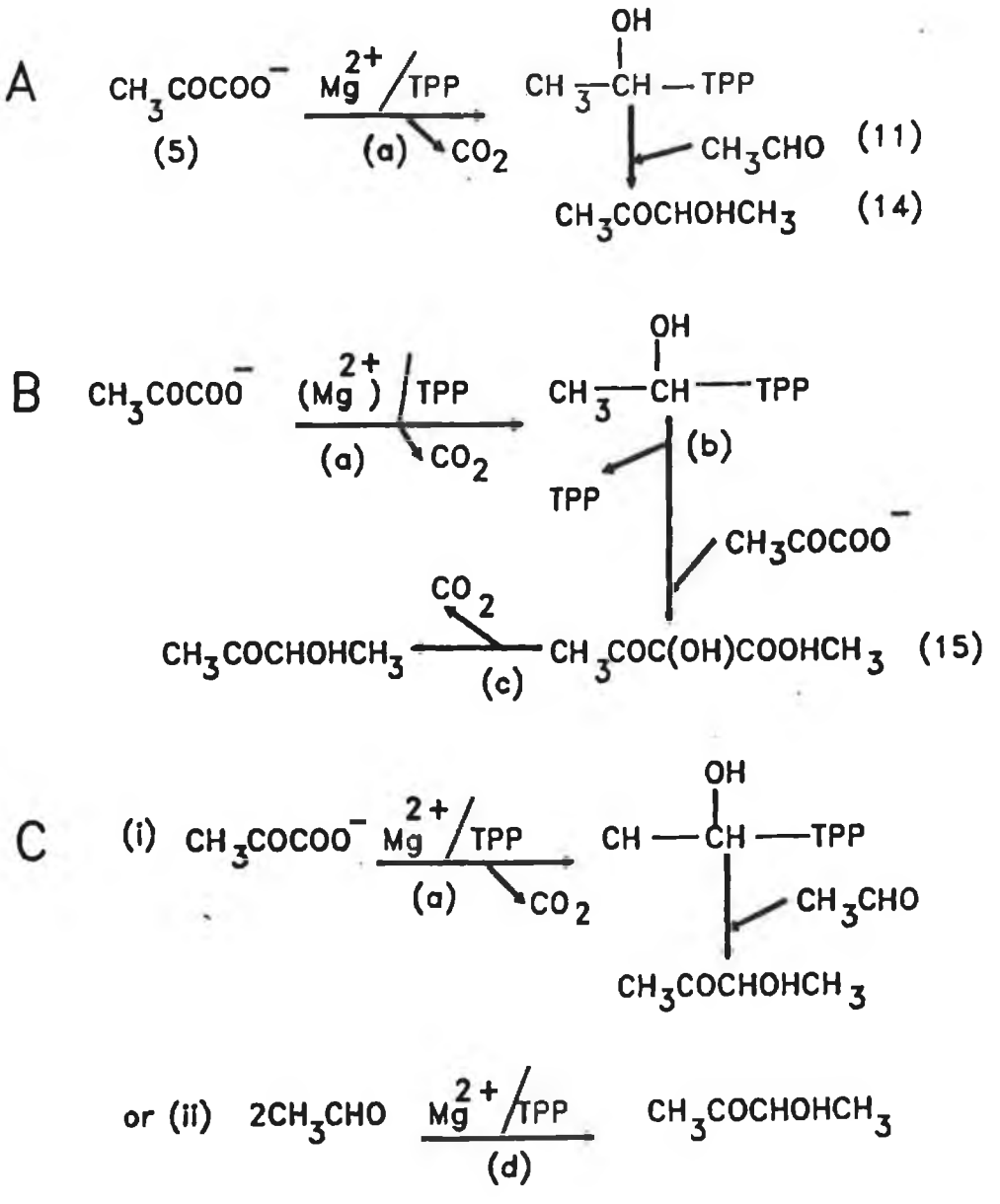


Figure 1.8 Mechanisms of formation of acetoin in A, Yeast; B, most bacteria and C, mammalian species. Compounds labelled 5, 11, 14, 15 are: (5) Pyruvate, (11) Acetaldehyde, (14) Acetoin and (15) α-Acetolactic acid. The enzyme systems involved are as follows: (a) Pyruvate decarboxylase, (b) α-acetolactate synthase, (c) α-acetolactate decarboxylase and (d) Pyruvate dehydrogenase.

A mechanism for the formation of acetoin in yeast has been proposed in the two-site theory of Juni (1961). According to this theory, 'active acetaldehyde' at site 1 is directly bonded to free acetaldehyde to form acetoin. The first site is implicated in acetoin synthesis because TPP is required and blocking the second site with acetaldehyde does not inhibit the rate of acetoin synthesis. The reaction is illustrated in Figure 1.9.

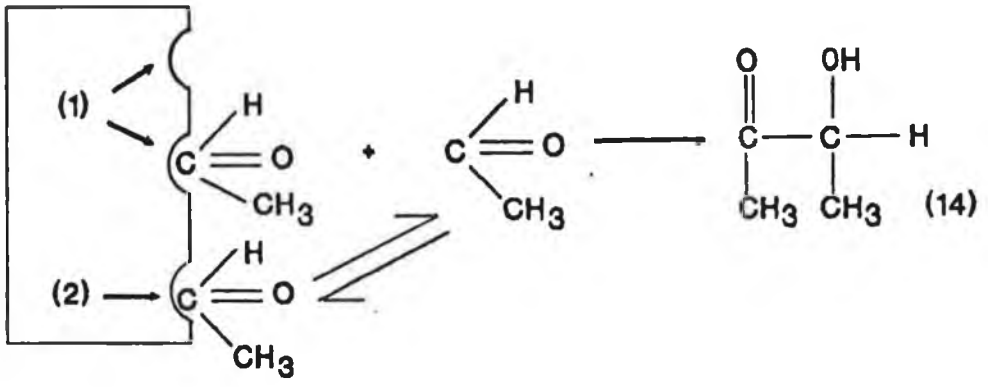


Figure 1.9 Two-site mechanism for acetoin (14) formation.

Soumalainen and Linnahalme (1966) identified the compounds formed from α-keto-monocarboxylic acids under anaerobic conditions by dried bakers' and brewers' yeast, (using paper chromatography, gas chromatography and colorimetric techniques). When pyruvic acid is metabolised by these yeasts considerable amounts of acetoin are formed. It was found that propioin was formed when α-ketobutyric acid or a mixture of pyruvic acid and α-ketobutyric acid was metabolised by brewers' yeast. In the latter case, both the

asymmetric 5-C acyloins, 3-hydroxy-2-pentanone and 2-hydroxy-3-pentanone appeared. Soumalainen and Linnahalme found that bisulphite completely inhibits the formation of acyloins by binding-free aldehyde. Juni (1961) looked at the decarboxylation of a limited number of α-keto acids by pyruvate decarboxylase under aerobic conditions. α-ketobutyrate is decarboxylated preferentially to pyruvate when both acids are present simultaneously in equal concentrations. Decarboxylation of trimethyl pyruvic acid could not be detected even when the amount of enzyme was increased thirty five fold.

U.S. patent no. L188,293 (1968) investigated the production of acyloin compounds of the formula $R_1CHOHCOCH_3$, where R_1 is a 3,4 disubstituted phenyl radical, in which the substituents are hydroxy, C_1-C_4 alkoxy or C_1-C_4 alkylenedioxy radicals, such compounds being in the L-configuration. Typical compounds of this formula are L(-) 3,4-dimethoxyphenylacetyl carbinol, L(-) 3-methoxy-4-hydroxyphenylacetyl carbinol and L(-) 3,4 - methyl-enedioxyphenylacetyl carbinol. These compounds are useful as intermediates in the stereospecific synthesis of L-α-methyl-3,4-dihydroxyphenylalanine, a valuable agent in the treatment of hypertension. The compounds are prepared by contacting an aldehyde of the formula R_1CHO where R_1 is as defined above, with a growing culture of an acyloin producing strain of Aerobacter aerogenes or Saccharomyces cerevisiae.

1.4.5 Pyruvate Metabolism in Bacteria

Pyruvate decarboxylase is an enzyme rarely found in bacteria but more commonly occurring in fungi and plants. Erwinia amylovora

was the first member of the family Enterobacteriaceae reported to possess a pyruvate decarboxylase which yields acetaldehyde and carbon dioxide from pyruvate (Haq, 1984). Ethanol is only a minor fermentation product of coli-aerogenes and other enteric bacteria, unlike yeast, they do not possess a pyruvate decarboxylase. On the other hand, E. amylovora produced yields of ethanol as high as 1.55 moles per mole of glucose fermented which is correlated with the presence of pyruvate decarboxylase. TPP and Mg^{2+} are cofactors of the E. amylovora enzyme.

In 1966 Dawes et al. provided enzymic evidence that Zymomonas mobilis used the Entner-Doudoroff pathway in the anaerobic conversion of glucose into ethanol and carbon dioxide. In doing so they confirmed the presence of pyruvate decarboxylase in this micro-organism. The activity of the enzyme in Z. mobilis is higher than in any other source (Welch and Scopes, 1984); even yeast do not have such high levels in their cytoplasm. The Zymomonas enzyme exhibits many properties similar to that from yeasts (Neale et al. 1987). The proteins tetrameric structure and size is like the yeast enzyme but there is no evidence of two types of subunit.

Hussain Quadri and Hoare (1973) studied pyruvate decarboxylase and acetoin formation in Athiorhodaceae. Cell free extracts of Rhodopseudomonas palustris strain Q decarboxylated pyruvate under anaerobic conditions forming carbon dioxide, acetaldehyde and acetoin. Pyruvate decarboxylase in photosynthetic bacteria requires only TPP as a cofactor and differs in this repsect from the enzyme in yeast and in other bacteria which require divalent

metal ions as well. Acetoin formation in the Athiorhodaceae, like that in yeast but unlike that in most bacterial species, involves the formation of acetaldehyde from pyruvate.

Several strains of the acetic acid bacteria Acetobacter and Gluconobacter contain a constitutive, soluble pyruvate decarboxylase which requires TPP and Mg^{2+} (DeLey and Schell, 1962). When grown on lactate a pyruvate decarboxylase is also present in the particulate fraction. Both D- and L-lactate are broken down by way of pyruvate and acetaldehyde to acetate.

The yeast, Torulopsis colliculosa and the bacterium, Enterobacter cloacae contain oxaloacetate and pyruvate decarboxylases (Yadav et al., 1980). Cell free extracts obtained from cells grown on glucose convert oxaloacetate and pyruvate but not citrate to acetoin. They indicate that acetoin formation in these organisms may be linked to the Krebs cycle as there is a dramatic increase in acetoin formation under aerobic conditions.

Dolin and Gunsalus (1951) investigated pyruvate metabolism in Streptococcus faecalis, by cell suspensions and vacuum-dried cells. They found the metabolism of pyruvate to be predominantly oxidative, yielding aerobically, acetate and carbon dioxide. Cell free extracts from these cells catalyse the breakdown of pyruvate predominantly to acetoin and carbon dioxide. Acetaldehyde is not an intermediate in acetoin formation but extracts contained an active α-acetolactate decarboxylase.

Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus delbrueckii, Lactobacillus lactis and Lactobacillus plantarum contain a pyruvate oxidase for the oxidation of pyruvate to acetyl phosphate and acetate (Hickey et al., 1983). L. plantarum produces acetoin as a major product (Rowatt, 1951; Nossal, 1952) through the enzymic decarboxylation of α-acetolactate (Hunt and Nossal, 1954). The metabolism of pyruvate in L. casei produces α-acetolactate which is then converted to diacetyl and acetoin (Branen and Keenan, 1972; Gardenas et al., 1980).

Lees and Jago (1976) investigated the formation of ethanol from glucose by lactic acid bacteria. Group N streptococci formed acetaldehyde and ethanol from glucose. Enzymic evidence showed that these organisms can reduce acetyl-CoA to acetaldehyde and convert acetyl-CoA to acetyl phosphate and acetate. Acetaldehyde could not be formed by direct decarboxylation of pyruvate as pyruvate decarboxylase is absent. This illustrates that some bacteria have an alternative route leading to the formation of acetaldehyde which does not involve either pyruvate or pyruvate decarboxylase.

In summary, TPP linked pyruvate decarboxylases have been characterised in mammalian, plant, some bacterial, yeast and other fungal species. Both glycolytic pathway and Krebs cycle intermediates are known to participate in acetoin biosynthesis. Acetaldehyde may serve as a sole carbon source for acetoin production by plant and animal tissues. In yeast, free

acetaldehyde condenses with 'active acetaldehyde' formed by decarboxylation of pyruvic acid to form acetoin. The majority of bacteria, however, exclusively require pyruvate or its precursor for acetoin production.

1.5 OXIDO-REDUCTASES

Oxido-reductases are a group of enzymes which catalyse oxidation-reduction reactions involving oxygenation, such as C-H--> C-OH, or overall removal or addition of Hydrogen atom equivalents as for $CH(OH) \rightleftharpoons C = O$ and $CH\text{-}CH \rightleftharpoons C = C$, (International Union of Biochemistry, 1979; Jones, 1986). In the latter reaction, coenzymes such as NAD(H), NADP(H) or FAD(H_2) may be used as a source/acceptor of the Hydrogen atom equivalents. A single micro-organism contains many oxido-reductases. In yeast, for example, the enzymes alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase play a major role in the metabolism of the organism, glyceraldehyde-3-phosphate dehydrogenase being a key enzyme in the glycolytic pathway. Other oxido-reductases present in yeast include, malate dehydrogenase, aldehyde reductases, which comprise the most rapidly evolving family of oxido-reductases (Davidson and Flynn, 1979), NAD- and NADP-isocitrate dehydrogenases (Satrustegui et al., 1983) and methylglyoxal reductase which catalyses the conversion of methylglyoxal into lactaldehyde in the presence of NADPH (Murata et al., 1986).

Oxido-reductases, in addition to other enzymes, are being used more and more as tools in organic synthesis. The abilities of enzymes to act as specific and chiral catalysts have been known for many years. The fact that most enzymes are specific with respect to the type of reaction they catalyse enables them to operate

independently on their own substrate in the presence of other enzymes and their substrates. This allows multiple, sequential, synthetic transformations to be carried out in one-pot reactions. Alcohols produced using yeast reductions have been used in synthesis of compactin, griseoviridin and a bee pheromone. Yeast is also employed in the prepartion of aromatic trifluoromethyl alcohols of value for inducing asymmetric reactions (Jones, 1986).

Alcohol dehydrogenases are ubiquitously distributed among plants, animals, yeasts, fungi and different types of bacteria (Branden et al., 1975). The enzyme catalyses the reaction

$$R\text{-}CHO + PNH + H^+ \rightleftharpoons RCH_2OH + PN^+$$

PN = pyridine nucleotide

(Sund and Theorell 1963). Sources of ADH which have been at least partially characterised, include yeast (YADH), Leuconostoc mesenteroides (Leuconostoc ADH), horse liver (HLADH), drosophila (DADH) and wheat germ. The alcohol dehydrogenases show considerable divergence in primary structure, quaternary structure, isozyme development, substrate specificity and other functional characteristics (Jornvall et al., 1984). YADH exhibits 23% sequence identity to HLADH (Jornvall , 1977; Eklund et al., 1985). The two most similar regions of the enzyme affect active site segments. In contrast, the structure of DADH has not revealed clear sequence similarities to mammalian ADH or YADH (Jornvall et al., 1981).

1.5.1 Yeast Alcohol Dehydrogenase

Bakers' yeast can produce at least three NAD-dependent alcohol dehydrogenases, one of which is associated with a particulate

fraction and two with the soluble portion of the cytoplasm (Heick et al., 1969). ADH I and ADH II are cytoplasmic enzymes while ADH III is a mitochondrial enzyme (Eltayeb and Berry, 1982).

ADH I, the classical fermentative isozyme, is responsible for the last step in the yeast glycolytic pathway, the reduction of acetaldehyde to ethanol (Racker, 1955; Lutsdorf and Megnet, 1968). ADH I has been considered to be produced constitutively (Schimpfessel, 1968; Lutstorf and Megnet, 1968; Fowler et al., 1972). However, Eltayeb and Berry (1977) presented evidence that ADH I may be induced by ethanol while Wills et al. (1980) indicated that ADH I is a repressible enzyme. Later Eltayeb and Berry (1982) showed that ADH I is absent from cells grown on acetate.

ADH II is considered to correspond to the enzyme isolated by Ebisuzaki and Barron (1957). The function of ADH II in the cell is to oxidise ethanol, formed during fermentation, to acetaldehyde, which can then be metabolised via the tricarboxylic acid cycle in the mitochondria and also serves as an intermediate in gluconeogenesis (Lutstorf and Megnet, 1968; Sugar et al., 1970; Wiesenfeld et al., 1975). ADH II is highly repressed by fermentative growth and is derepressed in the absence of a fermentable sugar such as glucose (Young and Pilgrim, 1985).

ADH III was discovered by Lutstorf and Megnet (1968). Sugar et al. (1970) confirmed its location in the mitochondrion. The metabolic function of ADH III is unknown, but indications are that it does not function fermentatively (Wills and Phelps, 1975). A role in respiratory metabolism has been suggested (Young and

Pilgrim, 1985). ADH III never contributes more than 10% of the total cellular alcohol dehydrogenase activity (Fowler et al., 1972; Wiesenfeld et al., 1975).

The three forms of ADH can be distinguished from each other by their heat sensitivity (Sugar et al., 1970). Thermal stability increases in the following order: ADH I, ADH II and ADH III. Inactivation of ADH III takes place only at temperatures above 70°C. ADH1 and ADH II can be distinguished by their activity with cinnamyl alcohol (Ebisuzaki and Barron, 1957). The isozymes of ADH can also be separated by gel electrophoresis. Three major bands were observed when the ioszymes were separated by discontinuous electrophoresis, corresponding to ADH I, II and III (Fowler et al., 1972). The isozymes have also been separated on starch and agarose gels (Sugar et al., 1971). Only minor differences between the mitochondrial and cytoplasmic isozymes were noted in the apparent Km's for NAD , NADH, ethanol and acetaldehyde (Wenger and Bernofsky, 1971).

1.5.2 Characterisation and Properties of Yeast Alcohol Dehydrogenases

The structures of the three isozymes of yeast alcohol dehydrogenase have been compared. The amino acid sequence of ADH I and II show only 22 differences out of 347 residues (Wills and Jornvall, 1979; Young et al., 1982) and the amino acid identity between ADH III and ADH I and ADH II is 79 and 80% respectively (Young and Pilgrim, 1985). Ganzhorn et al. (1987) used a three-dimensional model of yeast alcohol dehydrogenase, based on the homologous horse liver enzyme, to compare the substrate

binding pockets of the three isozymes. The active sites of ADH II and ADH III are the same, differing by only one residue (at position 294) from ADH I. Isozyme I from S. cerevisiae was found to correspond closely to the commercially available but taxonomically uncharacterised enzyme. After analysis of 86% of all positions, two amino acid differences were found among the 298 residues compared. The enzymes from these sources are thus about 99% identical (Wills and Jornvall, 1979). Most studies reported about yeast alcohol dehydrogenase have been performed with the fermentative isozyme (ADH I) or the commercial preparation.

Yeast alcohol dehydrogenase is a tetramer of molecular weight 140,000 to 150,000 as determined by ultra centrifugation and gel filtration (Branden et al., 1975). Despite the same apparent molecular weight, the native ADH III enzyme migrates in an unusual pattern on non-denaturing polyacrylamide gels (Wills and Phelps, 1975). Chelating agents,urea, sodium dodecyl sulphate and thiol oxidation have been used to dissociate the ADH I molecule into subunits. Most values observed for the molecular weight of the subunits are in the range 36,000 - 37,000 (Branden et al., 1975).

Studies on the Zn content, the number of S-H groups and coenzyme binding sites on the enzyme were performed using the fermentative enzyme (ADH I). It is generally accepted that the enzyme molecule contains four essential zinc atoms (Dickinson, 1970). Zinc binds the substrates and participates in an acid base system for removal of protons. A second zinc atom is also present in the catalytic domain, it has no known function (Jornvall et al., 1978). The enzyme possesses four active sites each of identical reactivity

(Hayes and Velick, 1954). It was thought that there were four coenzyme-binding sites on the enzyme but Dickinson (1970) showed using fluorescence measurements, gel filtration and a titration method that only three coenzyme-binding sites are available. This is in contrast with the established fact that the enzyme is composed of four subunits.

ADH I has 8 to 9 free S-H groups per subunit and is inhibited by thiol reagents (Branden et al., 1975). The enzyme is also inhibited by other compounds including inorganic and organic complex-forming agents, for example, o-phenanthroline which was also shown to inhibit ADH II (Ebisuzaki and Barron, 1957), heavy metals, NAD analogues, purine and pyridine derivatives and some drugs (Sund and Theorell, 1963).

1.5.3 Substrate Specificity

ADH I has been shown to be active toward a variety of substrates, it differs in many ways in its substrate specificity from HLADH (Branden et al., 1975). The isozyme oxidises primary straight-chain alcohols, but the reactivity with branched-chain alcohols as well as with secondary alcohols,hydroxy acids, polyalcohols and amino alcohols is very low (Sund and Theorell, 1963). Dickinson and Dalziel (1967) found that the yeast enzyme is completely inactive toward those secondary alcohols where both alkyl groups are larger than methyl and active with only one isomer of butan-2-ol and octan-2-ol. With the exception of methanol in the straight chain homologous series, the reactivity of ADH I decreases as the chain length increases (Sund and Theorell, 1963). The substitution of methionine with leucine in the

substrate binding site of ADH I produced a 7-10 fold increase in reactivity with butanol, pentanol and hexanol (Ganzhorn et al., 1987). They attributed this to tighter binding of the longer chain alcohols and to more rapid hydrogen transfer.

ADH II has a wide substrate specificity resembling but not identical to the classical alcohol dehydrogenase (ADH I), it is more able to oxidise the higher alcohols (Ebisuzaki and Barron, 1957). ADH II shows higher activity with n-propanol and n-butanol than ADH I (Ebisuzaki and Barron, 1957). The greatest difference of activity was illustrated with cinnamyl alcohol. Purified ADH II gave a C/E ratio of 0.4 whereas ADH I gave a ratio 0.04 to 0.075 (C/E ratio is the ratio of alcohol dehydrogenase activity with cinnamyl alcohol substrate relative to that obtained with ethanol). Wiesenfeld et al. (1975) looked at the substrate specificity of ADH III. The activity of the enzyme with alcohols and aldehydes was calculated relative to the activity with ethanol and acetaldehyde taken as 100%. They found that ADH III shows a preferential activity for alcohols with a double bond conjugated to the alcohol function.

Many authors studied the substrate specificity of commercial yeast alcohol dehydrogenase (Commercial ADH). Klinman (1972) found that this enzyme catalyses the reduction of a series of para-substituted benzaldehydes at different rates. The rate for the fastest benzaldehyde, p-Br, was 4% of the acetaldehyde rate, indicating that the benzaldehydes studied were relatively poor substrates of the enzyme. Klinman (1976) also studied the enzyme catalysed oxidation of aromatic alcohols. The rate of

oxidation of each para-substituted benzyl alcohol studied was approximately the same, at about 40% of the benzyl alcohol rate. Commercial ADH catalyses the conversion of trans-cinnamaldehyde to trans-cinnamyl alcohol (Bowen et al., (1986a). This alcohol is of significant value in the perfume industry. Bowen et al. (1986b) studied the inhibition of commercial ADH by aromatic and aliphatic aldehydes. Inhibition of the soluble enzyme varied from 92% for citral to 22% for benzaldehyde. o-tolualdehyde, p-totualdehyde and piperonaldehyde were not inhibitory. They state that none of these aldehydes are substrates for the enzyme.

1.5.4 Mechanism of Action of Alcohol Dehydrogenase

The mechanism of action of alcohol dehydrogenase has been studied in commercial preparations of the enzyme from various sources including liver and yeast (Cook and Cleland (1981a-c); Morris et al., 1980). Scharschmidt et al. (1984) studied the mechanism of the reaction catalysed by commercial ADH. The enzyme has a two-step chemical mechanism in which proton transfer from a zinc-bound alcohol to an acid-base catalytic group precedes hydride transfer. In a step prior to hydride transfer the N-1 of the nicotinamide ring of the nucleotide becomes pyramidal so that the aromaticity of the ring is destroyed and a carbonium ion forms at C-4. Hydride transfer occurs between a metal-bound alkoxide and a carbonium ion or, in the reverse reaction, between a metal-bound carbonyl group and the electron-rich dihydronicotinamide ring. Klinman (1976), when studying para-substituted benzaldehydes and benzyl alcohols as substrates for commercial ADH concluded that the binding of benzaldehydes to the enzyme depended on the electronic properties of the para-substituents, whereas

hydrophobic interactions were important for the binding of benzyl alcohol. The difference is surprising as benzaldehyde and benzyl alcohol are expected to bind at the same active site.

1.6 BIOTRANSFORMATIONS

Biotransformations should be considered as selective, enzymatic modifications of defined pure compounds into defined final products (Kieslich, 1984). Biotransformations can be accomplished with growing cells, cells in stationary phase, spores or even dried cells (Demain and Solomon, 1981). They are carried out using free or immobilised whole cells or isolated enzymes in crude or purified form. The scope of reactions which can be catalysed by microbial enzymes covers nearly all types of chemical reactions (Kieslich, 1976). Plimmer (1903), was among the first to organise reactions typical to biocatalysis into categories such as oxidations, reductions, hydrolysis, nitrification, denitrification, simple fermentations and the formation of optically active products.

Biotransformations have characteristics which are typical for enzyme reactions. The catalytic activity is usually restricted to a single reaction type, this prevents side reactions when only one enzyme is involved (Leuenberger, 1984). Microbial reactions can selectively introduce functional groups at a certain non-activated position in a molecule. An example is the 11-α-hydroxylation of progesterone with Rhizopus nigricans or Rhizopus arrhizus (Peterson and Murray, 1952). This is a crucial step in the synthesis of cortisone and it shortens the procedure from 37 steps (chemical synthesis) to 11. The microbial hydroxylation of

progesterone has economic consequences beyond abbreviating the chemical synthesis. The fermentation can be done at 37°C, with water as the solvent and at atmospheric pressure. Reactions under these conditions are much cheaper than those carried out under extremes of temperature and pressure and with solvents other than water, which had been required in the chemical synthesis of cortisone (Aharonowitz and Cohen, 1981). Due to their regioselectivity, biotransformations can differentiate between several functional groups of similar reactivity within the same molecule. Racemic mixtures which are often produced in chemical reactions are difficult to separate and pharmaceuticals whose action usually involves specific receptor-drug binding are usually stereospecific. Biotransformations provide the possibility to modify only one enantiomer of a racemic mixture. For example, several L-amino acids have been produced from acyl-DL-amino acids (Chibata et al. 1972; Eveleigh, 1981).

1.6.1 Design of Biotransformation Processes

For a successful cellular biotransformation, it is necessary that the substrate molecule comes into contact with the enzyme such that the catalytic capability of the micro-organism is not inactivated by the substrate or its product (Leuenberger, 1984). The substrate may be soluble in the biotransformation medium. If it is not, it may be dissolved in a non-toxic water miscible solvent; otherwise solubility may be enhanced by adding an emulsifying agent or by grinding crystalline substrates to micron size particles (Kondo and Masuo, 1961).

Many enzymes such as oxidoreductases and kinases, display their activity only in the presence of a coenzyme (e.g. NAD(H), NADP(H), FAD(H_2), ADP/ATP). The economic feasibility of such enzymes depends on an efficient coenzyme regenerating system. Several methods involving enzymatic, chemical or electrochemical regeneration of NAD(H) or NADP(H) have been reported (Wang and King, 1979). For example, in the formation of L-leucine from α-ketoisocaproate by the NADH dependent L-leucine dehydrogenase, the coenzyme (NADH) may be regenerated by running a parallel reaction in the same reactor: oxidation of formiate to CO_2 by formiate dehydrogenase which reduces NAD (Wichmann et al., 1982). Another enzymatic method to efficiently regenerate cofactors uses hydrogenase containing micro-organisms to catalyse the reduction of oxidised electron carriers with molecular hydrogen (Simon et al., 1974; Klibanov and Puglisi, 1980). For example, Klibanov and Puglisi employed immobilised whole cells of Alcaligenes eutrophus in the presence of hydrogen for the regeneration of nicotinamide and flavin coenzymes as well as for the reduction of artificial cofactors such as phenazine methosulphate, janus green, methylene blue and 2,6-dichlorophenol-indophenol.

A substrate may be added to the fermentation medium at the time of inoculation; this is favourable if the level of the enzyme responsible for the biotransformation is enhanced by induction. In many cases substrate is not added until maximum cell mass is obtained (batch culture), (Sariaslani and Rosazza, 1984). Cells produced by batch incubations may also be removed from the fermentation medium, harvested and resuspended in defined,

buffered medium. Biotransformations which belong to this category are those catalysed by commercially available bakers' yeast (Leuenberger, 1984).

Once a feasible biotransformation process has been established, it is necessary to try to improve the yield and reaction rate. Environmental conditions such as medium composition, temperature, pH and dissolved oxygen should be optimised (Sariaslani and Rosazza, 1984). (Optimum conditions for growth of cells with high enzyme activity may differ from those which are optimal for the biotransformation). Any one of these variables may be linked to physiological mechanisms which influence the types and amounts of products formed. Such mechanisms include catabolite repression and the transport of substrates to catalytic sites within microbial cells. Genetic strain improvement allows broad scope for increased productivity. Genetic modifications may be induced and mutants which may, for example, be constitutive for a specific enzyme can be selected (Demain, 1971). Other targets of genetic development may be enhancement of substrate/product tolerance, inactivation of enzymes catalysing side reactions and increase of membrane permeability facilitating substrate transport into the cell and product efflux from the cell. For example, in the production of monosodium glutamate (MSG) by Corynebacterium glutamicum, the product is released by making the cell membranes 'leaky'. This is done by the addition of saturated fatty acids, detergents or penicillin. Genetically altered strains could yield high levels of MSG without the need for such expensive additives (Eveleigh, 1981). Recombinant DNA technology provides new

prospects of strain improvement. An example is the construcion of a new Escherichia coli strain via recombinant DNA producing a 7 times increased level of penicillin acylase (Mayer et al., 1980).

Side reactions in a biotransformation process are observed if a substrate or its product is attacked by undesired enzyme activities present in the cell (Leuenberger, 1984). Selective inactivation of enzymes, which are responsible for side reactions, by physical and/or chemical treatment of biomass may be used to eliminate side reactions. For example, in the conversion of fumaric acid to L-aspartic acid (by aspartase) by E. coli cells, the fumaric acid is also converted to L-malic acid by the enzyme fumarase. This reaction is eliminated by treating the culture broth at pH 5 and 45°C for 1 h (Takamatsu et al., 1982). Another approach is mutagenisation and screening of mutants which have lost the ability to form enzymes responsible for side reactions. For example, Shibuya et al. (1981) isolated a Pseudomonas sp capable of deacylating glutaryl-7-aminocephalosporanic acid as a starting material for semisynthetic cephalosporins. This strain also contained β-lactamase activity which destroyed the antibiotic. After mutagenic treatment of the original strain with N-methyl-N-nitro-N'-nitrosoguanidine (NTG), this problem was avoided by selecting a β-lactamase-deficient mutant with high acylase activity.

Usually biotransformation products are extracellular compounds of low or medium molecular weight, which may be isolated from the fermentation medium after removal of the biomass. Product isolation involves subsequent concentration, fractionation and

final purification (Belter, 1979; Voser 1982). Lipophilic products are usually separated by extraction using a water immiscible organic solvent. Hydrophilic products not extractible by organic solvents can be isolated either on solid or liquid ion exchangers (Kunin and Winger, 1962) or by selective adsorption to polymeric resins. Final purification is accomplished in most cases either by crystallisation, drying, fractionated distillation or column chromatography.

1.6.2 Other Methods for Carrying Out Biotransformation Processes

Other methods for carrying out biotransformation processes include the use of purified enzymes, multiphase systems and immobilisation techniques utilising pure enzymes or cells (Demain and Solomon, 1981). Enzyme which are active in organic solvents are also of use in biotransformations.

Pure enzymes used for biotransformations can be obtained commercially or purified from a fermentation broth. These enzymes may be divided into two broad categories based on the relative complexities of the biotransformation system (Sariaslani and Rosazza, 1984). Some require no cofactors or additional components to catalyse the reaction. Examples include the hydrolases and glucose isomerase. The other group of enzymes is made up of complicated systems consisting of an enzyme, coenzymes and additional components often involved in the transfer or removal of electrons to and from substrates. The latter group of enzymes includes the monooxygenases, oxidoreductases, dioxygenases and those containing, for example, thiamine pyrophosphate. Enzyme

immobilization permits the continuous use of enzymes and the opportunity to run continuous reactors (Bowen et al., 1986a). Many reactions involving enzymes which require coenzymes are not economically feasible unless an efficient coenzyme regenerating system is run in parallel (Wang and King, 1979; Sariaslani and Rosazza, 1984).

The biotransformation of lipophilic substrates is limited by their solubility in water. However, biotransformations of substrates with low solubility in water have been successfully applied with two liquid phase systems consisting of water and a poorly water miscible solvent (Antonini et al., 1981). The micro-organism remains in the aqueous phase while the reaction can take place in this phase or at the interface between the two phases.
This technique has been used in various steroid biotransformations (Buckland et al., 1975; Fukui and Tanaka, 1981).

Kibanov (1986) found that many enzymes can act as catalysts in nearly anhydrous organic solvents (as distinct from multiphase systems). The only water required is a layer around the enzyme molecule to maintain its native, active conformation. He has found that lipases and horseradish peroxidase are from 10 - 100% as active in optimal organic media as they are in water. In such systems the equilibrium of reactions catalysed by hydrolytic enzymes is reversed from hydrolysis to the synthesis of organic compounds accompanied by water formation (Klibanov et al., 1977). An advantage of such a system is that organic substrates dissolve better in organic media than they do in water, while enzymes are insoluble in the organic media. This allows easy recovery and

reuse of the enzyme. Also, dissolved oxygen levels, important in many biocatalytic reactions, are higher in non-aqueous solvents than in water (Sariaslani and Rosazza, 1984).

1.6.3 Biotransformation with Immobilised Cells

Biotransformation with immobilised cells is attractive because the cells are more stable than those in free solution, they are more easily recovered from the reaction mixture and can be used repeatedly (Demain and Solomon, 1981). Immobilised cells have been used in continuous processes for the conversion of compounds like steroids, organic acids, sugars and antibiotics (Koscheyenko et al., 1981; Koscheyenko et al., 1983). When cells are immobilised the biotransformation can be continuously operated where a favourable substrate concentration can be maintained for a long time. An advantage of cell immobilisation over immobilisation of enzymes is that the enzyme remains in its natural environment within the cell and is therefore more stable (Chibata et al., 1986), on the other hand, whole cells are a disadvantage if other enzymes catalyse undesirable reactions.

Among the various methods of cell immobilisation, entrappment into polymerous matrix materials (for example, polyacrylamide gel or natural polymers such as carrageenan, alginate, collagen, cellulose and agar) have been most extensively applied for both small scale laboratory procedures and for large scale industrial applications. Technical application of entrapped cells has been used in Japan since around 1973. Escherichia coli cells with high aspartase activity have been immobilised in polyacrylamide gel (Sato et al., 1975) or in K-carrageenan (Sato et al., 1979). This process is

applied for the commercial production of L-aspartic acid from fumaric acid. Vieth and Venkatasubramanian (1976) used Streptomyces venezuelae and Bacillus species entrapped in a reconstituted collagen membrane for the isomerization of glucose. Methods of entrappment have also been applied to the biotransformation of steroids (Ohlson et al., 1979; Constantinides, 1980; Fukui and Tanaka, 1981).

This project will involve investigations of the bioconversion of aldehydes by the yeast Saccharomyces cerevisiae to the corresponding acyloin compound, in particular, the conversion of benzaldehyde to phenylacetyl carbinol (PAC). Initial work deals with standardisation of the bioconversion conditions and the setting up of various analytical techniques, including gas chromatography, high performance liquid chromatography and colorimetric and enzymic assays. With these basic tools, the main aims include: optimisation of conditions to give maximal initial rate of PAC production with a view to obtaining more prolonged high rates of conversion, investigation of the substrate specificity of the system, and study of the toxicity of substrate and products to the yeast cell. Yeast cell immobilization is briefly investigated as a means of protecting the cells. The role of the isozymes of yeast alcohol dehydrogenase in the conversion of benzaldehyde to the benzyl alcohol by-product is also studied.

2. MATERIALS AND METHODS

2.1 CHEMICALS

Agar (technical), malt agar, yeast extract, peptone (technical) and malt extract were obtained from Difco, Detroit, MI, U.S.A. Bacteriological peptone was obtained from Oxoid Ltd., Basingstoke, U.K. Acetyl benzoyl, creatine, bovine serum albumin, semicarbazide hydrochloride, glutathione (GSH), NAD, NADH, yeast alcohol dehydrogenase, yeast pyruvate decraboxylase, thiamine pyrophosphate, fatty acid standards, phenazine methosulphate, nitro blue tetrazolium, nondenatured protein molecular weight markers, boron trifluoride-methanol and glass distilled hexane were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. and St. Louis, MO, U.S.A. α-Naphthol, 3,5-Dinitrosalicylic acid, sodium pyruvate, acetaldehyde, propionaldehyde and sodium alginate were obtained from B.D.H. Ltd., Canada. Bradford reagent, acrylamide, N,N'-methylenebisacrylamide, N, N, N', N'-tetramethylethylene-diamine, ammonium persulphate, bromophenol blue and coomassie brilliant blue R were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Acetoin, methyl mandelate and all aromatic aldehydes and alcohols were obtained from Aldrich Chemical Co. Ltd., Milwaukee, WN, U.S.A. Invertase was obtained from Biocon Ltd., Ireland. H.P.L.C. grade methanol was purchased from Labscan Ltd., Ireland. All other chemicals used were of analytical reagent grade.

2.2 MICRO-ORGANISMS

2.2.1 Bakers' Yeast

Commercial, fresh pressed bakers' yeast, 30% w/v solids content, was

used throughout this work. It was obtained from Irish Yeast Products Ltd., Dublin, Ireland and East End Bakery Ltd., Kitchener, Ontario.

2.2.2 Yeast Cultures

Source of Strains

All other yeast strains were obtained from Labatts Brewing Co. Ltd., London, Ontario.

Growth and maintenance of cultures

Stock cultures were maintained on agar slopes of medium E (Wickerham, 1951), subcultured monthly and stored at 4°C. Cultures were incubated at 30°C and grew within 2-3 days. Cultures were grown in medium D and incubated as described in Section 2.2.4.

2.2.3 Bacterial Cultures

Source:

Lactobacillus plantarum and *Streptococcus faecalis* were obtained from the American Type Culture Collection, Rockville, MD, U.S.A. *Erwinia amylovora* and *Enterobacter aerogenes* were obtained from Northern Regional Research Laboratories, Preoria, Il, U.S.A.

Growth and maintenance of cultures

Bacteria were grown in the media and conditions described in Table 2.1. Cultures were maintained on agar slopes of the same media at 4°C.

TABLE 2.1 Optimum media and temperatures for the growth and maintenance of bacterial cultures. Cultures were grown on shaking incubators as described in Section 2.2.4 at the appropriate temperature.

Bacterium/Strain	Medium	Temperature
Lactobacillus plantarum ATCC 8014	Lactobacilli MRS	37°C
Erwinia amylovora NRRL B-127	Nutrient broth + 5% (w/v) sucrose	30°C
Enterobacter aerogenes NRRL B-494	Nutrient broth	30°C
Streptococcus faecalis ATCC 19433	Brain Heart infusion	37°C

2.2.4 Shake Flask Cultivation and Fermentations

Throughout the experimental work shake flask cultures were incubated at 30°C on an LH Engineering 2-tier orbital shaker (model MK 11/111) at 150 r.p.m. with a displacement of 50 mm.

2.2.5 Biomass Determination

Dry weight analysis

Yeast cells were removed from a measured volume of fermentation broth by centrifugation (7,500 g for 10 min.) and washed twice with 0.85% (w/v) NaCl. The centrifuged pellet was dried to a constant weight at 110°C in an aluminium foil dish. Dishes were coded in a vacuum dessicator before weighing. Determinations were carried out in duplicate.

Absorbance measurements

Biomass was also monitored by measuring the absorbance of the culture at 600 nm, in a 1 cm light path. A blank, containing the appropriate sterile medium, was used to zero the spectrophotometer.

2.2.6 Cell Viability Measurements

Viability of yeast populations was determined by pour-plating yeast suspensions, appropriately diluted in 0.85% (w/v) NaCl, with malt agar. Plates were incubated aerobically at 30°C for 48 h, and colony counted.

In the case of cells encapsulated in sodium alginate, 10 beads were dissolved in 15 ml of 0.1 M sodium citrate buffer pH 7.5 (Appendix A, 1) at 4°C. The resulting cell suspension was then diluted and viable cells counted as described above. All determinations were carried out in duplicate.

2.2.7 Fermentation and Growth Media

Medium A: (PAC Biotransformation medium I)

Sucrose (carbon source), 50 g/l; Citric acid, 10.5 g/l; Bacteriological peptone, 6 g/l, at pH 4.5.

Medium B: (PAC Biotransformation medium II)

Medium B was the same as medium A except that sodium pyruvate, 61.7 g/l, was used as the carbon source.

Medium C: (Biotransformation medium for encapsulated cells)

Sucrose, 50 g/l; bacteriological peptone, 6 g/l; succinic acid, 6 g/l; Calcium chloride, 0.15 g/l at pH 4.5.

Medium D: (Yeast growth medium)

Yeast extract, 10 g/l; peptone (technical), 20 g/l; glucose, 20 g/l at pH 4.5.

Medium E: (Yeast growth/maintenance medium)

Glucose, 40 g/l; peptone (technical), 5 g/l; yeast extract, 3 g/l; malt extract, 3 g/l at pH 5.5.

Source of media formulations

Media A, B and C were developed during the course of this work. Medium D is that described by Barrera and Corral (1980). Medium E is that of Wickerham (1951).

All media were adjusted to the appropriate pH with NaOH or HCl.

2.2.8 Sterilization Procedures

Growth media were sterilized in conical flasks at 121°C and 15 p.s.i. for 15-20 min.

2.3 ANALYTICAL PROCEDURES

2.3.1 Colorimetric Determination of L-Phenylacetyl Carbinol

PAC was estimated using the colorimetric method of Groger and Erge (1965). The method is based on the Voges-Proskauer reaction.

Reagent 1: 1 g creatine in 200 ml distilled water.

Reagent 2: 1 g α-naphthol in 20 ml 2.5 M sodium hydroxide.

Reagent 3: 50% (v/v) ethanol solution.

Reagent 2 must be freshly prepared before the assay.

Procedure:

A standard curve was prepared using a range of acetyl benzoyl solutions, 0.0-0.05 g/l. Unknown PAC solutions were determined within this concentration range using the appropriate correction factor (Section 3.2.3).

Method:

1. Tubes were prepared as follows:-

	Analytical	Reagent Blank
PAC sample/standard	2.5 ml	-
Distilled Water	-	2.5 ml
Reagent 1	1.0 ml	1.0 ml
Reagent 2	1.0 ml	1.0 ml
Reagent 3	2.5 ml	2.5 ml

2. Tubes were mixed well and placed in darkness for 30 min. for colour development.
3. The absorbance at 580 nm in a 1 cm light path was read using the reagent blank to zero the spectrophotometer.
4. The absorbance at 580 nm vs Acetyl benzoyl concentration (g/l) was plotted.
5. PAC concentrations of unknown solutions were determined from the standard curve using the correction factor (Section 3.2.2). All determinations were carried out in duplicate.

2.3.2 Estimation of Acetoin and other Aliphatic Acyloin Compounds

Aliphatic acyloin compounds were estimated using the method of Westerfeld (1945).

Reagent 1: 1 g of creatine dissolved in 200 ml of distilled water

Reagent 2: 1 g of α-naphthol dissolved in 20 ml of 2.5 M sodium hydroxide, prepared immediately before use.

Procedure:

A standard curve was prepared using a range of solutions containing between 1.0 and 12.0 µg of acetoin. Unknown acetoin solutions were determined within this concentration range.

Method:

1. Tubes were prepared as follows:-

	Analytical	Reagent Blank
Acetoin solution	5.0 ml	-
Distilled water	-	5.0 ml
Reagent 1	1.0 ml	1.0 ml
Reagent 2	1.0 ml	1.0 ml

2. Tubes were mixed and placed in the dark for 1 h.
3. The absorbance at 540 nm in a 1 cm light path was read using the reagent blank to zero the spectrophotometer.
4. The absorbance at 540 nm vs acetoin (µg) was plotted.
5. Acetoin concentrations of unknown concentration were determined from the standard curve. All determinations were carried out in duplicate.

2.3.3 Estimation of Sucrose

Sucrose concentration was estimated using the Dinitrosalicylic acid (D.N.S.) method (Miller, 1959). Results were expressed as reducing equivalents g/l using sucrose as standard.

D.N.S. reagent:

3, 5-dinitrosalicylic acid, 10 g/l; potassium sodium tartarate, 300 g/l; sodium hydroxide, 16 g/l were dissolved in 600 ml distilled water by heating, without boiling. The solution was cooled and diluted to 1 litre.

Procedure:

Before assaying, standards and samples were incubated with invertase enzyme (a 1 in 10 dilution of the stock enzyme was sufficient to hydrolyse the sucrose into its components, glucose and fructose). 1 ml of enzyme solution was added to 10 ml of sucrose solution and incubated for 30 min. in a water bath at 30°C.

A standard curve was prepared using sucrose solutions in the range 0.5-2.5 g/l. Unknown sucrose concentrations were determined within this concentration range.

Method:

1. Tubes were prepared as follows:-

	Analytical	Reagent Blank
Reducing sugar soln.	1.0 ml	-
Distilled water	1.0 ml	2.0 ml
D.N.S. reagent	2.0 ml	2.0 ml

2. Tubes were placed in a boiling water bath for 10 min., then cooled.
3. 10 ml distilled water were added to each tube and the contents mixed.
4. The absorbance at 540 nm in a 1 cm light path was read using the reagent blank to zero the spectrophotometer.
5. The absorbance at 540 nm vs sucrose concentration (g/l) was plotted.
6. Sucrose concentrations of unknown solutions were determined from the standard curve. All determinations were carried out in duplicate.

2.3.4 Estimation of Protein

Protein determinations for specific activity measurements were estimated using the Bradford method (Bradford, 1976).

Bradford Reagent:

The Bradford reagent, which contains Coomassie Brilliant Blue G 250, 0.01% (w/v); ethanol, 4.7% (w/v); and phosphoric acid, 8.5% (w/v) was obtained commercially (Section 2.1).

Procedure:

A standard curve was prepared using bovine serum albumin (BSA) as a standard. Standard solutions contained 10-100 µg of protein and unknown protein solutions were determined within this range. Protein solutions were prepared in the appropriate buffer.

Method:

1. Tubes were prepared as follows:-

	Analytical	Reagent Blank
Protein Solution	0.1 ml	-
Buffer	-	0.1 ml
Bradford Reagent	5.0 ml	5.0 ml

2. Tubes were mixed and the absorbance at 595 nm in a 1 cm light path was measured after 2 min. and before 60 min. against a reagent blank.
3. The absorbance at 595 nm vs protein concentration was plotted.
4. Protein concentrations of unknown solutions were determined from the protein (BSA) standard curve.

2.3.5 Estimation of Total Cellular Protein

Total cellular protein was determined using a modification of the Biuret method (Robinson and Hogden, 1940).

Method:

Yeast cells, removed from the fermentation broth, were washed thoroughly with a 0.85% (w/v) NaCl solution. 2 ml aliquots of washed cell suspension, containing 1-5 mg dry weight per ml and 1 ml of 3 M NaOH were measured into a 5 ml centrifuge tube and placed in a boiling bath for 5 min. When cool, 1 ml of 2.5% $CuSO_4$ was added, shaking thoroughly, and the reaction mixture was allowed to stand for 5 min. Standard BSA solutions were treated in the same manner (2.5-10 g/l). The precipitate formed was removed by centrifugation (7,000 g for 10 min.) and the supernatants were read against a reagent blank at 555 nm in a 1 cm light path in a

spectrophotometer. Protein contents of cell suspensions were determined from a curve of absorbance vs concentration of protein standards.

2.3.6 Estimation of Total Cellular Carbohydrate

Total cellular carbohydrate was determined using the method of Dubois (1956).

Reagent 1: 5% (w/v) solution of phenol in distilled water.

Reagent 2: Concentrated sulphuric acid (sp. gr. 1.84)

Procedure:

A standard curve was prepared using solutions containing 20-100 µg glucose. Unknown carbohydrate solutions were determined within this range.

Method:

1. Tubes were prepared as follows:-

	Analytical	Reagent Blank
Carbohydrate solution	1.0 ml	-
Distilled water	-	1.0 ml
Reagent 1	1.0 ml	1.0 ml
Reagent 2	5.0 ml	5.0 ml

   Reagent 2 should be added from a fast-flowing pipette, directing the stream of acid onto the surface of the liquid and shaking the tube simultaneously.

2. The tubes were allowed to stand for 10 min. shaken, and placed in a water-bath at 25°C to 30°C for 10 to 20 min.

3. The absorbance at 588 nm in a 1 cm light path was read using the reagent blank to zero the spectrophotometer.

4. The absorbance at 588 nm vs Glucose (µg) was plotted and unknown carbohydrate concentrations were determined from the standard curve.

2.3.7 Estimation of Ethanol

Ethanol was measured using a uv method (Beutler and Michal, 1977; Beutler, 1984). The test is based on the following principle:-

$$\text{Ethanol} + NAD^+ \overset{ADH}{\rightleftharpoons} \text{acetaldehyde} + NADH + H^+$$

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{\text{Al-DH}} \text{acetic acid} + NADH + H^+$$

NADH is determined by means of its absorbance at 340 nm in a 1 cm light path.

Note: ADH = alcohol dehydrogenase

Al-DH = aldehyde dehydrogenase

A test combination (Boehringer Mannheim GmbH, Germany) was used to estimate ethanol.

Reagent 1: Potassium diphosphate buffer, pH 9.0; NAD, approximately 1.3 mg/ml; aldehyde dehydrogenase, approximately 2.6 U/ml.

Reagent 2: Alcohol dehydrogenase, 6250 U/ml.

Measurements were made in glass or disposable cuvettes (1 cm light path) at 20-25°C.

Method:

1. Pipette into cuvettes

	Blank	Sample
Reagent 1	3.00 ml	3.00 ml
Distilled water	0.10 ml	-
Sample solution	-	0.10 ml

Mix, after approximately 3 min. the absorbances of the solutions were read against a water blank (A_1).

Reaction was started by addition of

Reagent 2	0.05 ml	0.05 ml

Mix, after completion of the reaction (approximately 5-10 min.), the absorbances of the solutions were read immediately one after the other (A_2).

2. The absorbance differences ($A_2 - A_1$) were determined for both blank and sample. The absorbance difference of the blank was subtracted from the absorbance difference of the sample.

$$\Delta A = \Delta A \text{ sample} - \Delta A \text{ blank}$$

For accurate results:-

$$0.50 \geq \Delta A \geq 0.10$$

Calculation:

According to the general equation for calculating the concentration in reactions in which the amount of NADH formed is stoichiometric with half the amount of substrate:

$$C = \frac{V \times MW}{\epsilon \times d \times v \times 2 \times 1000} \times \Delta A \text{ (g/l)}, \text{ where}$$

V = final volume (ml) (= 3.25 ml)
v = sample volume (ml)
MW = molecular weight of the substance to be assayed (g/mol)
d = light path (cm)
ϵ = absorption coefficient of NADH at 340 nm = 6.3 ($1 \times mmol^{-1} \times cm^{-1}$)

For Ethanol:

$$C = \frac{3.15 \times 46.07}{\epsilon \times 1 \times 0.1 \times 2 \times 1000} \times \Delta A = \frac{0.7256}{\epsilon} \times \Delta A \text{ g/l Ethanol}$$

2.3.8 Estimation of Alcohol Dehydrogenase Activity

Yeast alcohol dehydrogenase activity was determined as described by Bergmeyer (1983). The assay is based on the following principle:-

$$C_2H_5OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+$$

Enzyme activity is directly proportional to an increase in NADH concentration. This corresponds to an increase in absorbance at 340 nm (in a 1 cm light path).

Note: ADH = alcohol dehydrogenase

Method:

1. The assay mixture was prepared in a 3 ml glass or disposable cuvette (1 cm light path) as follows:-

		Conc. in Assay
2.50 ml	Sodium pyrophosphate buffer (0.1 M; pH 9; containing 1.67 mg glycine/ml)	85.5 mM 19.1 mM Glycine
0.10 ml	Semicarbazide hydrochloride (250 mg/ml; pH ca. 6.5)	6.2 mM
0.1 ml	Ethanol (96%)	0.6 M
0.01 ml	GSH (90 mg/ml)	1.0 M
0.20 ml	NAD (20 mg/ml)	1.8 mM
0.02 ml	Enzyme solution	

The assay was started by the addition of enzyme.

2. The rate of increase in absorbance at 340 nm was measured using

a chart recorder (American Scientific Instruments) set at a chart speed of 5 cm/min.

3. NADH concentration was calculated using an absorption coefficient of 6.3 l x $mmol^{-1}$ x cm^{-1} at 340 nm. One unit of activity produces 1 µmole of NADH per min. at pH 9 and 25°C.

2.3.9 Estimation of Pyruvate Decarboxylase Activity

Yeast pyruvate decarboxylase activity was determined according to the method of Ullrich et al. (1966). The assay is based on the following principle:-

$$\text{Pyruvate} \xrightarrow{\text{PyDC}} \text{Acetaldehyde} + CO_2$$

$$\text{Acetaldehyde} + \text{NADH} + H^+ \xrightarrow{\text{ADH}} \text{Ethanol} + \text{NAD}$$

Pyruvate decarboxylase activity is directly proportional to a decrease in NADH concentration, which corresponds to a decrease in absorbance at 340 nm (in a 1 cm light path).

Note: PyDC = pyruvate decarboxylase

ADH = alcohol dehydrogenase

Method:

1. The assay mixture was prepared in a 3 ml glass or disposable cuvette (1 cm light path) as follows:-

	Conc. in Assay
2.79 ml Citrate buffer (0.2M; pH 6.0)	0.186 M
0.10 ml Pyruvate, Na salt (100 mg/ml)	30 mM
0.08 ml NADH, Na salt (10 mg/ml)	0.32 mM
0.01 ml ADH, yeast (10mg/ml)	33 µg/ml
0.02 ml Enzyme solution in buffer	

The assay was started by the addition of the pyruvate decarboxylase enzyme.

2. The decrease in absorbance at 340 nm was measured using a chart recorder (Section 2.3.8).

3. One unit of activity produces 1 μmole of NAD per min. at pH 6 and 25°C.

2.3.10 Nuclear Magnetic Resonance Spectroscopy ('Hnmr)

'Hnmr spectra were recorded on a Perkin Elmer R-12B 60 MHz spectrometer. Solutions were prepared in deuteriochloroform with 1% tetramethylsilane (TMS) as internal standard.

2.3.11 Optical Rotation Measurements

Optical rotations were measured on an Optical Activity AA5 polarimeter, in a 1 cm^3 sample tube (path length 50 mm). For optical activity measurements L-3-methoxyphenylacetyl carbinol and L-4-methylphenylacetyl carbinol were dissolved in ethanol. L-4-chlorophenylacetyl carbinol was dissolved in chloroform.

2.4 GAS CHROMATOGRAPHY (GC)

2.4.1 Instrumentation

For the analysis of PAC, benzyl alcohol, and benzaldehyde by GC two gas chromatographs were used during the course of the work.

(a) Perkin Elmer F17 gas chromatograph with Flame ionization detector (FID) attached to a Carlo Erba SP 4720 integrator.

(b) Hewlett Packard 5830A gas chromatograph with FID.

Fatty acid methyl esters were analysed on a Hewlett Packard 5710A gas chromatograph with FID.

2.4.2 Analysis of L-Phenylacetyl Carbinol, Benzaldehyde and benzyl alcohol by GC

Column:

6 ft long x 0.25 in OD glass column packed with 30% silicone elastomer E301 on Chromosorb WHP 60-80. (Phase separations Ltd., Clwyd, Wales).

Conditions:

Injection temperature:	(a) 240°C (Hewlett Packard 5830A)
	(b) 225°C (Perkin Elmer F17)
Oven temperature:	(a) 155°C for 2 min, then changed at a rate of 3°C/min. to 195°C (Hewlett Packard 5830A)
	(b) Isothermal, 195°C (Perkin Elmer F17)
Detector temperature:	(a) 250°C (Hewlett Packard 5830A)
	(b) 195°C (Perkin Elmer F17)
Carrier gas (Nitrogen) flow rate:	35 ml/min.
Hydrogen pressure:	15 p.s.i.
Air pressure:	23 p.s.i.

Sample preparation:

Aqueous benzaldehyde, benzyl alcohol, and PAC standards and fermentation broth (10 ml) were extracted with ether (2 x 10 ml) and the ether extract made up to 20 ml. 1 µl volumes were injected. A sample chromatogram is presented in Appendix B.

2.4.3 Analysis of Fatty Acid Methyl Esters by GC

Column:

Supelcowax 10 column, 50 m by 0.25 mm internal diameter, film

thickness, 0.25 µm. (Supelco, U.S.A.).

Conditions:

Injection temperature: 300°C

Oven temperature: Changed from 130°C to 180°C at a rate of 2°C/min.

Carrier gas (Helium) flow rate: 2 ml/min.

Hydrogen pressure: 20 p.s.i.

Air pressure: 30 p.s.i.

Sample preparation:

Fatty acid methyl esters were prepared as described in Section 2.13.1. A sample chromatogram is presented in Appendix B.

2.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (H.P.L.C.)

2.5.1 Instrumentation

H.P.L.C. was carried out using the following Waters Associates (Milford, MA, U.S.A.) instruments: M600 pump; U6K injector; Model 440 absorbance detector. Peaks were detected using a Carlo Erba SP 4720 integrator.

2.5.2 Analysis of Aromatic Aldehyde and Aromatic Alcohol by H.P.L.C.

Column:

The column was a µBondapak TM/C18 reverse phase steel column (3.0 mm x 300 mm), obtained from Waters Associates, Milford, MA, U.S.A. It was stored in methanol.

Conditions:

Mobile phase: 40% (v/v) methanol in distilled water

Flow rate: 1 ml/min

Detector wavelength: Fixed at 254 nm

All solvents were filtered (0.45 µm filter, Sartorius, England) and degassed under vacuum.

Sample preparation:

Solutions containing aromatic aldehyde and/or aromatic alcohol were filtered through a 0.45 µm filter. 15 µl samples were injected. A sample chromatogram is presented in Appendix B.

2.6 PREPARATION OF PURE L-ACETYL AROMATIC CARBINOLS

2.6.1 Synthesis of Phenylacetyl Carbinol

(D-/L-)PAC was prepared using (D-/L-) methyl mandelate as a starting material. Although optically active methyl mandelate is commercially available, the less expensive racemic (D-/L-) form was used as the racemic form of PAC would show identical behaviour (GC, NMR) to the fermentation product, L-phenylacetyl carbinol, except for the absence of an optical rotation.

A solution of (D-/L-) methyl mandelate (10g) in ethanol (17 ml) was saturated with dry ammonia at room temperature initially, and then at 0°C. The crystals which separated after standing overnight were collected and washed with small portions of dry ether. The yield of (D-/L-) mandelamide was approximately 5.0 g.

A solution of the Grignard reagent, MeMgBr was prepared by adding methyl iodide (70 g) to magnesium (12 g) in dry ether (130 ml). To this solution (D-/L-) mandelamide (10 g) was added slowly. After complete addition the solution was retained at room temperature and then refluxed for 12 h. The solution was cooled on ice and dilute

sulphuric acid was added. The solution was extracted with ether (2 x 50 ml). The ether extracts were combined and the solvent removed under vacuum. The remaining liquid was distilled under vacuum to give (D-/L-) phenylacetyl carbinol (approximately 2 g).

2.6.2 Isolation of Pure L-Phenylacetyl Carbinol from a Fermentation Broth

L-phenylacetyl carbinol was extracted using a modification of the procedure reported by Smith and Hendlin (1953). This method does not separate aldehyde and carbinol. When the benzaldehyde substrate was depleted, the total fermentation broth was extracted with an equal volume of ether. The separated ether fraction was vacuum evaporated at 40°C to constant volume. This concentrate was added to 100 ml ether and extracted with an equal volume of aqueous sodium carbonate to remove any acids present. The ether fraction was then extracted three times with an equal volume of aqueous 10% (w/v) sodium metabisulphite which forms a complex with the carbinol (and aldehyde when present). The combined aqueous extract was washed with ether (2 x 100 ml), to remove any alcohol present. Solid $NaHCO_3$ was added to the aqueous extract until CO_2 evolution ceased resulting in production of free carbinol. The solution was extracted with ether (2 x 100 ml), and the combined ether extracts were dried using sodium sulphate. Removal of the ether gave a yellow oil identified as PAC (Section 2.3.10).

2.6.3 Isolation of Selected L-Acetyl Aromatic Carbinols from a Fermentation Broth

L-4-Chlorophenylacetyl carbinol, L-3-methylphenylacetyl carbinol and L-3-methoxyphenylacetyl carbinol were isolated following extraction

from a fermentation broth. Extracts, containing the L-acetyl aromatic carbinol and the corresponding aldehyde were prepared by the above procedure (Section 2.6.2). The carbinol and aldehyde were finally separated by application to 40 cm x 4 cm silica gel columns. L-3-methylphenylacetyl carbinol, L-4-chlorophenylacetyl carbinol and L-3-methoxyphenylacetyl carbinol were eluted with chloroform, 50:50 ether:chloroform and 50:50 methanol:dichloromethane respectively. Fractions containing the separated carbinols were combined and the purified carbinols recovered by vacuum evaporation.

2.7 CONVERSIONS USING PURE ENZYMES

2.7.1 Conversion of Pyruvate and Benzaldehyde to L-Phenylacetyl Carbinol using Pure Yeast Pyruvate Decarboxylase

The incubation mixture contained, yeast extract 20% (w/v) in 0.05 M sodium citrate buffer, pH 6 (Appendix A,1), benzaldehyde, 2 g/l; sodium pyruvate, 61.7 g/l and pyruvate decarboxylase, 1.43 U. Enzyme and substrates were incubated for a total of 5 h, a further 2 g/l benzaldehyde was added after 2 and 4 h respectively.

2.7.2 Enzymatic Conversion of Aromatic Aldehyde to Aromatic Alcohol by Yeast Alcohol Dehydrogenase (YADH)

Determination of the position of equilibrium in the benzaldehyde to benzyl alcohol reaction:

The reaction was measured in both directions so as to determine the position of equilibrium. Firstly, benzaldehyde (final concentration 10 mM) was incubated with NADH (10 mM) and YADH, 7 mg/ml (2520 U) in 2 ml 0.1 M sodium phosphate buffer, pH 7.5 (Appendix A, 3). The reaction was assayed by H.P.L.C. (Section 2.5) before addition of the

enzyme and after equilibrium had been reached. Benzylalcohol (10 mM), NAD (10 mM) and YADH (7 mg/ml) were similarly incubated and assayed.

Conversion of a range of aromatic aldehydes to the corresponding alcohols:

The reaction mixture contained aromatic aldehyde, 4 mM; NADH, 4 mM; sodium phosphate buffer, 0.1 M, pH 7 and purified YADH, 7 mg/ml. Incubations were carried out at 25°C for 6 h. The reaction was then terminated by boiling and the denatured heat coagulated enzyme was removed by centrifugation. A control which contained no enzyme was included in each case. Aromatic aldehyde and alcohol were determined by H.P.L.C. (Section 2.5).

2.7.3 Production of Acyloin Compounds from Aldehydes using Purified Yeast Pyruvate Decarboxylase or Soluble Fraction of Yeast Cell Homogenate

The incubation mixture contained acetaldehyde (0.55 M) or propionaldehyde (0.45 M), citrate-phosphate buffer, pH 6.8 (Appendix A, 5) and pyruvate decarboxylase (0.6 U) or soluble fraction of yeast cell homogenate (3.3 mg protein). In certain cases sodium pyruvate was added as a sole or co-substrate to a final concentration of 30 mM. The blank contained distilled water in place of aldehyde. In each case a control was included in which the enzyme was boiled for 5 min. before addition of aldehyde. Enzyme and aldehyde were incubated at 30°C for 30 min. and the reaction was stopped by boiling for 30s.

2.7.4 Enzyme Stability Measurements

The incubation mixture contained pyruvate decarboxylase, 260 µg/ml; or purified yeast alcohol dehydrogenase, 10 µg/ml; potassium phosphate buffer, 0.1 M, pH 7.0 (Appendix A, 4), containing 0.1 mM EDTA and 0.1 mg/ml BSA and the appropriate concentration of either benzaldehyde, benzyl alcohol or PAC. A control, containing only enzyme in buffer, was included in each experiment. In certain cases, yeast extract was included at a concentration of 20% (w/v). Incubations were carried out at 25°C for 6 h, with enzyme activity measurements at 1 h intervals.

2.8 METHODS OF CELL BREAKAGE

During this work two mechanisms of cell breakage were used. Cells were homogenised in a Bead beater (Biospec Products, U.S.A.), chamber volume 350 ml, using beads 0.4-0.5 mm in diameter (BDH Ltd., England). Cells were also homogenised in an MSK homogeniser (B. Braun, Melsungen, West Germany). Cells and beads (Braun Glasperlen, 0.45-0.5 mm diameter) were contained in glass bottles of 50 ml volume.

2.8.1 Investigation of Biotransformation Ability of Homogenised Cells

In evaluating the biotransformation abilities of homogenised cells, a cell suspension equilibrated for 1 h was homogenised in a Bead beater for 3 min at 4°C. Thiamine pyrophosphate and $MgCl_2$ were added to the homogenate to give final concentrations of 1.5 mmoles/l and 2.5 mmoles/l respectively. Biotransformation was allowed to proceed for 5 h.

2.8.2 Preparation of Mitochondrial and Soluble Fractions of Yeast Cell Homogenate

The homogenisation medium was the Tris-sorbitol-EDTA medium of Somlo (1968), which contained, Tris-Cl (final concentration 50 mM), pH 7.2 at 20°C; sorbitol (0.6 M) and EDTA (0.25 mM). Mitochondrial and soluble fractions were isolated by a modification of the method of Guarnieri et al. (1970).

Freshly harvested yeast (15 g wet weight) was mixed with 35 g glass beads (Braun Glasperlen, 0.45-0.5 mm diameter), and 25 ml homogenisation medium in a pre-cooled glass bottle. Cells were broken by shaking for 2 min. in a Braun homogeniser set at speed 1 (2,000 oscillations/min) at 4°C. The contents of the glass bottle were decanted free from glass beads and centrifuged in a bench centrifuge at maximum speed for 1.5 min. at 4°C. After transferring the supernatant to a cooled beaker, the pellet was resuspended in a further 25 ml of buffer and homogenised as before. This procedure was repeated once more. The three supernatants were combined together with a 20 ml aliquot of homogenisation buffer used to wash the glass beads after the final run, and centrifuged at 1000g for 10 min. (0°C) to remove cell debris, unbroken cells, and glass beads.

The crude supernatants from the above centrifugations were then centrifuged at 12,000 g for 10 min. (0°C) to give a pellet (P) and supernatant. The supernatant was centrifuged on a Beckman L-2 ultracentrifuge at 100,000 g for 1 h (0°C) to remove particulate material, providing the soluble fraction. The sedimented

mitochondria from the second centrifugation (P) were washed three times by resuspension in the homogenisation medium and finally suspended in an equal volume of this medium.

2.9 DISCONTINUOUS POLYACRYLAMIDE GEL ELECTROPHORESIS (DISCONTINUOUS PAGE)

2.9.1 Instrumentation

Electrophoresis unit:

Bio-Rad mini protein II comprising buffer tank, core to which gel plates are attached to form upper buffer reservoir, casting stand and gel plates (for slab gels, dim. 8 cm x 10 cm and 7 cm x 10 cm) and dividers. (Obtained from Bio-Rad, Richmond, California, U.S.A.).

Power supply:

Protein samples were electrophoresed using a Bio-Rad model 1000/500 power supply.

2.9.2 Preparation of Gels and Buffers

Isozymes of yeast alcohol dehydrogenase were separated on non-denaturing gels prepared by a modification of the method described by Jovin et al. (1964). The stacking gel (upper gel) contained 2.5% total acrylamide with 2.7% N,N'methylenebisacrylamide. The multiphasic buffer system (final pH 9.0) described by Rodbard and Chrambach (1971) was used. The method of preparation of solutions for gels and buffers is described in Tables 2.2 and 2.3.

TABLE 2.2 Components of stock solutions required to make the stacking (2.5%) and running (6%) gels for discontinuous PAGE.

Running gel Stock solutions			Stacking gel Stock solutions		
Volume Ratios[a]	Components/100ml		Volume Ratios	Components/100ml	
1	A	24.00g	2	A	5.00g
	Bis	0.65g		Bis	0.13g
1	1N HCl	24.00ml	1	1M H_3PO_4	12.80ml
	Tris	18.15g		Tris	2.85g
	Temed	0.23ml		Temed	0.23ml
		pH 8.9			pH 6.9
2	AP	0.14g	1	AP	0.28g

[a]Ratios in which stock solutions are mixed.

A = acrylamide
Bis = N,N'-methylenebisacrylamide
Temed = N, N, N', N'-tetramethylethylenediamine
AP = ammonium persulphate

TABLE 2.3 Components of upper and lower reservoir buffers for PAGE

Upper reservoir buffer Components/100ml		Lower reservoir buffer Components/100ml	
Glycine	0.37g	1N HCl	20 ml
Tris	0.63g	Tris	0.76g

1. The running gel was prepared by mixing the solutions in the appropriate ratios (Table 2.2), adding the ammonium persulphate last.
2. The gel was mixed gently and poured immediately.
3. A layer of water was carefully placed on top of the gel solution.
4. The gel was left to polymerise for approximately 30 min.

5. The solutions for the stacking gel were then mixed in the appropriate ratio (Table 2.2), adding the ammonium persulphate last.
6. The water was removed from the polymerised running gel and the comb put in place for the formation of sample wells.
7. The stacking gel was layered on top of the running gel until the wells in the comb were completely filled.
8. Water was carefully layered on the gel solution which was left to stand until polymerisation had occurred.

2.9.3 Sample Buffer

Sample buffer consisted of 1 ml stacking gel buffer, 1 ml glycerol and 1 ml water containing 0.25 mg bromophenol blue.

2.9.4 Preparation of Sample

All samples were diluted with an equal volume of sample buffer. Before mitochondrial fractions were diluted with sample buffer, the mitochondria were disrupted by mechanical breakage:- by freeze-thawing and osmotic shock. Firstly, the mitochondria were diluted 1:10 with water. This suspension was then frozen and thawed three times.

2.9.5 Electrophoresis

1. When the stacking gel had polymerised, the comb was removed and the water discarded from the gel surface.
2. Protein samples (15-300µg protein, standards, soluble fraction or mitochondrial fraction) were placed in the wells and over-layed with upper reservoir buffer.

3. The upper and lower reservoirs were filled with the appropriate buffer.
4. Gels were run at 4°C in the constant current mode operating at 48 mA for the stacking gel and 80 mA in the running gel until the marker dye was approximately 1 cm from the end of the gel. Two gels were run concurrently.
5. The gels were removed from the glass plates and the centre of the bromophenol blue dye front was marked by a small cut in the gel.

2.10 DETECTION OF ALCOHOL DEHYDROGENASE ACTIVITY

ADH was localized on non-denaturing gels by a modification of the method described by Lutstorf and Megnet (1968). After electrophoresis the gel was incubated for 1 hr at 25°C in the dark in the following solution: 60 mM pyrophosphate buffer, pH 8.8 (Appendix A,2); NAD, 0.4 mg/ml; phenazinemethosulphate (PMS), 25 µg/ml; Nitro Blue Tetrazolium (NBT), 0.4 mg/ml and 0.8% (w/v) ethanol or 0.8% (w/v) benzyl alcohol. A formazan band (insoluble dark blue precipitate) was formed at the site of enzyme activity. The reaction was stopped with 7.0% (v/v) acetic acid.

2.11 MOLECULAR WEIGHT DETERMINATION USING NON-DENATURING GELS

2.11.1 Procedure

The procedure used for determining molecular weights in a non-denaturing system is a modification of the methods of Bryan (1977) and Davis (1964). The proteins of unknown molecular weight, which were in the soluble fraction of yeast cell homogenate, were electrophoresed on a set of gels of various acrylamide concentrations. The Rf of each protein in each gel was determined. A

set of standards of known molecular weight was run in parallel. 100 log (Rf x 100) vs gel concentration was plotted for each band, (slope of plot = Retardation coefficient, Kr). In the case of the standards, - log slope vs log molecular wt. of standard was plotted. The molecular weights of the unknown proteins were determined from this.

2.11.2 Preparation of Gels

Gels were prepared as described in Section 2.9.2 except that the stock acrylamide/N,N'-methylenebisacrylamide (bis) solution for the running gel contained 40 g acrylamide and 1.08 g bis per 100 ml. This stock solution was diluted according to the percentage gel required. The stacking gel contained 2.5% total acrylamide in all cases.

The percentage gels used for standards and samples are shown in Table 2.4.

TABLE 2.4 Percentage gels run for standards and samples in determination of molecular weight

Protein	Molecular Weight	% Gels run
α-Lactalbumin	14,200	7, 8, 9, 10
Carbonic Anhydrase	29,000	6, 7, 8, 9
Albumin, Chicken Egg	45,000	7, 8, 9, 10
Albumin, Bovine Serum	66,000(monomer)	7, 8, 9, 10
	132,000(dimer)	4.5, 5, 5.5, 6
Sample	Unknown	6, 7, 8, 9

2.11.3 Fixative Solution

The fixative solution contained methanol, 400 ml; acetic acid, 70 ml and distilled water, 530 ml.

2.11.4 Staining Solution

Coomassie Brilliant Blue R, 0.5 g, was dissolved in 500 ml of fixative solution.

2.11.5 Electrophoresis

Standards and samples were prepared and electrophoresed as described in Sections 2.9.4 and 2.9.5 respectively.

2.11.6 Staining and Destaining

Standards:

1. Gels were immersed in fixative solution for approximately 2 h.
2. The gels were then stained in staining reagent for at least 6 h.
3. Gels were destained by diffusion against several changes of fixative solution, until the blue protein bands were visible against a clear background.
4. The gels were stored in a 7% (v/v) acetic acid solution.

Total protein present in the soluble fraction was also visualised in this way.

Samples:

Bands of interest for molecular weight determination, i.e. those proteins reacting with ethanol and benzyl alcohol, were detected as described in Section 2.10.

2.12 DETERMINATION OF INTRACELLULAR LEVELS OF SUBSTRATE AND METABOLITES

2.12.1 Sampling of Yeast

Yeast was sampled using the method of Saez and Lagunas (1976). A volume of culture containing 0.5-1.5 g of wet yeast was divided into aliquots. Each aliquot was filtered through a Millipore filter (0.45 μm pore size and 47 mm diameter). The yeast pellicle was washed with methanol-water (50/50 v/v) at -40°C, placed directly into liquid nitrogen and kept there for extraction.

2.12.2 Determination of Wet Weight

Wet weight of yeast was determined by vacuum filtration of 50 ml aliquots of culture through a 0.45 μm Millipore membrane. The yeast was resuspended in 50 ml of water, filtered again and immediately weighed.

2.12.3 Extraction of Cells

The combined filtered aliquots were mixed with 3 ml $HClO_4$ and 0.4 ml of 0.4 M Tris in a mortar previously cooled with liquid nitrogen. The mixture was ground to a powder in the presence of liquid nitrogen, transferred to a test tube and kept for 30 min. at -10°C with frequent vigorous shaking. The resultant liquid was frozen in liquid nitrogen and then allowed to thaw. This extraction procedure was repeated twice. The extract obtained was centrifuged for 10 min. at 10,000 g and the supernatent was adjusted to pH 6.5 with 10 N KOH at 0°C. After 15 min. at 0°C, the precipitated $KClO_4$ was removed by centrifugation. The supernatant was analysed for PAC,

benzaldehyde and benzyl alcohol. For the calculation of concentrations, it was assumed that 1.67 g wet yeast contains 1 ml cell sap (Conway and Downey, 1950).

2.13 EXTRACTION OF LIPIDS FROM YEAST CELLS

After harvesting (by centrifugation, 7,000 g for 10 min.), organisms were washed twice with 0.85% (w/v) NaCl and lipid was extracted by a modification of the procedure of Folch *et al*. (1957). Washed organisms (250 mg dry weight) were mixed with methanol (10 ml) and 150 µg of heptadecanoic acid (C 17:0), which was used as an internal standard. The suspension was shaken in a Braun glass bead homogeniser (Section 2.8) for two periods of 30 s after addition of 35 g glass beads (Glasperlen, B. Braun, Melsungen, West Germany; 0.45-0.5 mm diameter). Chloroform was then added to the suspension to give the ratio 2:1 (v/v) Chloroform/methanol and the suspension was stirred for 2 h at room temperature. The suspension was then clarified by centrifugation (7,500 g for 10 min.) and the extraction procedure was repeated on the residue. The combined extracts were washed with 0.25 vol. 0.88 % (w/v) KCl and the lower layer was dried on a rotary evaporator.

2.13.1 Preparation of Methyl Esters of Fatty Acids

Fatty acid methyl esters were prepared by a modification of the method of Morrison and Smith (1964). Boron trifluoride-methanol reagent (2 ml) was added to the lipid residue under nitrogen. The mixture, contained in a tube closed with a screw cap, was heated at 100°C for 1h. Esters were extracted by adding one vol. of distilled water and 2 vol. of glass distilled hexane, shaken briefly and centrifuging at 7,5000 g until both layers were clear. This

extraction was repeated and hexane-ester samples were pooled, giving a 97-99% extraction of esters suitable for gas chromatography. Esters of standard fatty acid solutions were similarly prepared.

2.14 IMMOBILISATION OF YEAST CELLS IN CALCIUM ALGINATE

Yeast cells were immobilised in calcium alginate by a method described by Chibata *et al*. (1986). Sodium alginate (50 ml; 4% (w/v)) was mixed with 50 ml of cell suspension (containing 25 g (wet weight) of cells) until homogenous. The mixture was dropped from a syringe (needle guage 21) into a 0.05 M calcium chloride solution (pH 6 to 8) at 37°C, forming beads of approximately 2 mm in diameter. The beads were then allowed to cure at 20-22°C for 2 h, washed with distilled water and stored overnight in 0.05 M calcium chloride at 4°C. Beads were rinsed again in distilled water before use.

The yeast cells used for encapsulation were obtained from a yeast cake (fresh pressed bakers' yeast 30% (w/w) solids content). Cells were equilibrated for 1 h in medium C (Section 2.2.7) before encapsulation. When directly comparing free and encapsulated cells, the free cells were also equilibrated for 1 h in medium C, recovered, and stored at 4°C until use.

2.15 ROUTINE MEASUREMENTS AND INSTRUMENTATION

pH was measured using either a Philips PW 9420 or an Orion pH meter. Spectrophotometric measurements were conducted on a Pye-Unicam SP6-550 u.v./vis. spectrophotometer or on an LKB Ultraspec II 4050 u.v./vis spectrophotometer with a 1 cm light path.

Balances used routinely included a Sartorins 1219 MP electronic balance (600 g ± 0.01 g) and a Precisa 80A electronic balance (30 g ± 0.0001 g).

Centrifugation was carried out using a bench-top Heraeus Christ model 600 centrifuge and a Sorvall RC-5B refrigerated superspeed centrifuge (DuPont Instruments).

3. RESULTS

3.1 PRELIMINARY STUDIES ON L-PHENYLACETYL CARBINOL

The following preliminary experiments were conducted to evaluate the effect of sucrose concentration, yeast dosage and pH on L-phenylacetyl carbinol (PAC) production. The results of these studies should allow a suitable medium for the biotransformation to be established.

Sucrose was chosen as the carbon source as bakers' yeast is grown commercially on molasses and it has been used as a medium substituent by previous workers (Neuberg and Liebermann, 1921; Neuberg and Hirsch, 1922; Ose and Hironaka, 1957; Becvarova et al., 1963; Netrval and Vojtisek, 1982). The optimal concentration of peptone, 6 g/l, in PAC production medium has previously been determined (Gupta et al., 1979).

The biotransformation medium in all cases contained, bacteriological peptone, 6 g/l, and KH_2PO_4, 13.6 g/l. Flasks (2 l Erlenmeyer), containing 1 l of medium were incubated as described in Section 2.2.4 for a total of 6 h (including an equilibration period of 1 h). Following the equilibration period, benzaldehyde was added to the medium at hourly intervals at a rate of 2 $gl^{-1}h^{-1}$ for 5 h.

3.1.1 Effect of Initial pH (pH_i) on L-Phenylacetyl Carbinol Production

The effect of pH on PAC production was investigated in the range pH 3-8. The fermentation medium, which contained 50 g/l sucrose, was inoculated with 50 g/l fresh pressed bakers' yeast (30% (w/v)

solids content). The concentrations of sucrose and yeast used were estimated from literature values as optimum levels for this system had yet to be determined.

PAC concentrations (Section 2.3.1) determined after the 6 h incubation are presented in Table 3.1. Initial and final pH values are also given.

Table 3.1 Relationship between initial pH and PAC production in a 5 h fermentation. The medium contained 50 g/l sucrose and was inoculated with 50 g/l fresh pressed bakers' yeast (30% (w/v) solids content).

Initial pH	Final pH	PAC (g/l)
3.0	3.0	3.3
4.0	3.6	4.0
5.0	4.0	2.8
6.0	4.5	2.9
7.0	5.5	1.6
8.0	6.0	1.4

Highest levels of PAC were produced at pH 4. Initial pH values were not maintained over the 6 h period. A significant drop in pH was evident in the range pH 5-8.

3.1.2 Effect of Sucrose Concentration on L-Phenylacetyl Carbinol Production

The effect of sucrose concentration on PAC production was determined in the range 0-80 g/l. The medium, initial pH 4, was inoculated with 50 g/l fresh pressed bakers' yeast (30% (w/v) solids content). PAC concentrations, determined following the 5 h

fermentation are presented in Figure 3.1. The amount of PAC produced increased to a maximum of 3.7 g/l at a sucrose concentration of 50 g/l.

3.1.3 Effect of Yeast Concentration on L-Phenylacetyl Carbinol Production

A medium containing 50 g/l sucrose at pH 4 was inoculated with 10, 20, 30, 40 and 50 g/l fresh pressed bakers' yeast (30% (w/v) solids content). The results are presented in Figure 3.2. PAC concentration following a 5 h incubation period increased with increasing yeast dose rate up to 30 g/l.

3.1.4 Comparison of Sodium Citrate and Sodium Phosphate as Buffering Agent in the Medium

The results presented in Table 3.1 show that even with KH_2PO_4 present in the medium, the initial pH is not maintained throughout the incubation period. A drop in pH is observed. Because of the optimum initial pH range observed, PAC production was compared in medium containing sodium citrate, which has a better buffering capacity in this range, and sodium phosphate.

The medium contained sucrose, 50 g/l, and either KH_2PO_4 13.6 g/l or citric acid 10.5 g/l, adjusted to pH 4 and also pH 5 with NaOH. Yeast concentration was 30 g/l (fresh pressed yeast, 30% (w/v) solid content).

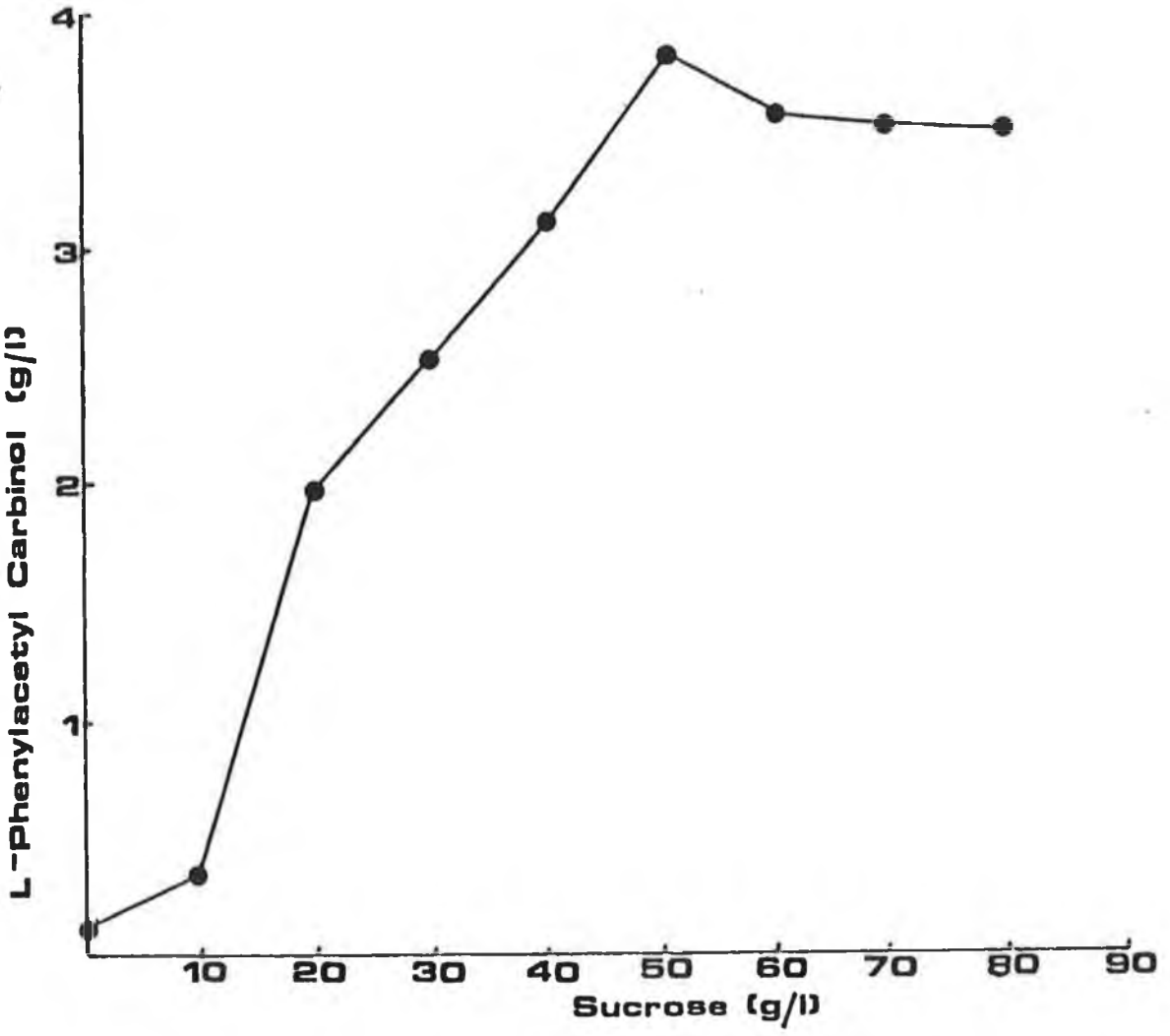


Figure 3.1. Relationship between initial sucrose concentration and PAC production in a 5 h incubation. The medium (pH 4), was inoculated with 50 g/l fresh pressed bakers' yeast (30% (w/v) solids content).

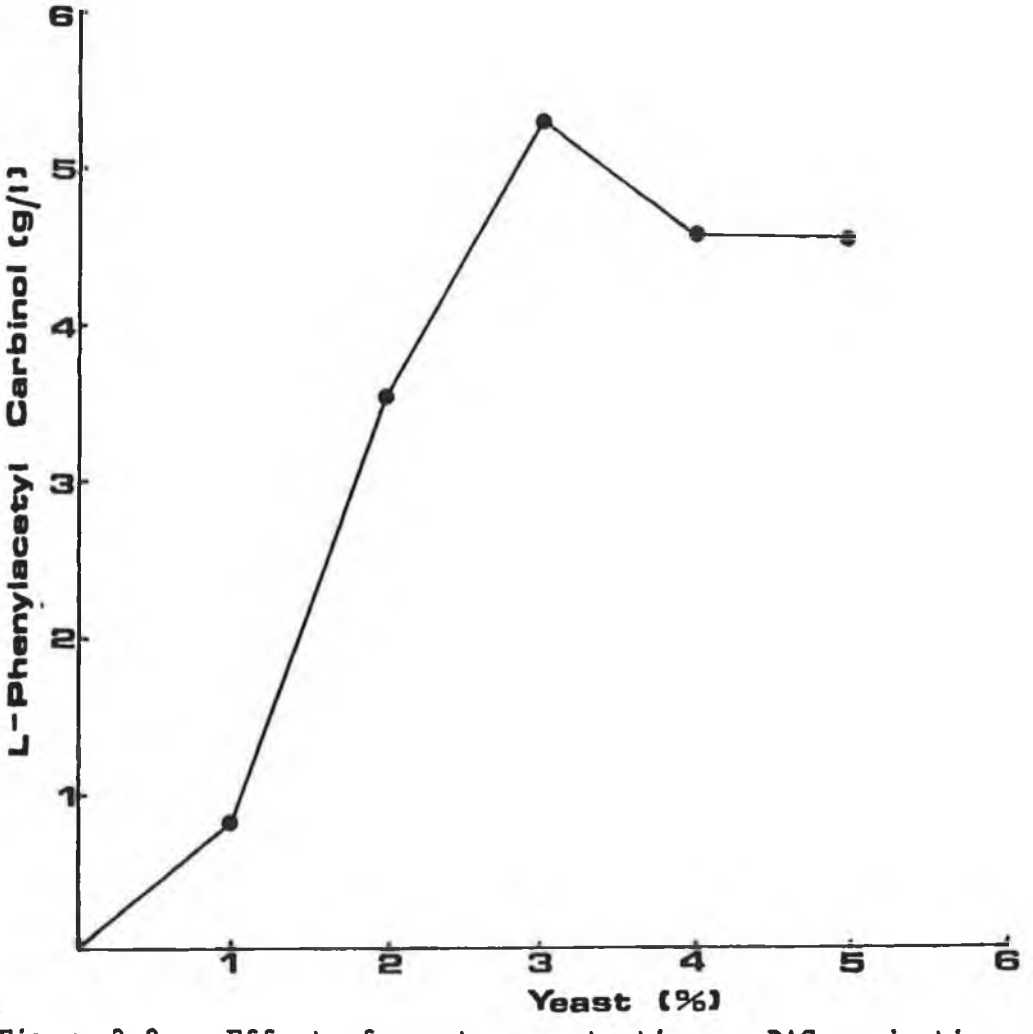


Figure 3.2. Effect of yeast concentration on PAC production. Medium, containing 50 g/l sucrose at pH 4 was inoculated with 10, 20, 30, 40 and 50 g/l fresh pressed bakers' yeast (30% (w/v) solids content).

The pH of the media buffered at pH 4 and 5 by KH_2PO_4 dropped by 0.5 and 1.0 pH unit respectively. The pH of the media buffered using sodium citrate remained at their initial values. The amount of PAC produced in all 4 flasks was approximately equal (approximately 4 g/l).

3.1.5 Medium A

Based on the above preliminary studies, a general medium (Medium A) consisting of sucrose, 50 g/l; bacteriological peptone, 6 g/l; citric acid 10.5 g/l, adjusted to pH 4.5, was established for PAC production. Using this medium an inoculum of 30 g/l fresh pressed bakers' yeast (30% (w/v) solids content) gave highest levels of PAC in a 5 h incubation. A pH of 4.5 was chosen as it is close to the pKa2 of citric acid which is 4.74, and is within the range of maximum PAC production.

3.1.6 Effect of Acetaldehyde on PAC Production

Other authors have found that addition of acetaldehyde to a PAC fermentation decreases the ratio of benzyl alcohol to PAC produced (Section 1.2). To examine the effect of acetaldehyde on PAC production, two flasks containing Medium A were each inoculated with 30 g/l fresh pressed bakers' yeast (30% (w/v) solids content). To one, 4 g/l of a 1:1 (v/v) solution of acetaldehyde: benzaldehyde was added hourly for 5 h. To the other, 2 g/l benzaldehyde was added hourly for 5 h. In each case, the ratio of benzyl alcohol to PAC was determined using 'Hnmr (Section 2.3.10). The 'Hnmr spectra are presented in Figure 3.3.

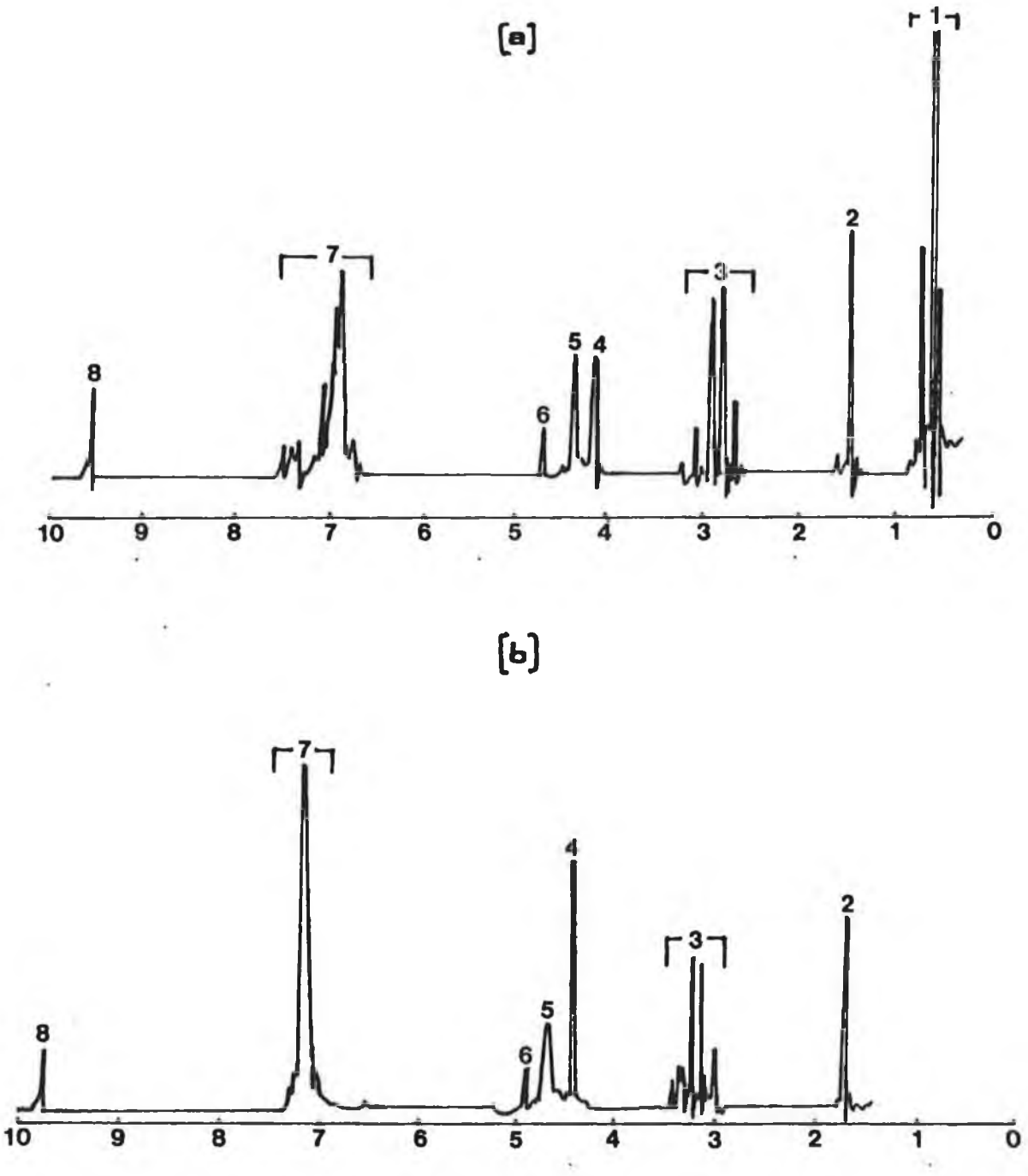


Figure 3.3. 'Hnmr spectra of (a) fermentation with added acetaldehyde and (b) fermentation without acetaldehyde. Peaks numbered 1 to 8 represent: 1, CH_3 of ethanol; 2, CH_3 of PAC; 3, CH_2 of ethanol; 4, CH_2 of benzyl alcohol; 5, OH of PAC and benzyl alcohol; 6, CH of PAC; 7, C_6H_5 of PAC and 8, CHO Benzaldehyde. Spectra were obtained as described in Section 2.3.10.

When acetaldehyde was added to the fermentation, the ratio of benzyl alcohol to PAC was approximately 1:1 while in the absence of added acetaldehyde, this ratio rose to 3:1 (These figures were obtained by comparing the ratio of the CH_2 peak (No. 4) of benzyl alcohol and the CH_3 peak (No. 2) of PAC. PAC was measured colorimetrically (Section 2.3.1) and was found to be the same in each case (approximately 4 g/l).

3.1.7 Comparison of Sucrose and Sodium Pyruvate as Co-Substrates in the L-phenylacetyl Carbinol producing system

The amount of PAC produced in Medium A, and Medium A in which the sucrose is replaced with sodium pyruvate, 61.7 g/l (Medium B), was compared after a 6 h incubation (including a 1 h equilibration period, (Section 2.2.4). The media were inoculated with 30 g/l fresh pressed bakers yeast (30% (w/v) solids content). A further 15 g/l yeast was added to each flask after 3 h. Benzaldehyde, 2 g/l was added to each flask hourly for 4 h. The results are presented in Table 3.2

Table 3.2 PAC produced in media containing sucrose and sodium pyruvate as co-substrate in a 5 h fermentation. Media were inoculated with 30 g/l fresh pressed bakers' yeast (30% w/v solids content), a further 15 g/l yeast was added after 3 h.

Co-Substrate	PAC (g/l)
Sucrose	3.2
Sodium Pyruvate	7.8

The amount of PAC produced with sodium pyruvate as co-substrate was more than twice that formed with sucrose.

3.1.8 Appraisal of Results

A basic biotransformation medium (Medium A) containing sucrose, bacteriological peptone and citric acid was established for PAC production. When sucrose was replaced by sodium pyruvate (Medium B) the amount of PAC produced almost doubled. The use of sodium pyruvate in the large-scale production of PAC is not really a viable alternative, however, as it is an expensive biochemical. The addition of acetaldehyde to a PAC fermentation reduced the ratio of benzyl alcohol to PAC produced but did not increase PAC titres.

3.2 PREPARATION OF PURE PHENYLACETYL CARBINOL AND VALIDATION OF THE COLORIMETRIC PAC ANALYTICAL METHOD

PAC is not available commercially. In routine analysis of PAC, acetylbenzoyl (1-phenyl-1,2-propanedione) was used as a standard. A small quantity of pure PAC was required to validate the use of acetyl benzoyl as a standard in the colorimetric assay and to determine the appropriate correction factor. Pure PAC was also required to allow the identification of the PAC peak in gas chromatograms of a fermentation broth.

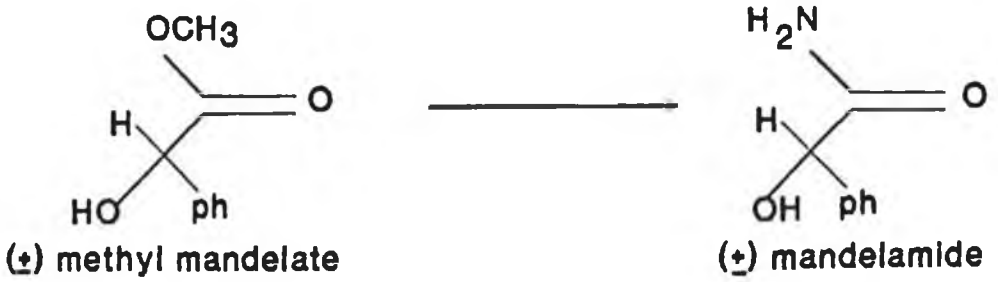
Two approaches were considered for the preparation of PAC

(a) Synthesis via established literature procedures

(b) Purification of PAC from the fermentation broth.

3.2.1 Synthesis of Phenylacetyl Carbinol

(D-/L-)PAC was prepared from (D-/L-)methyl mandelate in two steps. The first step involved conversion of (D-/L-) methyl mandelate to (D-/L-) mandelamide according to the procedure of McKenzie and Wren (1908) and is described in Section 2.6.1.



(±) methyl mandelate (±) mandelamide

(D-/L-) PAC was prepared from (D-/L-) mandelamide according to the procedure of Hey (1930), described in Section 2.6.1.

(±) mandelamide (±) P A C

The structure of the synthetic product was confirmed to be that of (D-/L-) PAC by 'Hnmr (Section 2.3.10). The 'Hnmr spectrum is shown in Figure 3.4.

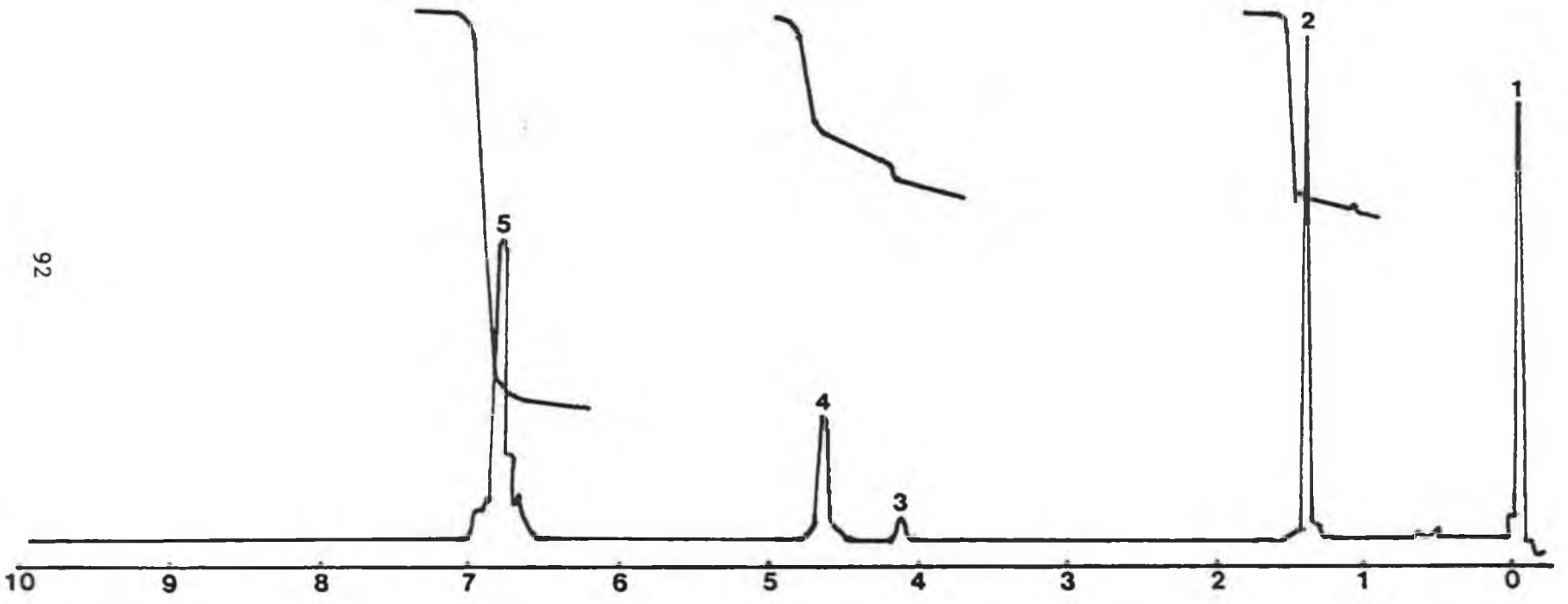


Figure 3.4. !Hnmr spectrum of pure L-phenylacetyl carbinol. Peaks numbered 1 to 5 represent: (1), TMS (internal standard); (2), CH_3; (3), OH; (4) CH and (5), C_6H_5 respectively. The 'Hnmr spectrum was obtained as described in Section 2.3.10.

GC analysis of the product showed one main peak (retention time 5.2 min.) together with very small peaks due to minor amounts of impurities (Figure 3.5(a)).

3.2.2 Isolation of Pure L-phenylacetyl Carbinol from a Fermentation Broth

The second approach to obtaining a pure sample of PAC was the use of a modification of a procedure of Smith and Hendlin (1953), (Section 2.6.2).

The product obtained gave an identical 'Hnmr spectrum to the synthetic sample of (D-/L-) PAC. The gas chromatogram, Figure 3.5(c), showed the presence of only one major peak, with an identical retention time to that of the synthetic sample (Figure 3.5(a).

Both the synthetic sample, (D-/L-) PAC and the purified L- PAC allowed unambiguous identification of the PAC peak in a gas chromatogram of the crude fermentation broth (Figure 3.5(b)).

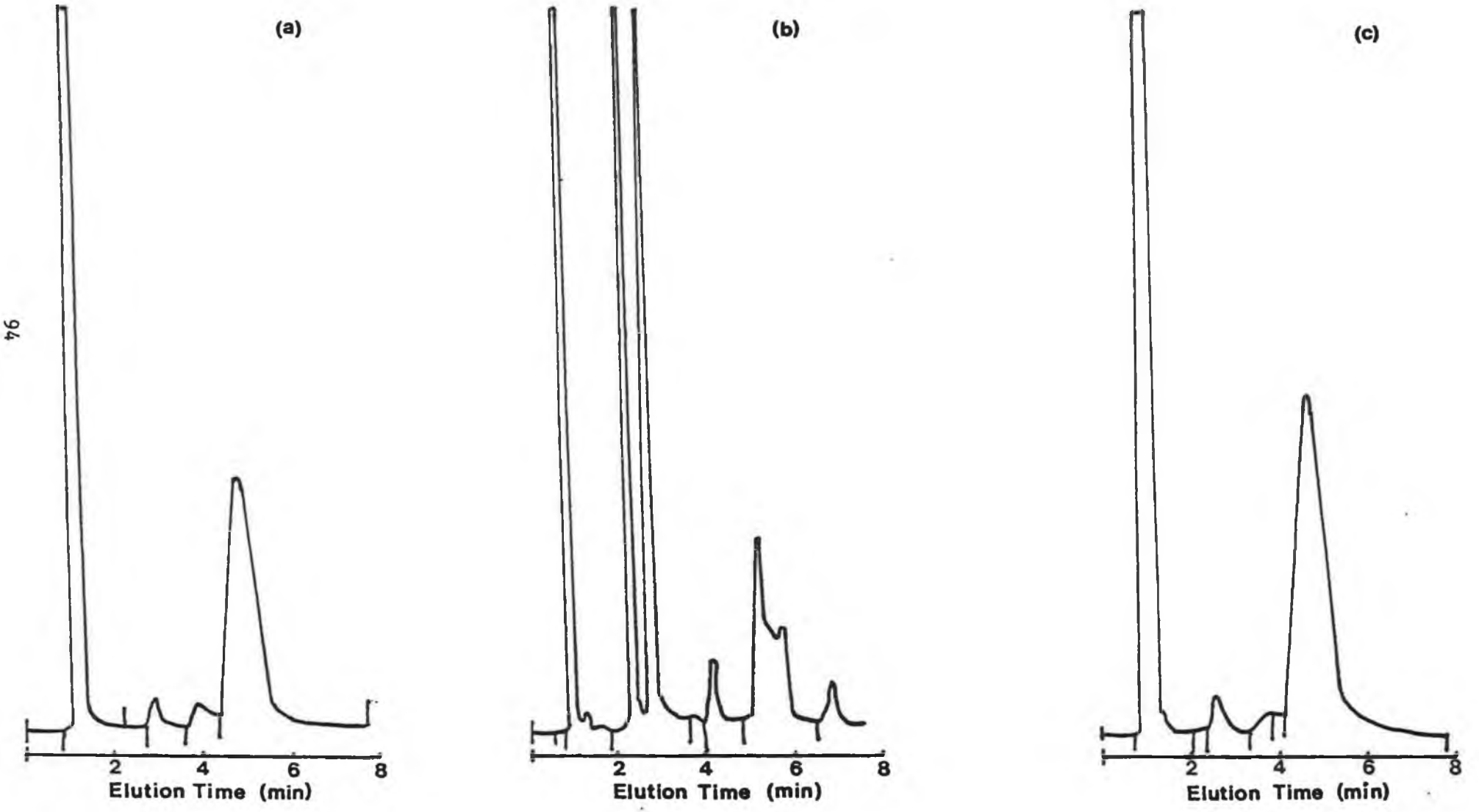


Figure 3.5. Gas chromagogram of (a) PAC synthesised as described in Section 3.2.1, (b) crude fermentation broth and (c) PAC purified from a crude fermentation broth (Section 2.6.2). (Perkin Elmer F17 GC).

3.2.3 Use of Purified PAC to Validate the Colorimetric Method of PAC Analysis

Acetyl benzoyl may be used as a standard for PAC analysis once the appropriate correction factor is established. To determine the correction factor, solutions containing 0-150 µg of PAC and acetyl benzoyl were assayed according to Groger and Erge (1965), (Section 2.3.1). The results are presented in Figure 3.6. Acetyl benzoyl absorbs more strongly than PAC at 580 nm. By measuring the slope of each line, a correction factor of 1.3 was calculated for conversion of acetyl benzoyl to PAC concentration. The PAC concentration as determined using this correction factor and the actual, known concentration of each standard were shown to correspond exactly.

Gas chromatography (GC) was used to further validate the use of the colorimetric assay. Solutions of PAC in the range 0-8 g/l were prepared and extracted as described in Section 2.4.2. The calibration curve constructed was linear in this concentration range. Two solutions containing 2 and 5 g/l PAC respectively were assayed using GC and the colorimetric method with acetyl benzoyl as a standard. The PAC concentration as determined by both methods was accurate. Benzaldehyde and benzyl alcohol do not interfere with the colorimetric assay.

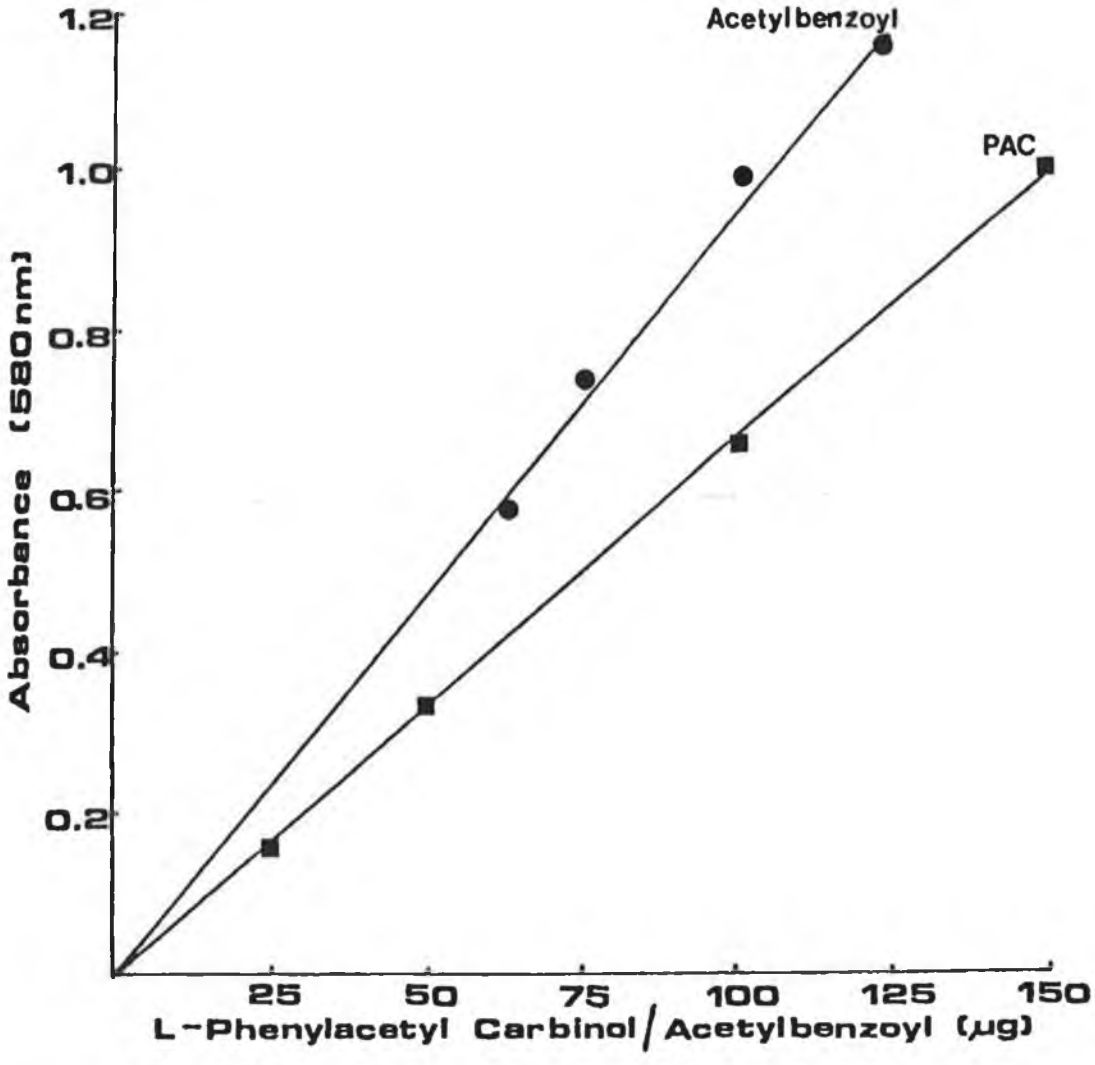


Figure 3.6. Absorbance of L-phenylacetyl carbinol (■) and Acetyl benzoyl (●) at 580 nm. A conversion factor for the conversion of acetyl benzoyl to PAC concentration was calculated as described in Section 3.2.3.

3.3 INVESTIGATIONS ON THE SPECIFICITY OF THE ACYLOIN FORMING SYSTEM

Investigations of the specificity of the acyloin forming system and the role of pyruvate decarboxylase and other enzymes in acyloin formation were carried out using whole yeast cells and using pure enzymes, comparing both aliphatic and aromatic aldehydes as substrates.

In a preliminary experiment selected aromatic and aliphatic aldehydes were added to fermentation Medium B. Medium B was used in the following experiments as results presented in Table 3.2 suggest that higher carbinol titres would be obtained using this medium, making it easier to detect carbinols formed from aldehydes which are poor substrates for the carbinol forming system. Flasks were inoculated with 30 g/l fresh pressed bakers' yeast (30% w/v solids content). A further 15 g/l yeast was added after 3 h. Flasks were incubated for a total of 6 h (including a 1 h equilibration period) as described in Section 2.2.4. Aldehyde was added in increments of 2 $gl^{-1}h^{-1}$ for 4 h. Following the 5 h fermentation, ether extracted cell free supernatants were qualitatively tested for the presence of acyloin product using ^{1}Hnmr (Section 2.3.20). Substituted aromatic aldehydes containing a CH_3, OCH_3 or Cl group in the *para* position acted as substrates as did the aliphatic aldehydes, acetaldehyde and propionaldehyde. *p*-Nitrobenzaldehyde, cinnamaldehyde and salicylaldehyde did not appear to produce carbinol products.

3.3.1 Conversion of Aromatic Aldehydes to L-Acetyl Aromatic Carbinols and Aromatic Alcohols by Saccharomyces cerevisiae

The conversion of a range of aromatic aldehydes to L-acetyl aromatic carbinols and aromatic alcohols by Saccharomyces cerevisiae was investigated. The reactions involved are illustrated in Figure 3.7.

Fermentations were carried out in 250 ml Erlenmeyer flasks, containing 150 ml of Medium B, inoculated with 4.5 g fresh bakers' yeast (30% w/v solids content). Flasks were incubated as described in Section 2.2.4. Following an equilibration period of 1 h, the fermentation was initiated by the addition of 2 g/l aromatic aldehyde. At 1,2,3 and 4 h intervals after fermentation initiation, aliquots equivalent to 2 g/l aromatic aldehyde were further added to each flask. After a fermentation period of 3 h an additional 2.25 g yeast (30% w/v solids content) was added per flask. After a fermentation period of 6 h the clarified broth was recovered for analysis. L-acetyl aromatic carbinols were determined colorimetrically (Section 2.3.1) and aromatic alcohols by GC (Section 2.4.2). The results are presented in Table 3.3.

The titres of L-acetyl aromatic carbinols and aromatic alcohols produced from benzaldehyde and substituted aromatic aldehydes containing a $-CH_3$, $-CF_3$, $-OCH_3$ or -Cl in either the ortho, meta or para ring position were determined. Highest carbinol yields were observed with benzaldehyde substrate. Aldehydes substituted in the ortho position were consistantly poor substrates for carbinol production. Aldehydes with $-CH_3$, $-CF_3$ and -Cl substituents

located in the para position produced higher carbinol yields than their meta counterparts. The opposite was the case with $-OCH_3$ substituent. Aromatic alcohol was produced from all substrates, where yields of carbinol were greater than 5 g/l levels of corresponding aromatic alcohols were relatively low, ranging from 3.5-14% (w/v) of carbinol yields. Only in the case where -Cl and $-CF_3$ were located in the ortho position and $-CF_3$ in the meta position did the yield of aromatic alcohol produced exceed that of carbinol produced at 6 h.

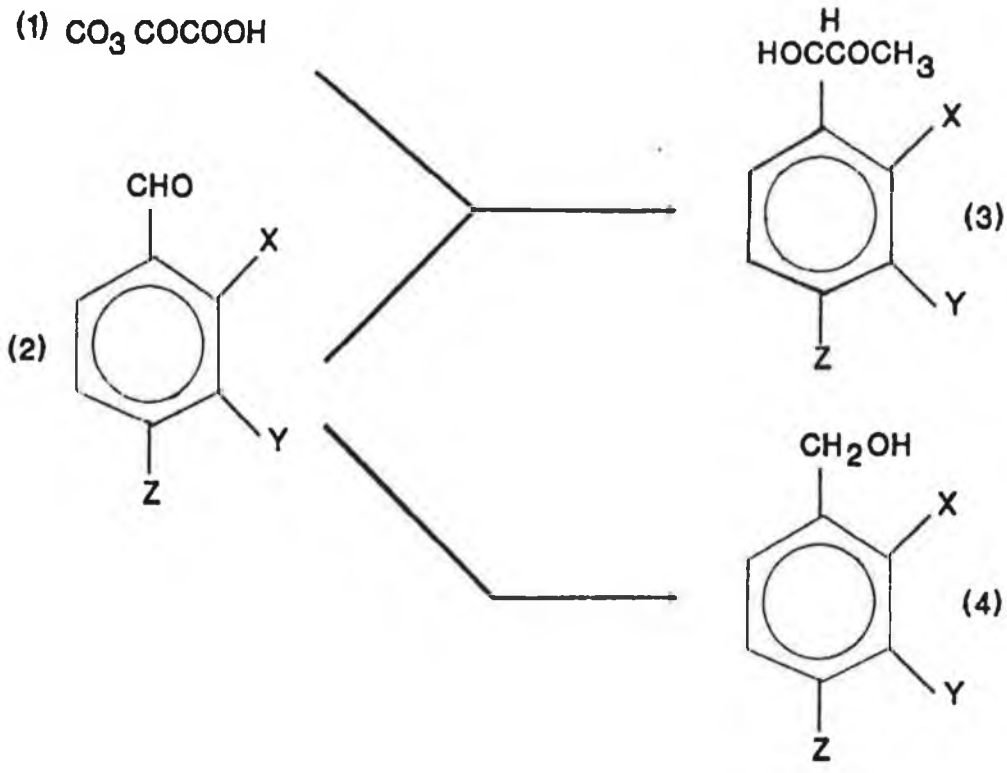


Figure 3.7 Schematic diagram of the conversion of substituted aromatic aldehyde (2) and pyruvate co-substrate (1) to L-acetyl aromatic carbinol (3) and aromatic alcohol (4).

Table 3.3 Conversion of aromatic aldehyde to L-acetyl aromatic carbinol and aromatic alcohol after a 6 h fermentation Bioconversion conditions are described in Section 3.3.1.

Substrate Aromatic aldehyde	Product 1 L-acetyl aromatic carbinol	Titre (g/l)	Product 2 Aromatic alcohol	Titre (g/l)	Location of Ring substituents (Refer Figure 1) X	Y	Z
Benzaldehyde	L-phenylacetyl carbinol	10.1-10.2	Benzyl alcohol	0.4	H	H	H
o-Tolualdehyde	L-2-Methylphenylacetyl carbinol	2.2-2.5	2-Methylbenzyl alcohol	1.0	CH_3	H	H
m-Tolualdehyde	L-3-Methylphenylacetyl carbinol	5.2-6.2	3-Methylbenzyl alcohol	0.4	H	CH_3	H
p-Tolualdehyde	L-4-Methylphenylacetyl carbinol	5.4-6.4	4-Methylbenzyl alcohol	0.3	H	H	CH_3
2-Chlorobenz-aldehyde	L-2-Chlorophenylacetyl Carbinol	0.6-0.7	2-Chlorobenzyl alcohol	0.9	Cl	H	H
3-Chlorobenz-aldehyde	L-3-Chlorophenylacetyl Carbinol	2.1-3.2	3-Chlorobenzyl alcohol	0.6	H	Cl	H
4-Chlorobenz-aldehyde	L-4-Chlorophenylacetyl carbinol	6.5-8.0	4-Chlorobenzyl alcohol	0.3	H	H	Cl
o-Anisaldehyde	L-2-Methoxyphenylacetyl carbinol	0.8-0.9	2-Methoxybenzyl alcohol	0.9	OCH_3	H	H
m-Anisaldehyde	L-3-Methoxyphenylacetyl carbinol	4.5-5.7	3-Methoxybenzyl alcohol	0.7	H	OCH_3	H
p-Anisaldehyde	L-4-Methoxyphenylacetyl carbinol	1.2-3.4	4-Methoxybenzyl alcohol	0.6	H	H	OCH_3
α,α,α-Trifluoro-o-tolualdehyde	L-2-(Trifluoromethyl)-phenylacetyl carbinol	0.2-0.3	2-(Trifluoromethyl)-benzyl alcohol	0.5	CF_3	H	H
α,α,α-Trifluoro-m-tolualdehyde	L-3-(Trifluoromethyl)-phenylacetyl carbinol	0.3-0.4	3-(Trifluoromethyl)-benzyl alcohol	0.5	H	CF_3	H
α,α,α-Trifluoro-p-tolualdehyde	L-4-(Trifluoromethyl)-phenylacetyl carbinol	0.5-0.8	4-Trifluoromethyl)-benzyl alcohol	0.8	H	H	CF_3

3.3.2 Purification of L-Acetyl Aromatic Carbinols

Three of the acetyl aromatic carbinols, L-3-methoxyphenylacetyl carbinol, L-4-chlorophenylacetyl carbinol and L-4-methylphenyl-acetyl carbinol were extracted and purified (Section 2.6.2). They were analysed by 'Hnmr (Section 2.3.20), polarimetry (Section 2.3.11) and colorimetrically (Section 2.3.1). The 'Hnmr data, presented in Table 3.4 were consistant with the structures of the L-acetyl aromatic carbinols. Specific rotation values for L-3-methoxyphenylacetyl carbinol, L-4-chlorophenylacetyl carbinol and L-4-methylphenylacetyl carbinol were -115^{o}, -185^{o} and -190^{o} respectively. The two sets of results were consistant and therefore validate the use of the colorimetric assay used in Table 3.3 for L-acetyl aromatic carbinol determination.

3.3.3 Conversion of Benzaldehyde and Pyruvate to Phenylacetyl Carbinol using Pure Yeast Pyruvate Decarboxylase

The reactivity of yeast pyruvate decarboxylase with benzaldehyde and sodium pyruvate as co-substrate was investigated. Enzyme and substrates were incubated as described in Section 2.7.1. PAC levels were measured colorimetrically at hourly intervals (Section 2.3.1) and pyruvate decarboxylase activity was measured at the beginning and end of the reaction (Section 2.3.9). The results are presented in Table 3.5.

Table 3.4 'Hnmr data for purified L-acetyl aromatic carbinols. The abbreviations, s, bs, and m stand for singlet, broad singlet and multiplet respectively and describe the nature of the peak(s). 'Hnmr spectra are obtained as described in Section 2.3.10.

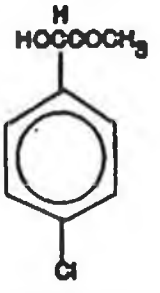
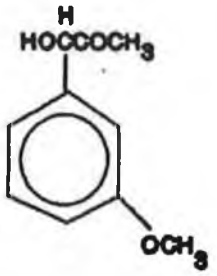
L-acetyl aromatic carbinol	$\delta(CDCl_3)$, (position of peak on 'Hnmr scale 0-10)
L-4-Methylphenylacetyl carbinol 	2.0 (3H, s, $\underline{CH_3}$ - CO -) 2.4 (3H, s, $\underline{CH_3}$ - Ph) 4.3 (1H, bs, $\underline{OH}$) 5.1 (1H, s, $\underline{CH}$ (OH)) 6.7-7.5 (4H, m, aromatic H)
L-4-Chlorophenylacetyl carbinol 	2.0 (3H, s, $\underline{CH_3}$ - CO-) 4.3 (1H, bs, $\underline{OH}$) 5.1 (1H, s, $\underline{CH}$ (OH)) 6.9-7.6 (4H, m, aromatic H)
L-3-Methoxyphenylacetyl carbinol 	2.0 (3H, s, $\underline{CH_3}$ - CO -) 3.7 (3H, s, $\underline{OCH_3}$) 4.3 (1H, bs, $\underline{OH}$) 5.1 (1H, s, $\underline{CH}$ (OH)) 6.7-7.5 (4H, m, aromatic H)

Table 3.5 Biotransformation of benzaldehyde and pyruvate to PAC by pure yeast pyruvate decarboxylase and residual enzyme activity after 5 h. Incubation conditions are described in Section 2.7.1.

Time (hr)	PAC (μ moles)	Specific Activity Pyuvate Decarboxylase	%
0	0.0	9.1	100
1	1.1	N.D.	
2	1.1	N.D.	
3	1.2	N.D.	
4	1.2	N.D.	
5	1.2	2.1	23

Note: N.D. = Not determined

Pure yeast pyruvate decarboxylase converts benzaldehyde to PAC in the presence of pyruvate as co-substrate. Greater than 90% of the PAC formed is produced in 1 h (1.1 - 1.2 μmoles). During the 5 h incubation period, 77% of enzyme activity was denatured.

3.3.4 Production of Acyloin Compounds by Yeast Pyruvate Decarboxylase and by Soluble Fraction of Yeast Cell Homogenate from Aliphatic Aldehyde Substrates

The results obtained in Section 3.3.3 show that yeast pyruvate decarboxylase catalyses the conversion of benzaldehyde and pyruvate to PAC. The following experiments were conducted to try and ascertain if other enzymes, apart from pyruvate decarboxylase, catalyse the formation of acyloin compounds, in particular PAC, in yeast cells. Morimoto et al. (1988) describe a 'new' enzyme in bakers' yeast, propioin synthase, which produces propioin from propionaldehyde without Keto-acid. This reaction is illustrated in Section 1.4.3. This enzyme also catalyses the formation of acetoin

from acetaldehyde in the absence of pyruvate (Morimoto et al., 1988). The conversion of acetaldehyde to actoin by propioin synthase is illustrated in Section 1.4.3. Pyruvate decarboxylase can also catalyse the formation of these acyloin compounds but only in the presence of the α-ketoacid (Juni, 1961).

In the following preliminary experiments the aliphatic aldehydes, acetaldehyde and propionaldehyde were incubated with pure pyruvate decarboxylase and soluble fraction of yeast cell homogenate in the presence and absence of pyruvate. One would expect that pyruvate decarboxylase would catalyse the formation of acyloin only in the presence of pyruvate, while the formation of acyloin from aldehyde alone would indicate the presence of an alternative acyloin-forming enzyme - perhaps propioin synthase. If significant propioin synthase activity were found, the next step would be to investigate its role in PAC formation.

Soluble fraction of yeast cell homogenate was prepared as described in Section 2.8.2. Aldehydes with and without pyruvate were incubated with both pure pyruvate decarboxylase and soluble fraction as described in Section 2.7.3. In the case of each substrate or combination of substrates, a control was included in which the enzyme was boiled for 5 min. before addition of aldehyde.

Qualitative estimation of acyloin compounds was by the Voges-Proskauer reaction as described by Westerfeld (1945), (Section 2.3.2). The appearance of a pink colour in this method indicates the presence of acetoin or a 5-carbon ketol, while a green colour is an indication of the presence of the 6-carbon ketol

propioin. Acyloins could not be determined quantitatively in this case, as the aldehydes inhibit colour development in the Westerfeld procedure (Juni, 1961). Acyloin formation was indicated by an increased absorbance in the analytical over the control. The results are presented in Tables 3.6 and 3.7.

Table 3.6 Qualitative estimation of acyloin compounds produced from selected aliphatic aldehydes in the presence and absence of pyruvate using pure yeast pyruvate decarboxylase. Substrate concentrations and incubation conditions are described in Section 2.7.3.

	Absorbance at 540 nm		
Substrate(s)	Analytical	Control	Acyloin (+/-)
Propionaldehyde	0.061	0.083	-
Acetaldehyde	0.301	0.170	+
Pyruvate	0.823	0.294	+
Propionaldehyde + Pyruvate	0.228	0.112	+
Acetaldehyde + Pyruvate	1.764	0.284	+
H_2O	0.139	0.107	-
Std. 5 µg Acetoin	0.131	0.000	+

Table 3.7 Qualitative estimation of acyloin compounds produced from selected aliphatic aldehydes in the presence and absence of pyruvate using soluble fraction of yeast cell homogenate. Substrate concentrations and incubation conditions are described in Section 2.7.3.

	Absorbance at 540 nm		
Substrate(s)	Analytical	Control	Acyloin (+/-)
Propionaldehyde	0.007	0.014	-
Acetaldehyde	0.780	0.179	+
Pyruvate	0.951	0.100	+
Propionaldehyde + Pyruvate	0.193	0.035	+
Acetaldehyde + Pyruvate	2.020	0.347	+
H_2O	0.041	0.057	-
Std. 5 µg Acetoin	0.118		+

All positive results obtained were reflected by the presence of a pink colour which implies that propionaldehyde was not converted to propioin by either pyruvate decarboxylase or by soluble fraction of yeast cell homogenate. The formation of acyloin (acetoin) with acetaldehyde as substrate for pure yeast pyruvate decarboxylase is difficult to explain as yeast pyruvate decarboxylase does not form acetoin from acetaldehyde alone (Neuberg and Simon, 1933). However, small quantities of acetoin can be formed spontaneously in acetaldehyde solutions. The formation of acyloin when soluble fraction was incubated with acetaldehyde cannot be conclusively attributed to propioin synthase activity, (a) because propioin was not formed when propionaldehyde was incubated under the same conditions and (b) because a positive result was obtained with pure pyruvate decarboxylase. There may have been some pyruvate in the soluble fraction which would give rise to production of some acetoin in the presence of acetaldehyde.

Pyruvate alone, and the combinations of propionaldehyde and pyruvate, and acetaldehyde and pyruvate all give rise to the production of acyloin compounds by both pure pyruvate decarboxylase and soluble fraction. The pink colour obtained in the Voges-Proskauer reaction would suggest that the acyloin produced in the case of acetaldehyde and pyruvate mixed, and pyruvate alone, was acetoin. When pyruvate and propionaldehyde were co-substrates, the pink colour obtained would suggest that the product was the 5 carbon ketol formed by the condensation of propionaldehyde with 'active acetaldehyde'.

3.3.5 The Effect of Acetaldehyde and Propionaldehyde on Colour Development in the Westerfeld Colorimetric Assay

The degree to which acetaldehyde and propionaldehyde interfere with colour development in the Westerfeld colorimetric assay was investigated. This experiment was performed to ensure that the concentrations of these aldehydes used in the reactions in Section 3.3.4 were not totally inhibiting the detection of any acyloin compound that might be formed. Solutions containing 10 µg of acetoin, with and without various concentrations of propionaldehyde and acetaldehyde was assayed according to Westerfeld (1945) as described in Section 2.3.2. The results are presented in Table 3.8.

Table 3.8 Effect of acetaldehyde and propionaldehyde on colour development in the Westerfeld assay (1945). Solutions, containing 10 µg acetoin with and without various concentrations of these aldehydes were assayed.

	Alphatic Aldehyde		
	(µl)	µmoles	% Inhibition of Colour Development
Propionaldehyde	(20)	275	82
	(15)	207	79
	(10)	138	62
	(5)	69	42
	(2)	27	11
Acetaldehyde	(20)	354	44
	(15)	265	42
	(10)	177	34
	(5)	88	22
	(2)	35	9
	(0)	0	0

Propionaldehyde inhibited colour development by almost 50% more than acetaldehyde. The presence of 275 µmoles of propionaldehyde resulted in only 18% of maximum colour development, this increased to 89% when the aldehyde was reduced ten fold. In the case of acetaldehyde, 354 µmoles inhibited colour development by 44%. This decreased to 9% in the presence of 35 µmoles of acetaldehyde.

3.3.6 Appraisal of Results

The broad substrate specificity of the carbinol forming system was illustrated by the conversion of ortho, meta and para substituted aromatic aldehydes to the corresponding L-acetyl aromatic carbinols by Saccharomyces cerevisiae. The aldehyde was also converted to the aromatic alcohol in all cases. Pure yeast pyruvate decarboxylase converts benzaldehyde and pyruvate to PAC. The presence of another acyloin forming enzyme in bakers' yeast, propioin synthase, was not confirmed. Detection of an alternative acyloin-forming activity was not quantitative due to the inhibition of the assay by the aldehyde substrates but it may be concluded from the results that propioin synthase does not play a significant role in acyloin formation in the yeast cell.

3.4 CONVERSION OF AROMATIC ALDEHYDE TO AROMATIC ALCOHOL BY YEAST ALCOHOL DEHYDROGENASE

The incubation of Saccharomyces cerevisiae with a range of substituted aromatic aldehydes led to the formation of the corresponding aromatic alcohol in all cases (Section 3.3.1). The role of yeast alcohol dehydrogenase in this conversion was investigated by incubating a range of substituted aromatic aldehydes with pure yeast alcohol dehydrogenase and assaying for alcohol

formation. The conversion of benzaldehyde to benzyl alcohol by the isozymes of yeast alcohol dehydrogenase was examined and compared to the corresponding acetaldehyde to ethanol reaction using polyacrylamide gel electrophoresis.

3.4.1 Conversion of Benzaldehyde to Benzyl Alcohol: Determination of Position of Equilibrium of the Reaction

The reaction was measured in both directions so as to determine the position of equilibrium. Firstly, benzaldehyde was incubated with enzyme as in Section 2.7.2. The reaction was assayed by high performance liquid chromatography (Section 2.5) before addition of the enzyme and after equilibrium had been reached. Secondly, benzyl alcohol with NAD in place of NADH, was similarly incubated and assayed. The results showed that in the alcohol to aldehyde reaction 97.8% of the benzyl alcohol remained at equilibrium, whereas, in the case of aldehyde to alcohol, only 1.5% of the benzaldehyde remained at equilibrium. In this case the equilibrium lies on the side of alcohol formation. This is the expected result as the equilibrium constant for ethanol formation is 8.0×10^{-12}M (Bergmeyer, 1983).

3.4.2 Aromatic Aldehydes as Substrates for Yeast Alcohol Dehydrogenase

The titres of aromatic alcohols produced from benzaldehyde and substituted aromatic aldehydes containing a $-CH_3$, $-CF_3$, $-OCH_3$ or -Cl in either the ortho, meta or para ring position were determined after a 6 h incubation with NADH and purified yeast alcohol dehydrogenase (Section 2.7.2). The results are presented in Table 3.9.

Table 3.9 Conversion of aromatic aldehyde to aromatic alcohol after a 6 h incubation with yeast alcohol dehydrogenase. Incubation conditions are described in Section 2.7.2.

Substrate Aromatic aldehyde	Product Aromatic alcohol	Product Yield m mole/l	Location of ring substituents (Refer Figure 1)		
			X	Y	Z
Benzaldehyde	Benzyl alcohol	2.4	H	H	H
o-Tolualdehyde	2-Methylbenzyl alcohol	0.3	CH_3	H	H
m-Tolualdehyde	3-Methylbenzyl alcohol	1.4	H	CH_3	H
p-Tolualdehyde	4-Methylbenzyl alcohol	2.0	H	H	CH_3
2-Chlorobenzaldehyde	2-Chlorobenzyl alcohol	1.3	Cl	H	H
3-Chlorbenzaldehyde	3-Chlorobenzyl alcohol	2.0	H	Cl	H
4-Chlorbenzaldehyde	4-Chlorobenzyl alcohol	2.8	H	H	Cl
o-Anisaldehyde	2-Methoxybenzyl alcohol	0.6	OCH_3	H	H
m-Anisaldehyde	3-Methoxybenzyl alcohol	3.0	H	OCH_3	H
p-Anisaldehyde	4-Methoxybenzyl alcohol	3.3	H	H	OCH_3
α, α, α-Trifluoro-o-tolualdehyde	2-(Trifluoromethyl)benzyl alcohol	*	CF_3	H	H
α, α, α-Trifluoro-m-tolualdehyde	3-(Trifluoromethyl)benzyl alcohol	0.7	H	CF_3	H
α, α, α-Trifluoro-p-tolualdehyde	4-(Trifluoromethyl)benzyl alcohol	0.7	H	H	CF_3

* Standard insoluble

Corresponding aromatic alcohols were produced from all substrates tested. In general, ortho substituted aldehydes were poor substrates for the enzyme. With the exception of CF_3 containing substrates, which produce low product titres, alcohol levels obtained from meta and para substituted aldehydes were 54-134% of the level produced from the benzaldehyde control.

3.4.3 The Role of Isozymes of Yeast Alcohol Dehydrogenase (YADH) in the Conversion of Benzaldehyde to Benzly Alcohol

The results presented in Table 3.9 show that YADH is instrumental in the conversion of aromatic aldehyde (including benzaldehyde) to the corresponding aromatic alcohol. The role of the isozymes of YADH in the conversion of benzyl alcohol was investigated. In the following experiments the activity of YADH isozymes from yeast cell free extracts towards benzyl alcohol and ethanol were compared. The reactivity of these substrates with a commercial preparation of ADH was also examined. The role of mitochondrial ADH (ADH III) in the conversion of benzaldehyde to benzyl alcohol was investigated.

A crude, cell free extract (soluble fraction) of Saccharomyces cerevisiae was prepared as described in Section 2.8.2. Extracts were applied to polyacrylamide gels and the isozymes were separated by non-denaturing discontinuous polyacrylamide gel electrophoresis (disc PAGE), (Section 2.9). In a parallel experiment, purified, commercial YADH was electrophoresed under the same conditions. Enzyme activity was visualised by the tetrazolium method (Section 2.10) using both ethanol and benzyl alcohol as substrates. Under physiological conditions the equilibrium of the reaction lies on the

side of alcohol formation (Section 3.4.1). In using the tetrazolium method, to visualise enzyme activity the reaction is driven in the opposite direction as electrons are passed from NADH to an electron acceptor (Phenazine methosulphate) which in turn reduces the nitro blue tetrazolium to a blue insoluble formazan. The electrophoretic patterns (zymograms) for crude extract and pure enzyme are presented in Figure 3.8.

Figure 3.8 (Overleaf) Polyacrylamide gels illustrating the reactivity of ADH isozymes in yeast cell homogenate and of commercial ADH with ethanol and benzyl alcohol substrates. All running gels contain 6% acrylamide.

(a) Crude extract (200 µg) Benzyl alcohol substrate.

(b) Commercial ADH (150 µg) Benzyl alcohol substrate

(c) Mitochondrial ADH Benzyl alcohol substrate
((i) 100 µg (ii) 200 µg (iii) 300 µg)

(d) Crude extract (100 µg) Ethanol substrate

(e) Commerical ADH (15 µg) Ethanol substrate

(f) Mitochondrial ADH Ethanol substrate
((i) 50 µg (ii) 100 µg (iii) 150 µg)

(g) Total protein in crude extract

(h) Crude extract (100 µg) No substrate in tetrazolium mix (Control)

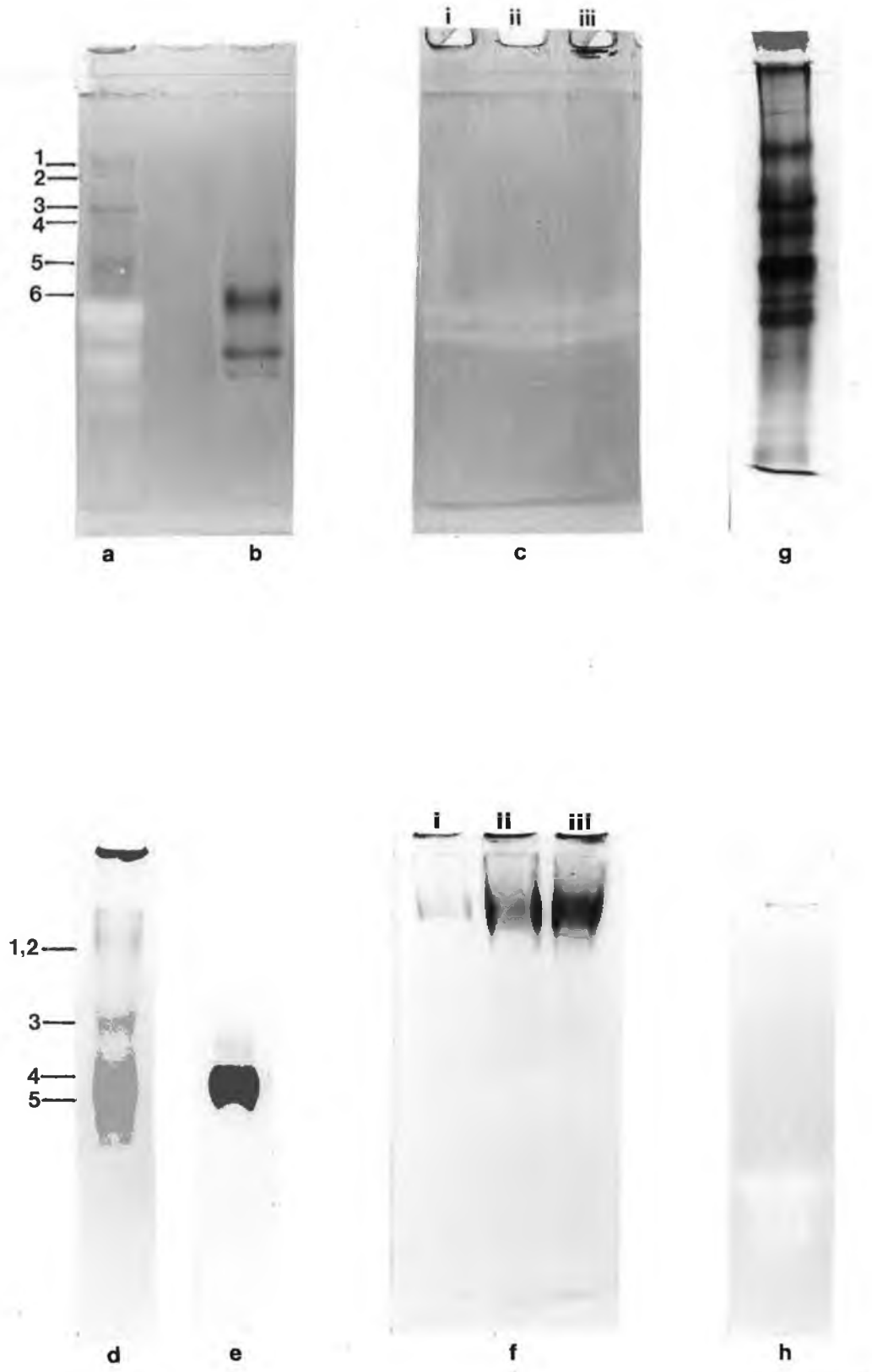


Figure 3.8

When ethanol was used as a substrate (Figure 3.8(d)), 3 major bands were observed, band (1,2), band 3 and a very dense band 5. Band (1,2) was composed of two sub-bands which were difficult to distinguish due to the diffuse nature of the bands. Band 5 was a major band which corresponded exactly to the one and only band due to commercial ADH activity. It has previously been shown that isozyme ADH I corresponds closely to the commercially available but taxonomically uncharacterised enzyme (Wills & Jornvall, 1979). Therefore, band 5 is assumed to be ADH I.

When benzyl alcohol was used as a substrate, three major bands of equal intensity were obtained. (Bands 1, 3 and 5 in Figure 3.8(a)). In addition, 3 bands of a lesser intensity were visualised (bands 2, 4 and 6). As was the case for the ethanol substrate, bands 1 and 2 appear to be associated, band 4 may be a sub-band of band 3, while band 6 corresponds exactly to the pure ADH standard (commercial enzyme) and is assigned ADH I.

In Figure 3.8, the reactivity of benzyl alcohol and ethanol with the isozymes of ADH was compared, yet the quantity of protein loaded on the gel was different in each case. In an experiment where equal quantities of protein were used (100 µg), the exact same banding patterns were obtained, but in the case of benzyl alcohol the bands were less clear and more difficult to distinguish. The total protein present in the crude extract was stained using Coomassie Blue (Section 2.11.6) and is illustrated in Figure 3.8(g).

3.4.4 The Role of Mitochondrial ADH in the Conversion of Benzaldehyde to Benzyl Alcohol

To investigate the role of mitochondrial ADH in the conversion of benzaldehyde to benzyl alcohol, yeast mitochondria were isolated as described in Section 2.8.2. The reactivity of mitochondrial ADH with benzyl alcohol and ethanol was compared.

The mitochondrial fraction was run on non-denaturing polyacrylamide gels as described for the crude extract (Section 2.9). Before samples were loaded on the gels, the enzyme was at least partially dissociated from the membranes by mechanical disruption of the mitochondria (Section 2.9.4). The results are presented in Figure 3.8 (c & f). Activity of benzyl alcohol with mitochondrial ADH was not detected Figure 3.8(f). Concentrations of up to 300 µg mitochondrial protein were tested. A broad, distorted band was observed with ethanol as a substrate Figure 3.8(f).

Activity was detected with as low as 50µg of protein. The distorted appearance may be attributed to the membraneous nature of the preparation. Young and Pilgrim (1985) suggest charge heterogeneity on the subunits or formation of heterotetramers synthesised by two different genes (or both) as possible reasons for the unusual pattern on the gels.

3.4.5 Molecular Weight Determination of Proteins, reacting with Benzyl Alcohol and Ethanol Substrates

Due to the close similarity in banding patterns obtained when benzyl alcohol and ethanol were compared as substrates for soluble yeast

enzymes (Figure 3.8), it is difficult to ascertain whether these bands of reactivity are due to the same protein (which may differ in its degree of reactivity with the 2 substrates) or to different proteins. The following experiment was carried out in order to characterise selected bands obtained with each substrate with regard to molecular weight. Bands selected were numbers 1, 2, 3, 4 and 5 in the case of benzyl alcohol (Band 6 was not included as it corresponded to the major band obtained with commercial ADH which in turn corresponded with band 5 for ethanol). With ethanol as a substrate (Figure 3.8(d)) the molecular weight of bands (1,2), 3, 4 and 5 was determined.

The molecular weight of a given protein was determined as described in Section 2.11. The yeast crude extract was electrophoresed on a set of gels of various polyacrylamide concentrations and the Rf (electrophoretic mobility) of each protein in each gel was determined; 100 log (Rf x 100) was plotted against the percent gel concentration for each protein. Such a plot is called a Ferguson plot (Ferguson (1964)), (Figure 3.9) and its slope is the Retardation Coefficient (K_R). In parallel, a set of standards of known molecular weight were run on various gel concentrations. Individual slopes (K_R) were determined for each standard (Table 3.10) using linear regression analysis and the logarithm of the negative slope was plotted against the logarithm of the molecular weight of each protein. This produces a linear plot from which the molecular weights of the unknown proteins were determined. The results are presented in Table 3.11.

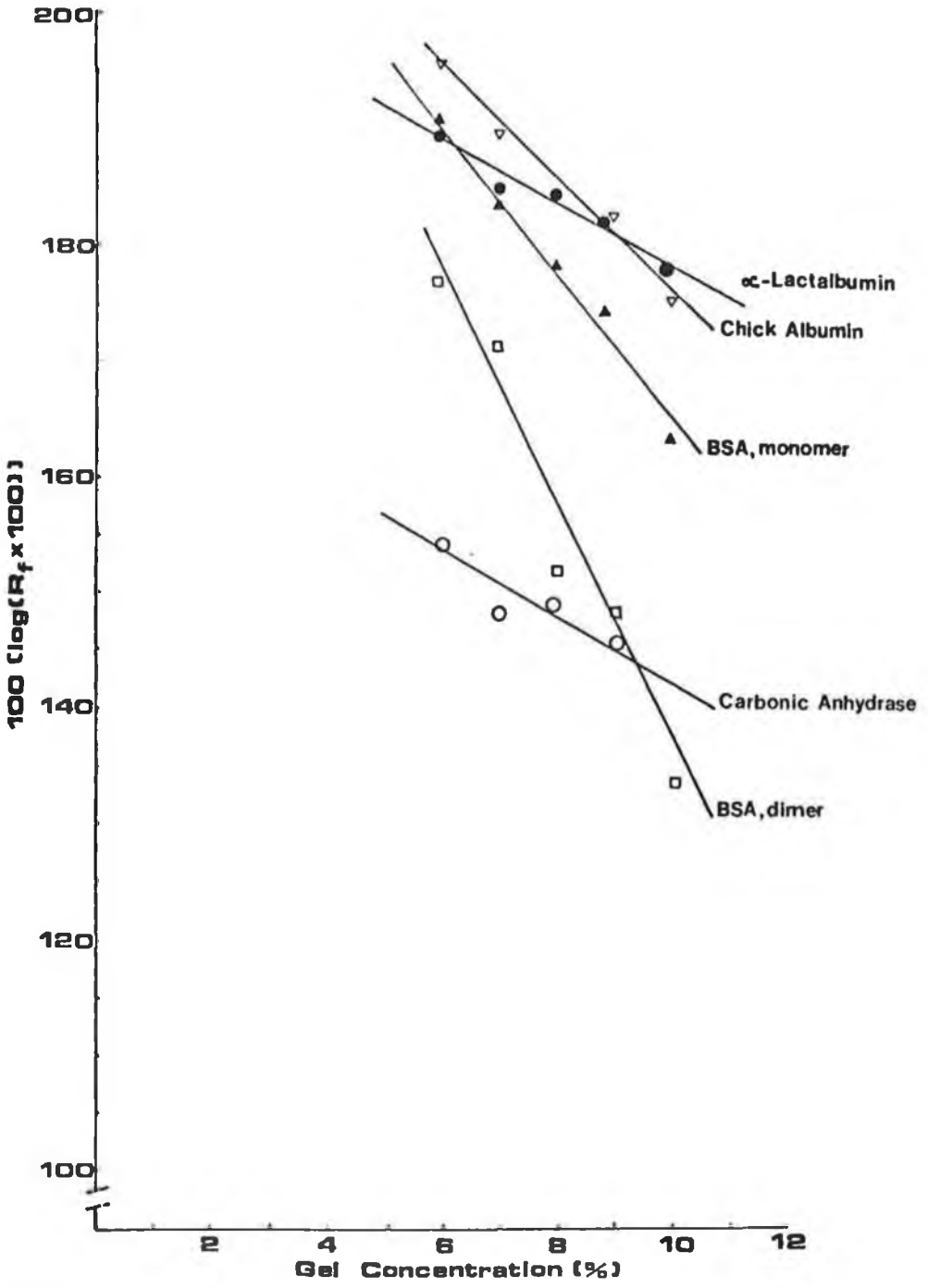


Figure 3.9. Ferguson plots for standards of known molecular weight.

Bands 1 and 2 (Benzyl alcohol) and band 1,2 ethanol all have molecular weights in the region of 150,000 and may correspond to the same protein. Bands 3, 4 and 5 (benzyl alcohol) had molecular weights of 55,000, 45,000 and 45,000 respectively, while bands 3, 4 and 5 (ethanol substrate) had molecular weights of approximately twice those figures.

Table 3.10 Retardation coefficients of molecular weight standards. These were calculated from the slope of the Ferguson Plot (Figure 3.9).

Standard	Molecular Wt.	Calculated K_R
α-Lactalbumin	14,200	-2.48
Carbonic Anhydrase	29,000	-2.36
Albumin, Chicken egg	45,000	-4.47
Albumin, Bovine Serum	6,000 (monomer)	-11.02
	132,000 (dimer)	-6.32

Table 3.11 Molecular weights of proteins on zymograms of yeast crude extract using benzyl alcohol and ethanol as substrates. Molecular weights were determined as described in Section 3.4.5.

Substrate	Band No.	Calculated K_R	Calculated Mol. Wt.
Benzyl alcohol	1	-13.2	160,000
	2	-12.0	147,500
	3	-5.3	55,000
	4	-4.5	45,000
	5	-4.4	45,000
Ethanol	1,2	-11.8	147,500
	3	-9.6	110,000
	4	-8.5	98,000
	5	-9.0	105,000

3.4.6 Appraisal of Results

It was shown by using a range of aromatic aldehyde substrates and pure enzyme that YADH converts these aldehydes to the corresponding alcohol in whole cell systems. Polyacrylamide gel electrophoresis of yeast cell free extracts indicated that a multiplicity of proteins could convert benzaldehyde to benzyl alcohol. These proteins were further characterised by molecular weight analysis (by polyacrylamide gel electrophoresis). In total 5 bands were characterised, 2 had a molecular weight of approximately 150,000, the published molecular weight for YADH. The other 3 bands were shown to have molecular weights of 55,000, 45,000 and 45,000 respectively. When ethanol was compared to the benzyl alcohol substrate, one band had a corresponding molecular weight of approximately 150,000 while the other three bands had molecular weights of 110,000, 98,000 and 105,000 respectively. Ethanol was a substrate for mitochondrial ADH but no reactivity was observed with the benzyl alcohol substrate even with up to 6 times more protein. The results are difficult to explain in terms of the whole cell system, at least one isozyme of YADH converts benzaldehyde to benzyl alcohol. The other proteins are different to those catalysing the acetaldehyde-ethanol reaction which have a molecular weight of approximately one third the published value for YADH. They could possibly be active fragments of the YADH enzyme or even totally different dehydrogenase enzymes.

3.5 BIOTRANSFORMATION OF AROMATIC ALDEHYDES BY SACCHAROMYCES CEREVISIAE: INVESTIGATION OF REACTION RATES

The following experiments were carried out to investigate the optimum conditions for the enzymic reaction involved in the biotransformation of aromatic aldehyde to L-acetyl aromatic carbinol and to examine the rate of aromatic alcohol by-product formation under these conditions. In preliminary studies (Section 3.1) optimum conditions for the biotransformation of benzaldehyde over a 5 h period were determined. These conditions are not necessarily optimum for the enzymes involved but rather prolong product formation. As with any enzymic reaction, optimum conditions for enzyme activity were determined by measuring initial reaction rates, in this case, in a cellular system.

Rate of production of L-phenylacetyl carbinol by Saccharomyces cerevisiae in reaction mixtures containing benzaldehyde with sucrose as co-substrate was investigated in short 1 h incubations. To investigate 'initial rates' for these biotransformations, shorter reaction periods are impractical because of possible delays in diffusion of substrate and product into and from the cell respectively. The effect of yeast dose rate, sucrose and benzaldehyde concentration, pH and biotransformation products on the rate of reaction was determined.

The basic medium used for the experiments was Medium A (optimum sucrose concentration and pH were determined). Erlenmeyer flasks (250 ml), containing 150 ml of medium were inoculated with fresh pressed bakers' yeast (30% (w/v) solids content) and incubated as described previously (Section 2.2.4). Following an equilibration

period of 1 h the fermentation was initiated by addition of aromatic aldehyde. The shake flask reaction was carried out for 1 h. L-acetyl aromatic carbinols were determined colorimetrically (Section 2.3.1) and aromatic alcohols by GC (Section 2.4.2).

3.5.1 The Effect of Benzaldehyde Concentration on the Rate of L-Phenylacetyl Carbinol Production

The effects of benzaldehyde concentration (0-8 g/l) was investigated in a 1 h reaction time. Medium A (pH 4.5) was inoculated with 30 g/l fresh pressed bakers' yeast (30% (w/v) solids content). Incubation conditions are described in Section 3.5. The results are presented in Figure 3.10. Rate of PAC production increases with increasing benzaldehyde concentration up to 6 g/l where 1.3 g/l PAC is produced. Between 6 and 8 g/l benzaldehyde there is a sharp decline in the rate of PAC production.

3.5.2 The Effect of Yeast Dose Rate on the Rate of L-Phenyl-Acetyl Carbinol Production

Using a benzaldehyde concentration of 6 g/l the effect of yeast dose rate on PAC production was investigated. Yeast dose rate was varied from 10-70 g/l. All other conditions were as described in Section 3.5. The results are presented in Figure 3.11. The rate of PAC production increased with increasing dose rate up to 60 g/l which produced 2.7 g/l PAC.

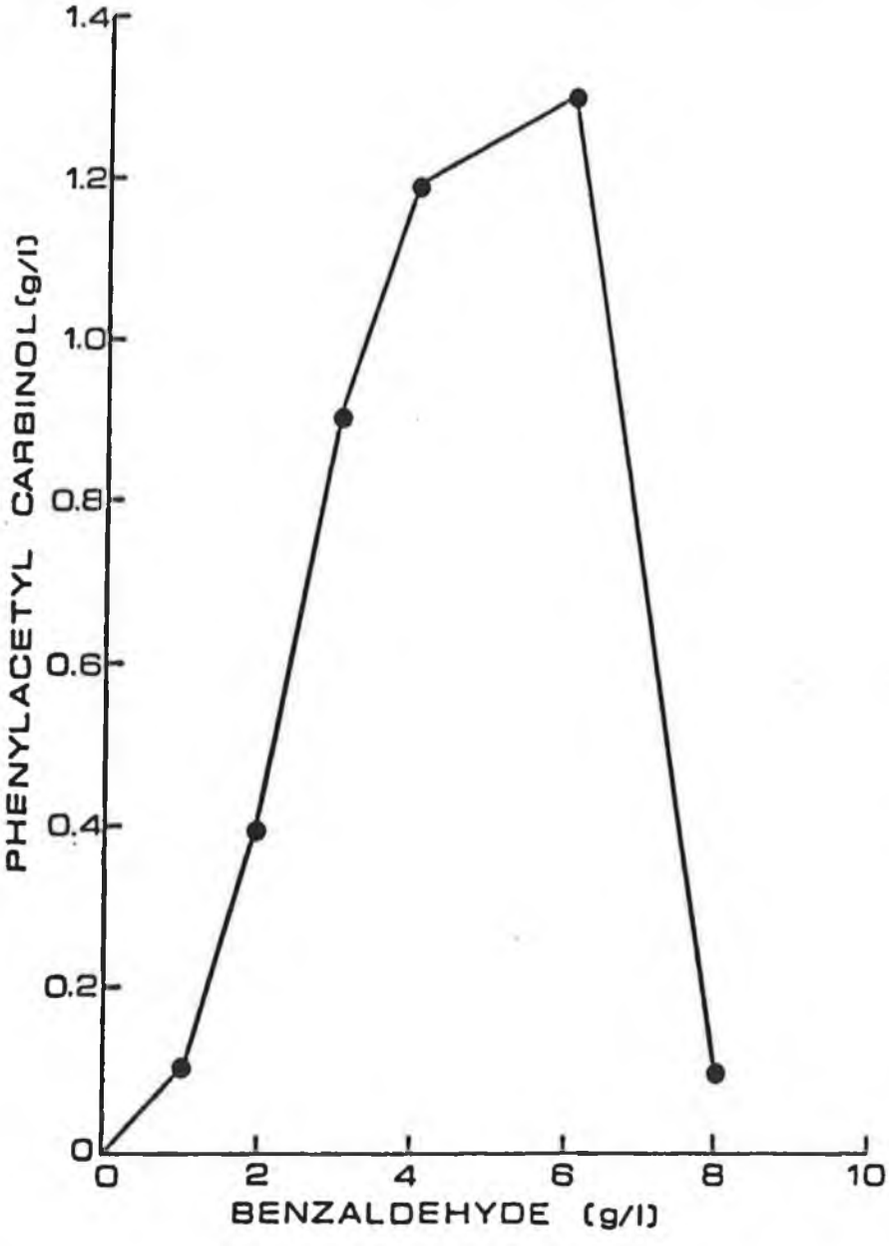


Figure 3.10. Effect of benzaldehyde concentration on the rate of L-phenylacetyl carbinol production in a 1 h biotransformation. Medium A was inoculated with 30 g/l fresh pressed bakers' yeast (30% (w/v) solids content). Conditions are described in Section 3.5.

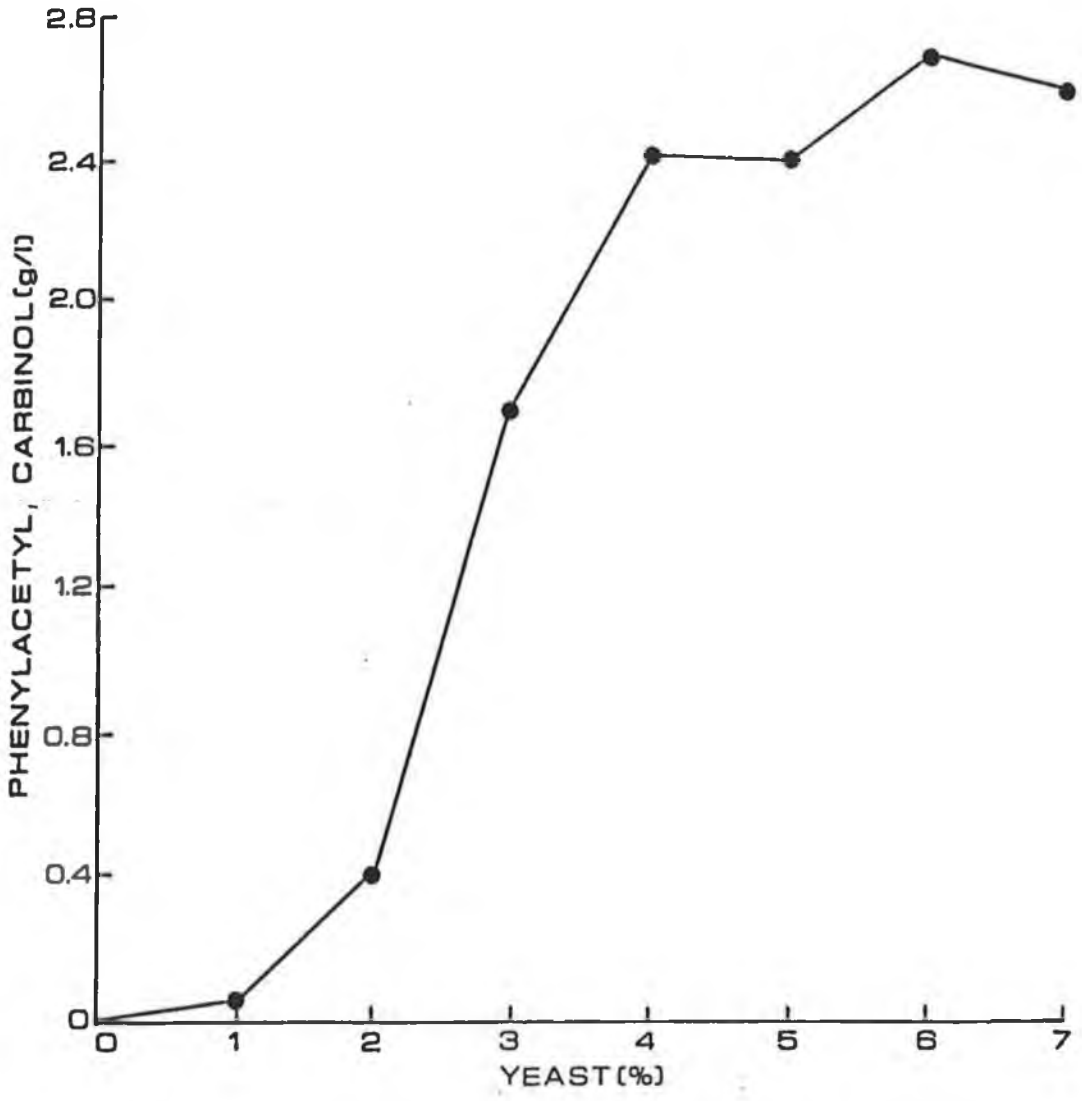


Figure 3.11. Effect of yeast dosage on rate of L-phenylacetyl carbinol production in a 1 h biotransformation. Reaction conditions are described in Section 3.5.2.

3.5.3 The Effect of Sucrose Concentration on the Rate of L-Phenylacetyl Carbinol Production

Using a benzaldehyde concentration of 6 g/l and a yeast dose rate of 60 g/l, the effect of sucrose concentration on PAC production was determined. The results are presented in Figure 3.12. Rate of PAC formation increased with increasing sucrose concentration and was maximum at 50 g/l when 2.8 g/l PAC was produced in 1 h.

3.5.4 The Effect of pH on the Rate of L-Phenylacetyl Carbinol Production

The effect of pH on reaction rate was determined by varying the initial pH in the range pH 2-6. Medium A was inoculated with 60 g/l fresh pressed bakers' yeast (30% (w/v) solids content). 6 g/l benzaldehyde was added following the 1 h equilibration period. The results are presented in Table 3.12. Substantial conversion rates were observed in a broad pH range, 3-6, with a maximum bioconversion rate of 2.5 $gl^{-1}h^{-1}$ at pH 4.5.

Table 3.12 Effect of pH on production of phenylacetyl carbinol in a 1 h biotransformation. Medium A was inoculated with 60 g/l fresh pressed bakers' yeast (30% (w/v) solids content). Conditions are described in Section 3.5.4.

Initial pH	Final pH	Phenylacetyl carbinol (g/l)
2.0	2.3	0.1
3.0	3.0	2.0
4.0	4.1	2.5
4.5	4.4	2.5
5.0	4.9	2.1
5.5	5.4	2.3
6.0	5.7	2.0

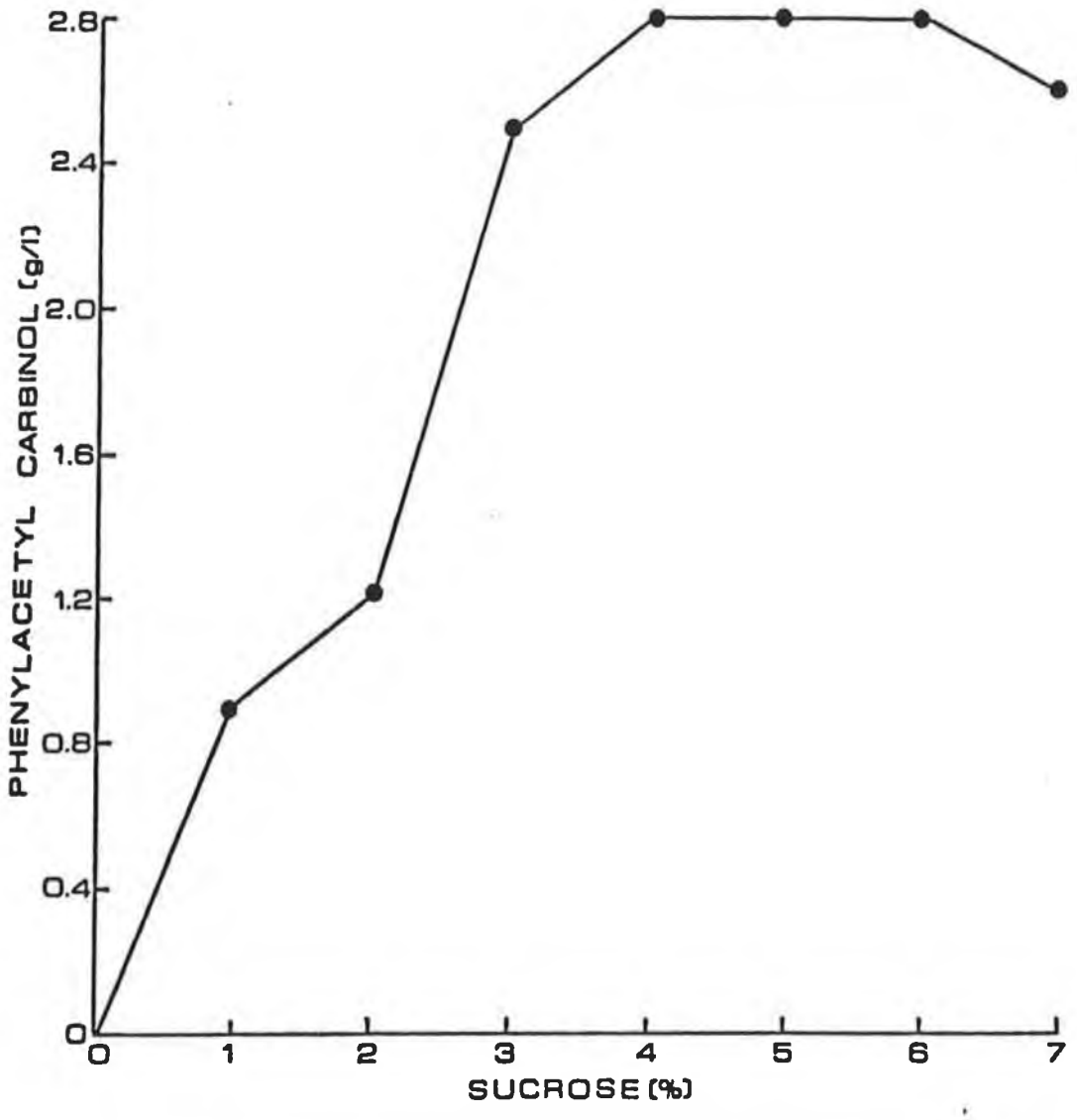


Figure 3.12. Effect of sucrose concentration on L-Phenylacetyl carbinol production in a 1 h biotransformation. Medium A, was inoculated with 60 g/l fresh pressed bakers' yeast (30% (w/v) solids content). 6 g/l benzaldehyde was added following a 1 h equilibration period.

3.5.5 Rates of Bioconversion of Benzaldehyde and Selected Substituted Aromatic Aldehydes

In Section 3.3.1 a range of aromatic aldehydes were investigated as substrates for the L-acetyl aromatic carbinol forming system. The results obtained indicated whether or not these aldehydes were substrates for the system but they did not reflect the rate of the enzymic reaction taking place. This experiment was carried out to investigate the initial rates of the reactions involved in L-acetyl aromatic carbinol and alcohol formation.

Comparative reaction rates for benzaldehyde and selected substituted aromatic aldehydes using both sucrose and sodium pyruvate as co-substrate were investigated. General reaction conditions were the same as those found optimal for the initial rate of PAC formation (Sections 3.5.1 to 3.5.4). Both Medium A and Medium B were used with initial aromatic aldehyde concentrations of 6 g/l. The results are presented in Table 3.13. Rate of aromatic alcohol production was much higher when sucrose was used as co-substrate rather than pyruvate. o-tolualdehyde and 2- and 3-chlorobenzaldehyde were poor substrates for aromatic carbinol formation although 2-chlorobenzaldehyde produced significant aromatic alcohol in sucrose containing medium. These results are consistent with the findings in Section 3.3.1 where it was found that aldehydes substituted in the ortho position were poor substrates for carbinol production. Overall, aldehydes with substituents in the para position seem to produce carbinols at a faster rate than their ortho and meta substituted counterparts, an observation also consistent with earlier findings (Section 3.3.1).

Table 3.13 Rate of formation of aromatic carbinol and aromatic alcohol from aromatic aldehyde and sucrose or pyruvate. Initial aldehyde alcohol concentration was 6 g/l. Reaction conditions are described in Section 3.5.5.

	Sucrose		Pyruvate	
	Aromatic Carbinol	Aromatic Alcohol	Aromatic Carbinol	Aromatic Alcohol
Benzaldehyde	2.74	2.72	3.80	0.39
o-Tolualdehyde	0.00	0.32	0.10	0.06
m-Tolualdehyde	0.45	0.87	1.35	0.20
p-Tolualdehyde	1.71	1.78	1.86	0.08
2-Chlorobenzaldehyde	< 0.05	1.00	< 0.10	0.18
3-Chlorbenzaldehyde	0.43	0.95	0.10	0.20
4-Chlorobenzaldehyde	1.46	1.90	0.64	0.17

3.5.6 Effect of Products, Benzyl Alcohol and L-Phenylacetyl Carbinol on the rate of L-Phenylacetyl Carbinol Production

The rate of an enzyme reaction can be affected by substrate and product concentration. The effect of substrate concentration on the rate of PAC formation has been examined (Section 3.5.1). Flasks, containing medium A (25 ml in 250 ml Erlenmeyer flask) and the appropriate concentration of product were inoculated with 60 g/l fresh pressed bakers' yeast (30% (w/v) solids content) and incubated for 1 h as described in Section 2.2.4. PAC concentration was measured colorimetrically (Section 2.3.1) 1 h following the addition of 6 g/l benzaldehyde.

3.5.7 Effect of L-Phenylacetyl Carbinol Concentration on the Rate of L-Phenylacetyl Carbinol Production

Initial PAC concentration was varied in the range 0-15 g/l. PAC levels were measured before inoculation, following the 1 h equilibration period and 1 h after the addition of benzaldehyde.

The results are presented in Table 3.14. Rate of PAC production decreases with increasing PAC concentration. Rate of PAC production declined from 3.44 $gl^{-1}h^{-1}$ to zero when the initial PAC concentration was raised from zero to 14.3 g/l.

Table 3.14 Effect of L-Phenylacetyl Carbinol concentration on rate of L-phenylacetyl carbinol production. Flasks containing medium A and the appropriate concentration of PAC were inoculated with 60 g/l fresh pressed bakers' yeast (30% (w/v) solids content). Reaction conditions are described in Section 3.5.6.

[PAC] before Inoculation (g/l)	[PAC] following Equilibration (g/l)	[PAC] 1 h after 6 g/l Benzaldehyde (g/l)	Net PAC Produced (g/l)
0.00	0.00	3.44	+ 3.44
5.01	4.43	6.88	+ 2.45
9.54	9.26	9.99	+ 0.73
14.30	14.50	14.30	0.00

3.5.8 Effect of Benzyl Alcohol Concentration on L-Phenylacetyl Carbinol Production

The effect of benzyl alcohol concentration in the range 0-8 g/l on PAC production was investigated. PAC concentration was measured 1 h following the addition of 6 g/l benzaldehyde. The results are presented in Figure 3.13. PAC production decreases with increasing initial concentrations of benzyl alcohol in the range 2-8 g/l.

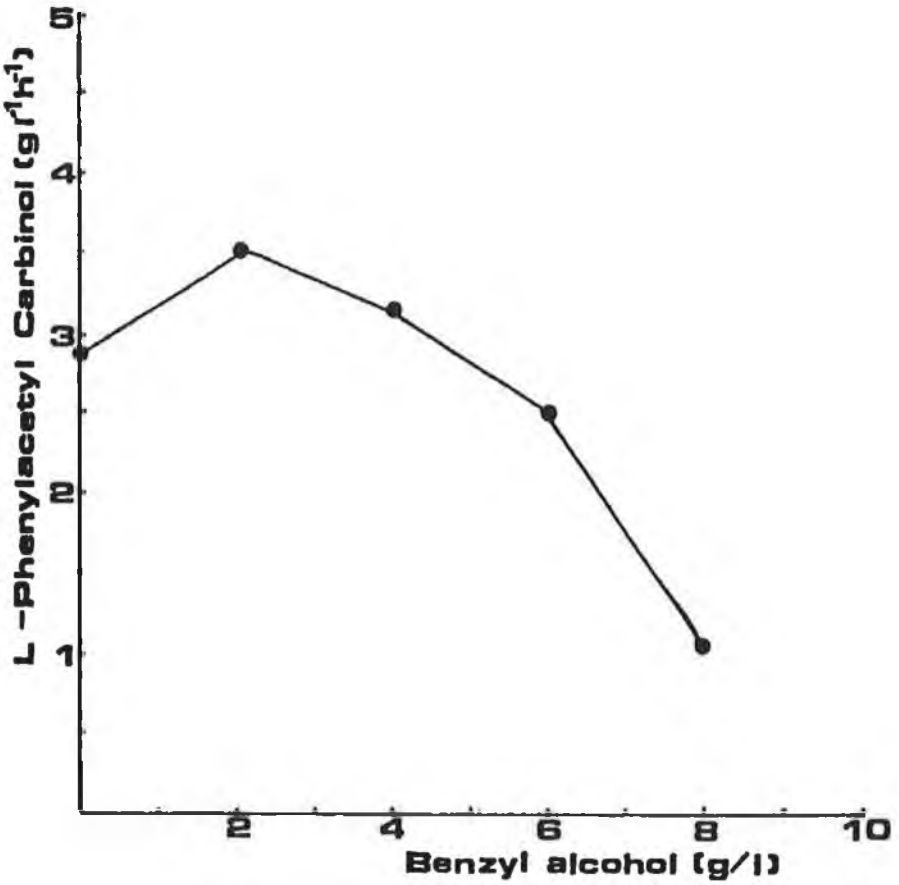


Figure 3.13. Effect of benzyl alcohol concentration on the rate of L-phenylacetyl carbinol production. Medium A, containing the appropriate concentration of benzyl alcohol was inoculated with 60 g/l fresh pressed bakers' yeast (30% w/v) solids content). Reaction conditions are described in Section 3.5.6.

3.5.9 Appraisal of Results

Investigation of reaction rates using benzaldehyde and selected substituted aromatic aldehydes confirmed the broad substrate specificity of the carbinol forming system, while also illustrating the differences in reaction rates depending on the position and nature of the ring substituent. Rate of aromatic alcohol production was higher with sucrose as co-substrate than with a pyruvate co-substrate.

Highest initial rates of PAC formation were observed in medium A containing 6 g/l benzaldehyde (substrate) and 60 g/l fresh pressed bakers' yeast (30% (w/v) solids content). This indicates that a higher concentration of substrate and enzyme than was used previously (Section 3.1) is required for maximal rates of product formation. As benzaldehyde concentration increased above 6 g/l, the rate of PAC production decreased. Increase in product (PAC and benzyl alcohol) concentration also resulted in lower rates of PAC formation. This decrease in reaction rate may be due to inhibition of the enzymes involved by substrate and/or products, the attainment of a position of equilibrium or an overall inhibition of cellular metabolism due to toxicity effects of substrate and/or products.

3.6 BIOTRANSFORMATION OF BENZALDEHYDE BY SACCHAROMYCES CEREVISIAE: CHARACTERISATION OF THE FERMENTATION AND TOXICITY EFFECTS OF SUBSTRATES AND PRODUCTS

In Section 3.5 it was observed that as the concentration of biotransformation substrate and products increases the rate of

product formation decreases. It is possible that the substrate and products, which are all organic molecules, have a toxic effect on the yeast cell, perhaps by interaction with membrane components or by denaturation of one or several key enzymes. Ethanol, a yeast metabolite, can be toxic to yeast cells at certain levels. The degree of toxicity depends on a number of factors including the yeast strain, temperature, osmotic pressure and intracellular ethanol (D'amore and Stewart, 1987).

In the following experiments the profiles of three model fermentations (F1, F2 and F3) were analysed. The effect of substrates and products on yeast viability, cellular protein, carbohydrate and lipid and key enzymes in the biotransformation were examined. The effect of substrates and products on yeast cell permeability and the ability of homogenised and non-viable cells to make PAC was also investigated.

3.6.1 Three Model Fermentations for Production of L-Phenylacetyl Carbinol

Fermentations were carried out in 2 l flasks containing 1 l of medium A which were incubated under standard conditions (Section 2.2.4). At intervals during the fermentation and after 10 h cells were removed by centrifugation for analysis of the broth. Yeast dose rates for F1, F2 and F3 were 30, 60 and 60 g/l respectively. For F1 and F2, benzaldehyde was added to the fermentation in 6 aliquots of 2 $gl^{-1}h^{-1}$ for 6 h. In the case of F3, benzaldehyde was added in 2 aliquots of 6 g at the start of the biotransformation and after 4 h. Benzaldehyde, phenylacetyl carbinol, benzyl alcohol, residual sucrose concentration and yeast

viability was monitored at intervals throughout the fermentation (Sections 2.4.2, 2.3.1, 2.4.2, 2.3.3 and 2.2.6 respectively). The results are presented in Figures 3.14, 3.15 and 3.16.

There was a higher initial rate of production of PAC in F3 containing the high starting benzaldehyde concentration. However, PAC concentration peaked at 3.3 g/l after 2 h and was accompanied by a massive reduction in yeast viability. When benzaldehyde was pulse fed at the rate of 2 $gl^{-1}h^{-1}$ (F1 and F2) higher overall titres of PAC were achieved after 4-5 h. Number of viable yeast cells increased initially and then in the 3-5 h period dropped dramatically with a concomitant rise in benzaldehyde levels. The results suggest that increasing benzaldehyde levels decreases yeast cell viability and that a cessation of PAC production follows the major drop in yeast cell viability. The increase in viable counts observed in the early stages of the F1 and F2 fermentations indicates that at the lower benzaldehyde concentrations observed in these cases, yeast growth can occur.

Sucrose is rapidly metabolised as benzaldehyde level is depleted in F2, which contains 60 g/l yeast. In F1, which contains 30 g/l and F3, which contains 60 g/l yeast but a high initial benzaldehyde concentration, sucrose metabolism appears to be inhibited. Patterns of benzyl alcohol production were fairly similar in the three model fermentations.

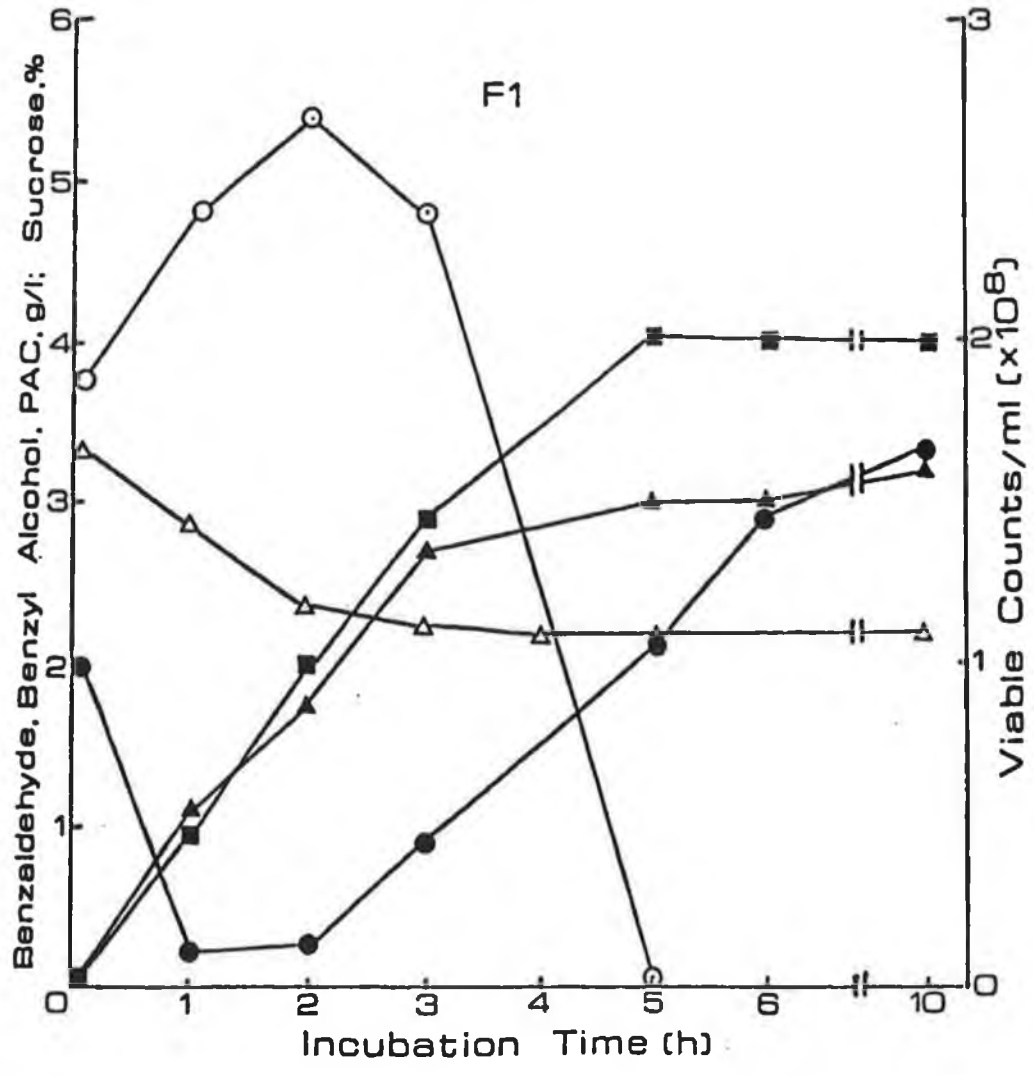


Figure 3.14. Profile of model fermentation F1. Yeast dose rate was 30 g/l, benzaldehyde was added to the fermentation in 6 aliquots of 2 g l^{-1} h^{-1}. Conditions for the fermentation are described in Section 3.6.1. For Figures 3.14 to 3.16, O represents viable counts; △, residual sucrose; ●, benzaldehyde; ■, PAC and ▲, benzyl alcohol.

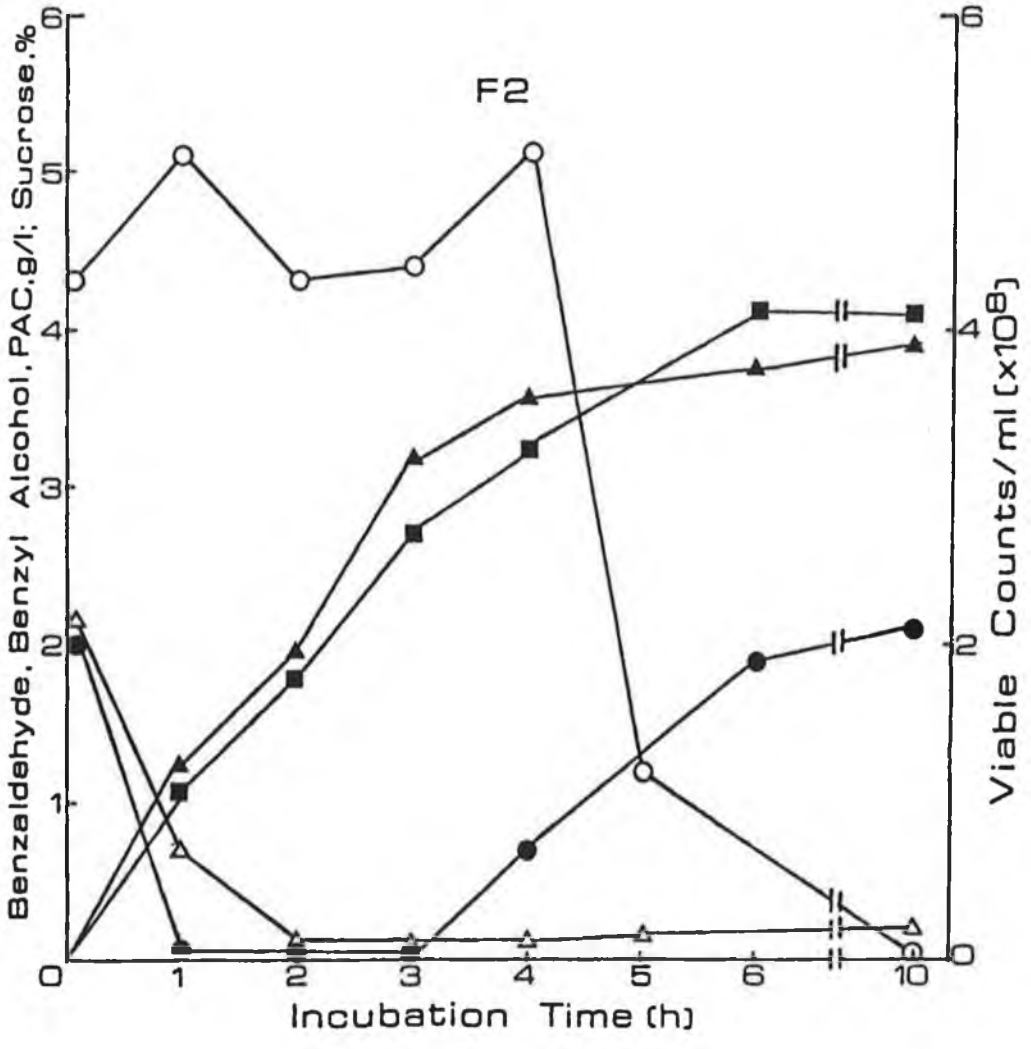


Figure 3.15. Profile of model fermentation F2. Yeast dose rate was 60 g/l, benzaldehyde was added to the fermentation in 6 aliquots of 2 $gl^{-1}h^{-1}$. Conditions for the fermentation are described in Section 3.6.1.

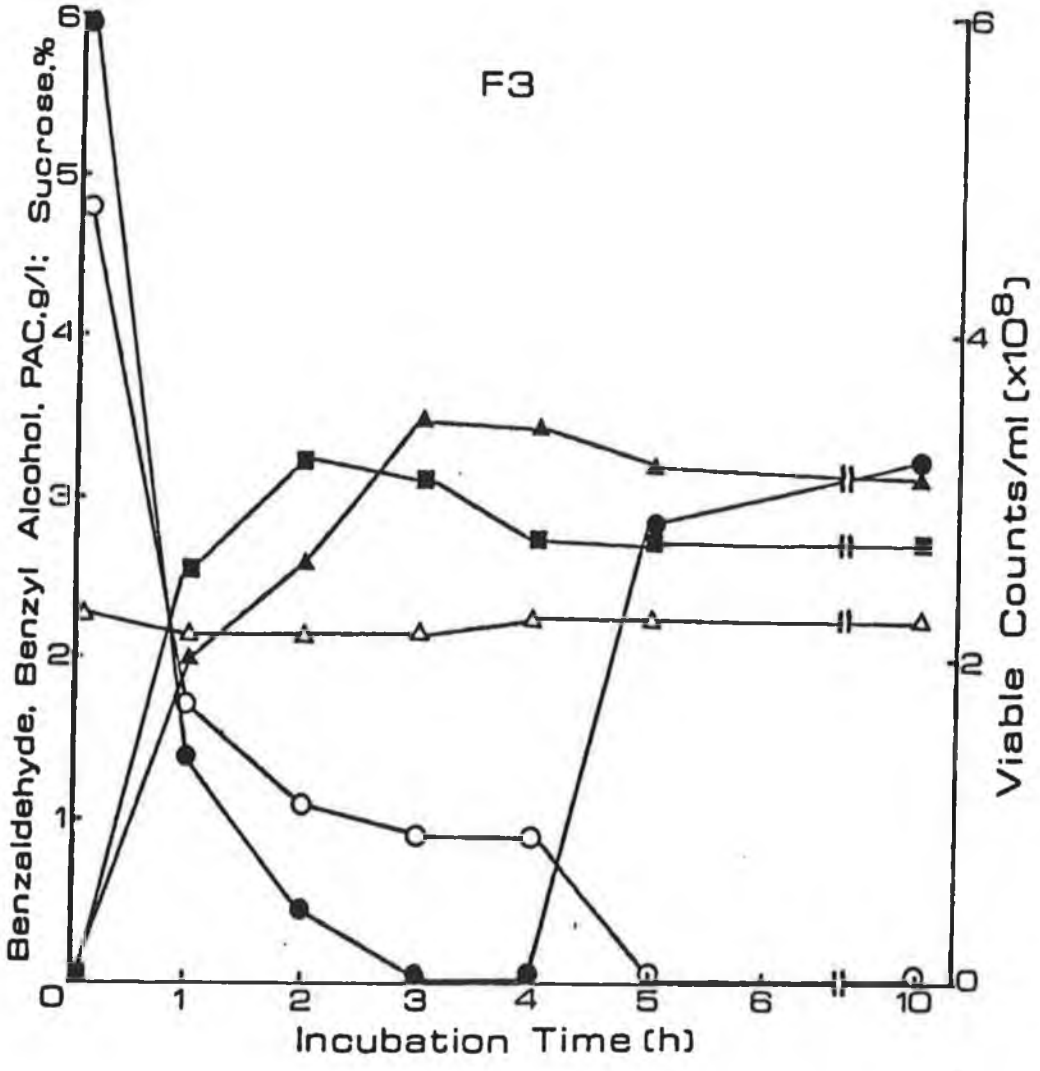


Figure 3.16. Profile of model fermentation F3. Yeast dose rate was 60 g/l, benzaldehyde was added to the fermentation in 2 aliquots of 6 g at the start of the biotransformation and after 4 h. Conditions for the fermentation are described in Section 3.6.1.

3.6.2 Production of L-Phenylacetyl Carbinol by Non-Viable Yeast Cells

Yeast cells, which had lost viability after a 5 h incubation in F1 (<0.01% of viable cell number remains) were recovered and re-inoculated into medium A. The medium contained either sucrose or sodium pyruvate and other cofactors or medium constituents as indicated in Table 3.15. Benzaldehyde was added at a rate of 2 $gl^{-1}h^{-1}$ for 2 h. PAC and benzyl alcohol were measured after a 1 and 2 h incubation. The results obtained are presented in Table 3.15.

The sucrose concentration used was 25 g/l rather than 50 g/l as this reflected the approximate sucrose concentration in F1 after 5 h and would therefore minimise osmotic shock effects. Only negligible levels of PAC and benzyl alcohol were detected as compared with the initial whole yeast bioconversion rates in F1 (Figure 3.14).

Table 3.15 Production of phenylacetyl carbinol by non-viable yeast cells. Yeast cells which had lost viability after 5 h incubation in F1, were reinoculated into medium A. Incubation condiditions are described in Section 3.6.2.

*Medium Variables	Incubation Time (h)	Phenylacetyl Carbinol (g/l)	Benzyl Alcohol (g/l)
Sucrose - 25 g/l	1	0.02	0.05
	2	0.02	0.05
Sodium pyruvate - 30 g/l	1	0.07	0.05
	2	0.08	0.04
Sucrose - 25 g/l	1	0.03	0.05
NAD - 1 mM	2	0.03	0.06

* All media contained: $MgCl_2$, 25 mM; thiamine pyrophosphate, 1.5 mM.

3.6.3 Intracellular and Extracellular Concentrations of Biotransformation Substrate and Products

In order to investigate the possible toxic effect of benzaldehyde towards yeast cells and intracellular proteins/enzymes, intracellular and extracellular concentrations of benzaldehyde, benzyl alcohol and PAC were determined (Sections 2.4, 2.4 and 2.3.1 respectively) in biotransformations having a high (F3) and low (F1) initial benzaldehyde dose rate. (F1 was chosen in preference to F2 because the sucrose concentration in F2 was depleted). Yeast was sampled and intracellular metabolites extracted as described in Sections 2.12.1 and 2.12.3 respectively. Results are presented in Table 3.16.

Where initial benzaldehyde concentrations were low, intracellular concentrations of this substrate and the biotransformation products were substantially lower than extracellular concentrations. With the high initial benzaldehyde concentration, intracellular substrate concentrations were substantially higher than the extracellular levels and similar concentrations of biotransformation products were observed inside and outside of cells. At lower benzaldehyde concentrations, the cell membrane may protect cellular constituents from benzaldehyde by maintaining a gradient between extracellular and intracellular benzaldehyde levels. The results indicate that at higher concentrations of benzaldehyde, this permeability barrier is not maintained. The observation that the intracellular benzaldehyde concentration actually exceeds the extracellular concentration cannot be

explained simply by free diffusion and suggests that benzaldehyde may become concentrated in the cell by association with intracellular lipid or other material.

Table 3.16 Effect of initial benzaldehyde level on intracellular and extracellular concentrations of substrate and products. Substrate/Product concentrations were determined in biotransformations having a high and low initial benzaldehyde dose rate.

Initial Benzaldehyde	Substrate/Product Concentration (g/l)		
		Intracellular	Extracellular
6.0*	Benzaldehyde	7.60	2.75
	Benzyl alcohol	2.45	1.80
	Phenylacetyl carbinol	1.20	1.55
2.0**	Benzaldehyde	0.15	1.10
	Benzyl alcohol	0.45	1.60
	Phenylacetyl carbinol	0.65	1.80

*, ** Substrates, products measured 1 h* (after benzaldehyde addition) and 0.5 h** (after 2nd benzaldehyde addition).

The above results are an average of 2 separate trials.

3.6.4 Effect of Biotransformation Substrate and Products on Yeast Pyruvate Decarboxylase and Alcohol Dehydrogenase

The effect of benzaldehyde, benzyl alcohol and PAC on enzyme stability was investigated. Purified yeast alcohol dehydrogenase and pyruvate decarboxylase were incubated in various concentrations of substrate and products and residual activity monitored as described in Sections 2.3.8 and 2.3.9. Enzymes were incubated as pure protein solutions and in the presence of 20% yeast extract, which might mimic possible stabilising effects of yeast intracellular protein. Reaction mixtures and incubation conditions are described in Section 2.7.4.

3.6.4.1 Effect of benzaldehyde concentration on yeast alcohol dehydrogenase and pyruvate decarboxylase activity:

In order to test the possible effect of observed increased intracellular benzaldehyde concentrations on enzyme stability, purified yeast alcohol dehydrogenase and pyruvate decarboxylase were incubated in various benzaldehyde concentrations and residual activity monitored. The results are presented in Table 3.17.

In the absence of yeast extract, alcohol dehydrogenase was rapidly denatured at higher benzaldehyde levels. At a benzaldehyde concentration of 7 g/l the enzyme only retained 33% of its original activity after 6 h. Pyruvate decarboxylase was substantially resistant to benzaldehyde denaturation. Yeast extract significantly retarded denaturation of alcohol dehydrogenase.

Table 3.17 Effect of benzaldehyde concentration on stability of purified yeast alcohol dehydrogenase and pyruvate decarboxylase. Reaction mixtures and incubation conditions are described in Section 2.7.4.

Benzaldehyde Concentration mg/ml	Yeast Extract Supplement (% w/v)	Residual Activity after 6 h (%)	
		Alcohol Dehydrogenase	Pyruvate Decarboxylase
0.0	0	90.6	89.0
0.2	0	82.1	89.0
1.0	0	76.3	93.0
3.0	0	33.6	100.0
7.0	0	0.0	87.6
0.0	20	74.2	No data
1.0	20	77.9	No data
3.0	20	68.7	No data
7.0	20	61.0	No data

3.6.4.2 Effect of L-phenylacetyl carbinol and benzyl alcohol on yeast alcohol dehydrogenase and pyruvate decarboxylase activity:

The effects of increasing concentrations of the biotransformation products, benzyl alcohol and PAC (95% pure by GC), on alcohol dehydrogenase and pyruvate decarboxylase were examined. The results are presented in Table 3.18.

Benzyl alcohol had a greater denaturing effect on pyruvate decarboxylase, the enzyme only retaining less than half its original activity. On the other hand, PAC was more toxic to alcohol dehydrogenase. At a concentration of 3 g/l, benzyl alcohol caused greater denaturation of pyruvate decarboxylase than benzaldehyde. In contrast, a concentration of 3 g/l benzyl alcohol caused relatively little denaturation of alcohol dehydrogenase over a 6 h period. Partial denaturation of pyruvate decarboxylase by PAC occurs in the absence of yeast extract. However, in the presence of 20% yeast extract, residual activity in solutions containing up to 2 g/l PAC, were approximately equal to the control after a 6 h incubation. Yeast extract also has a significant protecting effect on alcohol dehydrogenase against PAC denaturation.

Table 3.18 Effect of biotransformation products benzyl alcohol and phenylacetyl carbinol on stability of purified yeast alcohol dehydrogenase and pyruvate decarboxylase. Reaction mixtures and incubation conditions are described in Section 2.7.4.

Biotransformation Product	Concentration g/l	Yeast Extract Supplement (% w/v)	Residual Activity after 6 h (%) Alcohol Dehydrogenase	Pyruvate Decarboxylase
Benzyl alcohol	0.0	0	96.2	72.6
	0.5	0	83.3	94.8
	1.5	0	89.2	62.6
	3.0	0	91.0	45.9
	0.0	20	ND	78.8
	3.0	20	ND	65.4
Phenylacetyl carbinol	0.0	0	92.7	79.7
	0.5	0	82.1	78.6
	1.0	0	62.6	73.1
	2.0	0	44.7	70.8
	0.0	20	90.2	78.8
	0.5	20	91.0	ND
	1.0	20	79.8	ND
	2.0	20	75.3	78.8

ND = No data

3.6.5 Effect of Biotransformation Substrate and Products on Biomass and Total Cellular Protein and Carbohydrate

Initial and final yeast dry weight, total cellular protein and carbohydrate content and culture absorbance values (600 nm) were determined on samples from F1, F2 and F3 described previously (Section 3.6.1) before and after a 10 h biotransformation. Procedures are described in Sections 2.3.5, 2.3.6 and 2.2.5 respectively. The results are presented in Table 3.19. Reductions in total dry weight ranged from 22% in F1 to 34% in F3. The pattern of reductions in total cellular protein was similar, ranging from 23% in F1 to 32% in F3. Culture absorbance reductions were somewhat higher, ranging from 31-42%. Reductions in biomass and protein were greatest in F3 where benzaldehyde was added in two larger aliquots of 6 g/l. Similar reductions in yeast carbohydrate content were not observed.

Table 3.19 Yeast dry weight, total cellular protein, carbohydrate content and culture absorbance (600 nm) before and after a 10 h biotransformation. Conditions for F1, F2 and F3 are described in Section 3.6.1.

		ANALYSIS		
Component		Before Biotransformation (mg/ml)	After Biotransformation (mg/ml)	Decrease %
Total dry weight:	F1	8.2	6.3	23
	F2	16.0	12.5	22
	F3	4.6	10.8	34
Protein:	F1	4.61	3.56	23
	F2	9.73	7.24	26
	F3	9.58	6.50	32
Carbohydrate:	F1	2.13	2.32	- 9
	F2	4.66	4.42	9
	F3	4.24	3.97	6
Culture absorbance (1/50 dilution)	F1	0.582	0.400	31
	F2	1.090	0.701	36
	F3	1.079	0.628	42

3.6.6 Effect of Biotransformation Substrate and Products on Yeast Fatty Acid Content

Total fatty acid content was determined on yeast recovered from F1, before and after a 10 h biotransformation. Extraction of lipids, and preparation and analysis of fatty acid methyl esters are described in Sections 2.13, 2.13.1 and 2.4.3 respectively. The results are presented in Table 3.20. Yeast fatty acid content was reduced by approximately 30% which paralleled the reduction in biomass. The proportions of the various fatty acids remained the same.

Table 3.20 Total fatty acid content and proportions of fatty acids in yeast recovered from F1 before and after a 10 h biotransformation. Conditions for F1 are described in Section 3.6.1.

Component		Before	After
Fatty acid % distribution	16:0	11.2	11.4
	16:1	36.6	38.1
	18:0	8.1	7.9
	18:1	41.3	36.6
	18:2	1.9	2.2
	18:3	0.9	0.8
Total yeast fatty acids mg/ml culture		0.51	0.36
Total yeast biomass mg/ml culture		8.80	6.60
Total fatty acids mg/g yeast		57.50	54.00

3.6.7 Production of L-Phenylacetyl Carbinol by Homogenates of Viable Yeast Cells

The biotransformation ability of whole yeast cells and homogenates of viable cells, with and without added cofactors was compared. Yeast cells (30 g/l) were inoculated into 150 ml of medium B in

250 ml Erlenmeyer flasks and incubated under normal conditions (Section 2.2.4). Cells were homogenised in the medium as described in Section 2.8.1 and replaced in flasks with and without the added cofactors listed in Table 3.21. Benzaldehyde was added to flasks containing broken or whole cells at a rate of 2 $gl^{-1}h^{-1}$ for 3 h. Benzyl alcohol and PAC concentrations were monitored over a 4 h period. The results are presented in Table 3.21.

The results indicate that homogenised yeast cells can produce similar PAC levels to whole cells only when supplemented with thiamine pyrophsphate and magnesium. Whole cells produced almost 50% more benzyl alcohol than broken cells, the addition of cofactors to cell homogenates did not affect the amount of benzyl alcohol produced.

Table 3.21 Comparison of the biotransformation activities of whole and homogenised yeast cells. 30 g/l fresh pressed bakers' yeast (30% (w/v) solids content) was used in each case. Incubation conditions are described in Section 3.6.9.

Yeast Cells	Cofactors	Incubation Time (h)	Phenylacetyl Carbinol (g/l)	Benzyl alcohol (g/l)
Whole	-	1	0.18	0.06
		2	0.81	ND
		3	2.87	ND
		4	5.00	0.37
Homogenised	-	1	0.35	0.04
		2	0.73	ND
		3	1.26	ND
		4	1.70	0.22
Homogenised	TPP, Mg*	1	0.53	0.05
		2	1.67	ND
		3	3.34	ND
		4	5.23	0.23

*Thiamine pyrophosphate 1.5 mM/l; $MgCl_2$ 2.5 mM/l
ND = No data

3.6.8 Ethanol Production in F1, F2 and F3

Ethanol is produced by yeast cells under fermentative conditions (Section 1.3.1). This experiment was conducted to investigate how much ethanol is produced by yeast in the presence of benzaldehyde. Conditions for F1, F2 and F3 were as described in Section 3.6.1. The results are presented in Figure 3.17.

At least half the ethanol produced in a PAC fermentation is produced during the equilibration period, i.e. the hour before the first aldehyde addition where levels of 4-8 g/l ethanol were observed (in the case of F2 and F3 almost all the ethanol produced was in this period). Final titres of ethanol were between 7 and 13 g/l.

In the following experiments the effect of ethanol on PAC production was investigated. Reaction conditions were as described for F1 (Section 3.6.1).

3.6.9 The effect of Ethanol Concentration on L-Phenylacetyl Carbinol Production

Medium A supplemented with 10 g/l ethanol and an unsupplemented control were inoculated with 30 g/l fresh pressed bakers' yeast (30% (w/v) solids content). The fermentation was initiated immediately by the addition of benzaldehyde, i.e. there was no equilibration period. Levels of PAC, ethanol and benzyl alcohol were monitored over a 10 h period (Sections 2.3.1, 2.3.7 and 2.4.2 respectively). The results are presented in Figure 3.18.

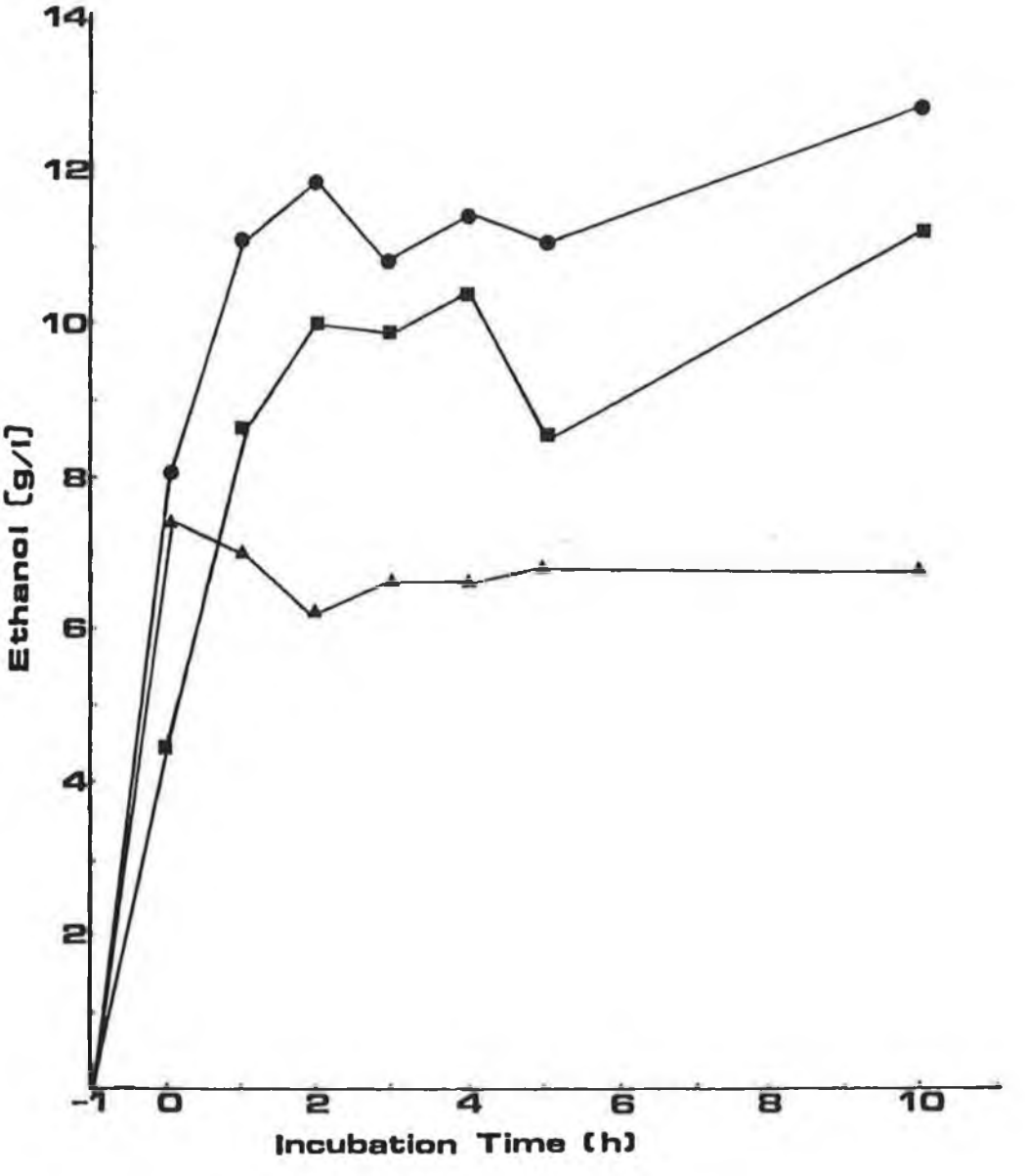


Figure 3.17. Ethanol produced in model fermentations F1 (■) F2 (●) and F3 (▲). Fermentation conditions are described in Section 3.6.1. The time -1 to 0 h represents the equilibration period.

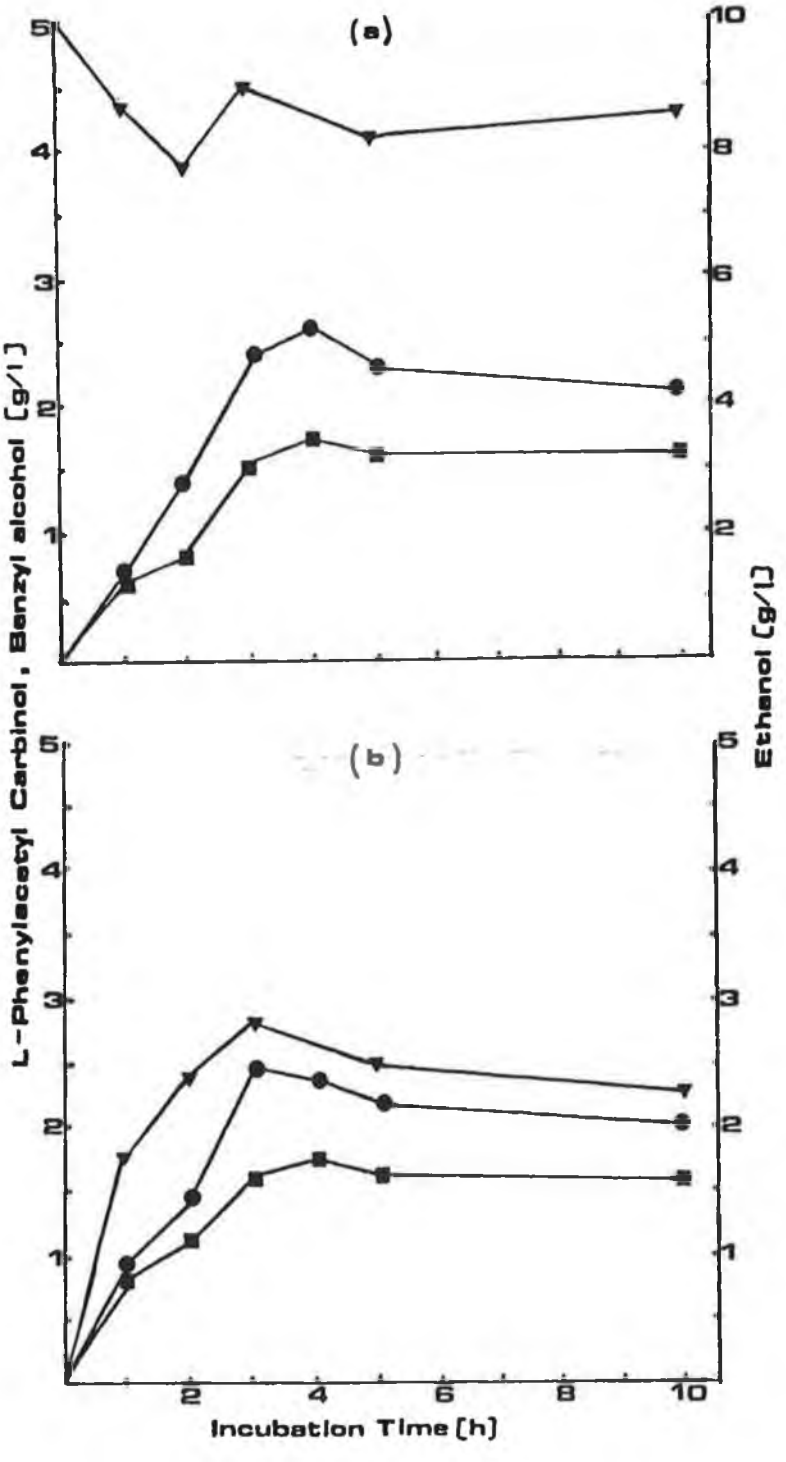


Figure 3.18. Production of L-phenylacetyl carbinol (●), ethanol (▼), and benzyl alcohol (■) in fermentation. Medium A, supplemented with 10 g/l ethanol (Figure 3.18a) and an unsupplemented control (Figure 3.18b) were inoculated with 30 g/l fresh pressed bakers' yeast (30% (w/v) solids content). Reaction was initiated immediately by addition of aldehyde. Reaction conditions were as described for F1 (Section 3.6.1).

Ethanol concentration dropped in the ethanol supplemented flask from 10 to 8 g/l in 2 h, no net increase was evident over the 10 h period. A maximum of 2.5 g/l ethanol was produced in the control. PAC and benzyl alcohol titres were approximately equal in both cases but lower than in the corresponding fermentation with an equilibration period (F1, Section 3.6.1). The results suggest that PAC and benzyl alcohol production is independant of ethanol concentration.

3.6.10 The Effect of Ethanol Produced in PAC Biotransformation Media on Yeast Cell Viability

This experiment was conducted to investigate if ethanol, in the concentrations produced in a PAC fermentation, could be a factor in the loss in yeast cell viability observed in Section 3.6.1. Medium A was inoculated with 30 g/l fresh pressed bakers' yeast (30% (w/v) solids content) and incubated as for F1 (Section 3.6.1) but in the absence of added benzaldehyde (which may interfere with yeast cell viability). Ethanol produced and yeast cell viability were monitored over a 10 h period (Sections 2.3.7 and 2.2.6). The results are presented in Figure 3.19.

In the absence of benzaldehyde, ethanol levels reached approximately 15 g/l in 10 h (which is even greater than those attained in Section 3.6.10). In that period yeast cell counts approximately doubled, indicating that ethanol concentration is not a factor in loss of yeast cell viability during PAC production.

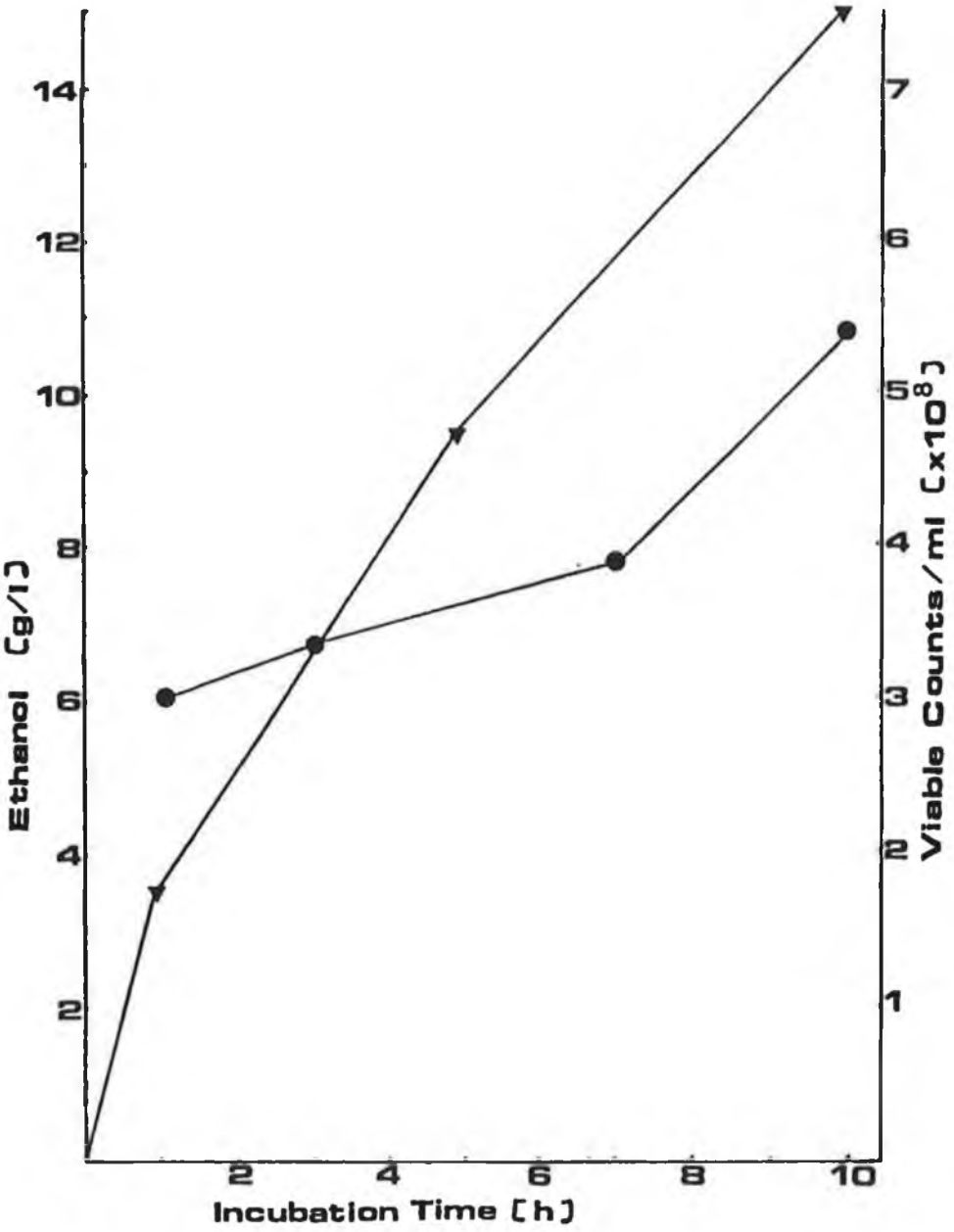


Figure 3.19. Relationship between ethanol concentration (▼) and yeast cell viability (●). Medium A was inoculated with 30 g/l fresh pressed bakers' yeast (30% (w/v) solids content) and incubated as for F1 (Section 3.6.1) but in the absence of added benzaldehyde.

3.6.11 Appraisal of Results

High initial rates of PAC formation were observed in fermentations containing a high starting benzaldehyde level, but a huge reduction in yeast viability was observed resulting in early cessation of product formation. Pulse feeding to maintain lower benzaldehyde concentrations resulted in a lower initial reaction rate, but prolonged yeast viability and the biotransformation. This resulted in higher overall product titres. Benzaldehyde appeared to alter the cell permeability barrier to substrates and products. Effect of benzaldehyde and the biotransformation products on yeast pyruvate decarboxylase and alcohol dehydrogenase stability was determined. In the absence of yeast extract, alcohol dehydrogenase was rapidly denatured at high benzaldehyde levels, while pyruvate decarboxylase was fairly resistant to benzaldehyde denaturation. Pyruvate decarboxylase and alcohol dehydrogenase were relatively stable in the presence of their products, PAC and benzyl alcohol respectively. PAC had a denaturing effect on alcohol dehydrogenase as had benzyl alcohol on pyruvate decarboxylase in the absence of yeast extract. Both enzymes were significantly more stable in the presence of a 20% (w/v) yeast extract solution.

3.7 PRELIMINARY STUDIES ON IMMOBILISATION OF YEAST CELLS IN CALCIUM ALGINATE

A sharp fall in yeast cell viability coincides with the levelling off in PAC production (Section 3.6.1). Immobilisation of the cells in calcium alginate was investigated in batch and continuous biotransformation systems as a means of protecting the cells and thus prolonging PAC production. Yeast cels were encapsulated in the calcium alginate matrix as desctibed in Section 2.14.

3.7.1 Comparison of PAC production by free and encapsulated cells in batch culture

The objective of this experiment was to investigate if encapsulation of yeast cells altered their PAC producing capacity or prolonged cell viability when compared to free cells. Medium C, 500 ml in a 1 L flask, was inoculated with 30 g/l fresh bakers' yeast (30% (w/v) solids content) or with 30 g/l yeast encapsulated in calcium alginate. Following a 30 min. equilibration period, 2 g/l benzaldehyde was added at 1 h intervals for 6 h. PAC and yeast cell viability was measured (Sections 2.3.1 and 2.2.6). The results are presented in Figure 3.20.

Slightly higher rates of PAC production were observed in non-encapsulated cells. When PAC was being produced at a linear rate encapsulated cells grew at a slower rate than free cells. However, as PAC production levels off and benzaldehyde concentration subsequently increases (F1, Section 3.6.1), cell viability in the beads decreased at an equal rate to that of free cells. The calcium alginate matrix offers little protection to the cells from potentially toxic levels of benzaldehyde.

Diffusion of substrate to cells in the inner regions of the beads is not a limiting factor in product formation as it has been shown that bead size does not greatly influence levels of PAC produced. Using beads of sizes 3, 2 and 1-1.5 mm in diameter, PAC levels of 2.93, 3.14 and 3.22 g/l respectively were obtained.

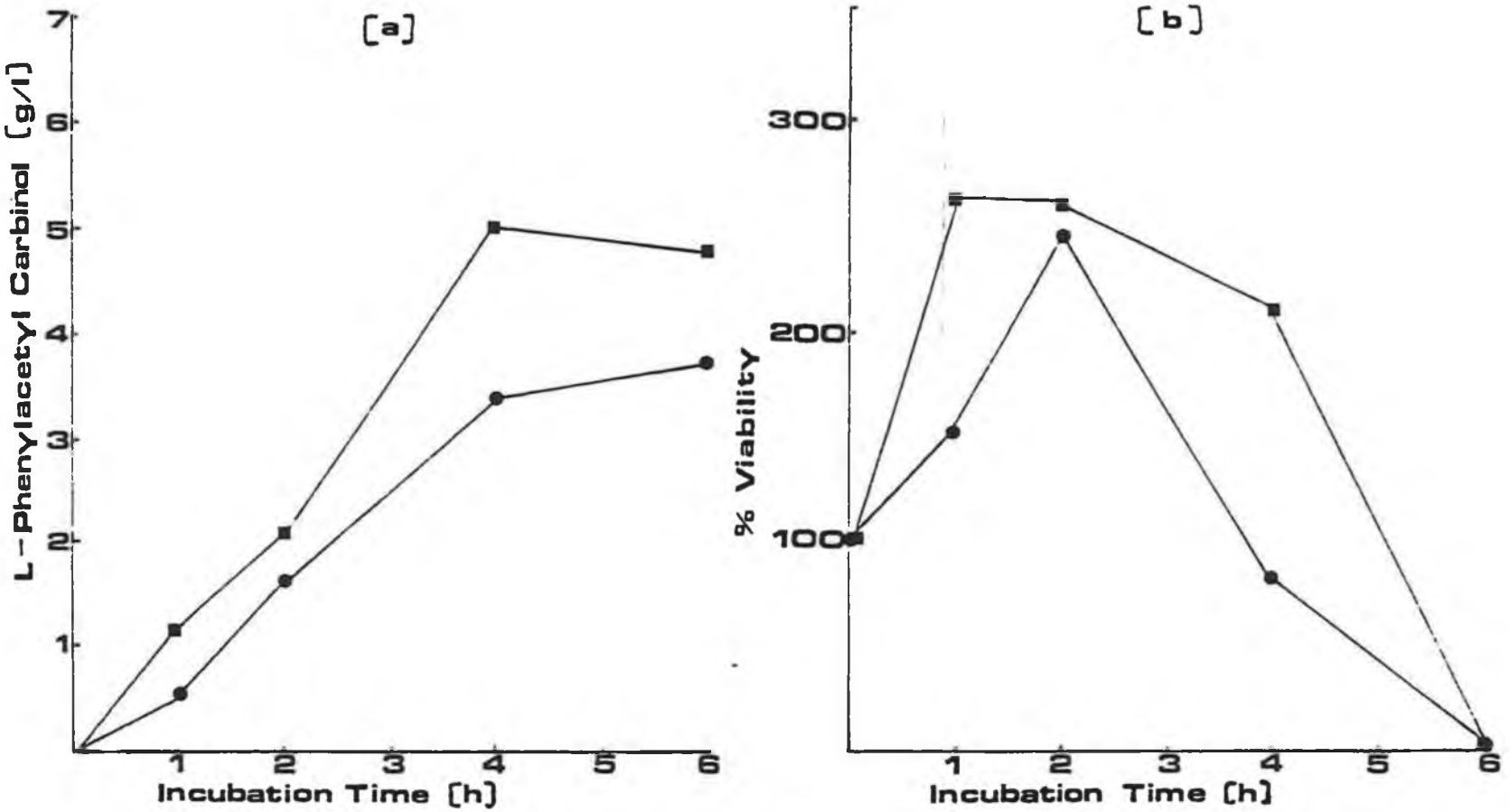


Figure 3.20. (a) L-Phenylacetyl carbinol production by encapsulated (●) and non-encapsulated (■) yeast cells, (Bioconversion conditions are described in Section 3.7.1) and (b) yeast cell viability of encapsulated (●) and non-encapsulated (■) cells during fermentation.

3.7.2 Production of L-Phenylacetyl Carbinol by Encapsulated Yeast Cells in a Continuous Reactor

A continuous system allows greater control over the benzaldehyde concentration to which the cells would be exposed. For the following experiments a small scale reactor was set up as illustrated in Figure 3.21. A constant volume was maintained in the reactor which contained 30 g/l of yeast cells (fresh bakers' yeast, 30% (w/v) solids content) encapsulated in calcium alginate. Medium C, containing the appropriate concentration of benzaldehyde was pumped through the vessel to give the desired dilution rate. Samples were taken from the outlet at hourly intervals for 6 h and analysed for PAC (Section 2.3.1). The temperature of the reactor was maintained at 30°C using a circulating water bath.

The effect of dilution rate (D) in the range 0.5-1.25 v/h on PAC production was investigated. Benzaldehyde concentration in the medium was 2 g/l. Results are presented in Table 3.22.

Table 3.22 Relationship between dilution rate (D) and L-phenylacetyl carbinol production by yeast cells encapsulated in calcium alginate. Benzaldehyde concentration in the medium was 2 g/l Reaction conditions are described in Section 3.7.2.

D (v/h)	Rate PAC Production ($gl^{-1}h^{-1}$)
0.5	0.27
0.75	0.28
1.0	0.42
1.25	0.47

The rate of PAC formation increased with increasing dilution rate. When the dilution rate was doubled from 0.5 to 1.0 v/h the rate of PAC production increased by 30%.

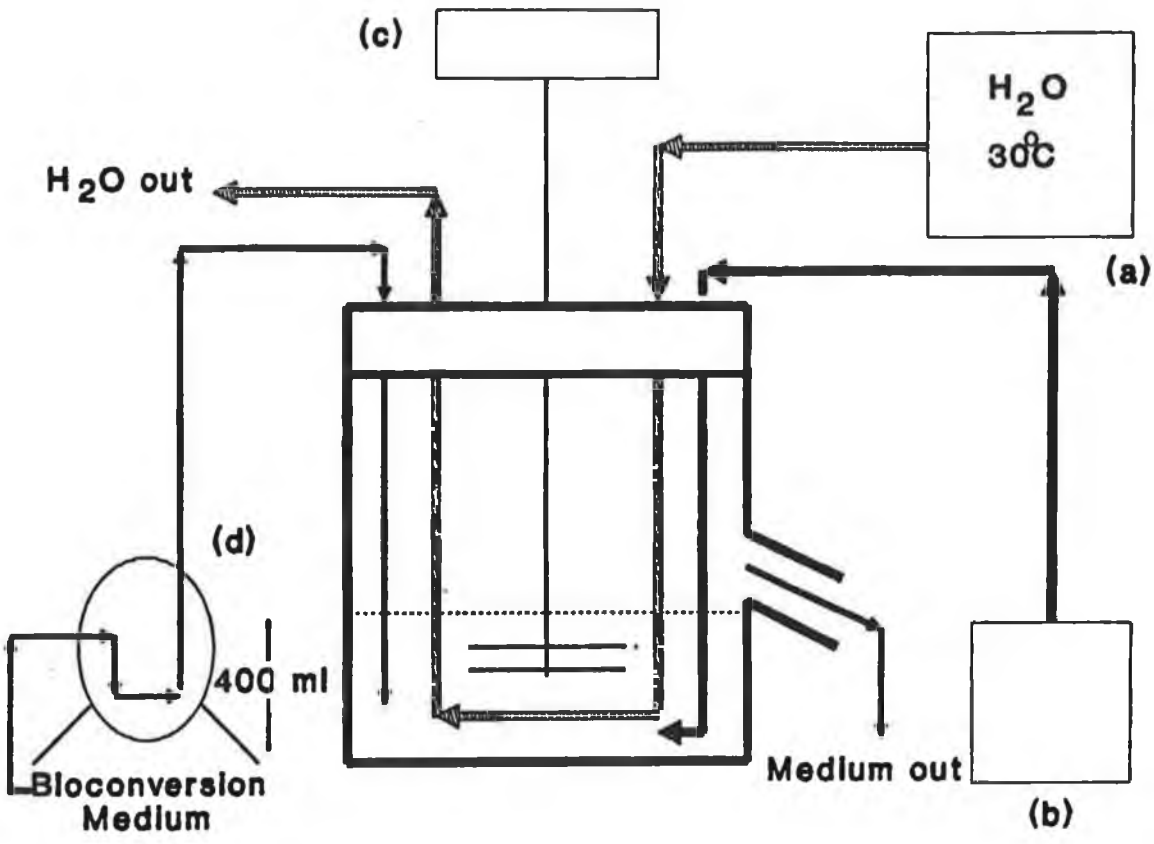


Figure 3.21. Small scale reactor for production of PAC by encapsulated yeast cells in a continuous system. Conditions are described in Section 3.7.2. The objects labelled (a) to (d) are: (a), circulating water bath; (b), fish tank aerator; (c), Biolafitte stirring motor and (d) peristaltic pump.

3.7.3 Effect of Benzaldehyde Concentration on L-Phenylacetyl Carbinol Production in a Continuous System

Using a dilution rate of 1 v/h the effect of benzaldehyde concentration on PAC production was investigated. Medium C, containing 2, 3 and 4 g/l benzaldehyde was pumped through the system and the rate of PAC production was determined. The results are presented in Table 3.23.

Table 3.23 Effect of benzaldehyde concentration on L-phenylacetyl carbinol production in a continuous system. Dilution rate (D) was 1 v/h. Reaction conditions are described in Section 3.7.2.

Benzaldehyde (g/l)	Rate PAC production ($gl^{-1}h^{-1}$)
2	0.42
3	0.63
4	0.96

The highest rate of PAC production (0.96 $gl^{-1}h^{-1}$), was at a benzaldehyde concentration of 4 g/l.

3.7.4 Appraisal of Results

The results of these preliminary experiments indicate that encapsulation of yeast cells does not enhance PAC production or maintain yeast cell viability when compared to free cells. Levels of PAC produced were independent of bead size. Slightly lower levels of PAC were produced in encapsulated cells. Substrate and/or products may concentrate in the calcium alginate matrix and decrease the rate of product formation (Sections 3.5.7 and 3.5.8). Rate of PAC produced in a continuous system increased with increasing benzaldehyde concentration up to 4 g/l. Concentrations

above 4 g/l were not used as encapsulation does not protect against a decrease in cell viability (Section 3.7.1) and an increasing benzaldehyde concentration is concommitant with a decrease in yeast cell viability (Section 3.6.1).

3.8 GROWTH OF YEAST IN MEDIA CONTAINING BENZALDEHYDE

The effect of benzaldehyde on the rate of PAC production by yeast cells (most of which are in the stationary phase of growth) has been examined (Section 3.5.1). It has been observed that an increase in benzaldehyde concentration corresponds with a decrease in viability of these yeast cells (Section 3.6.1). In the following set of experiments the effect of benzaldehyde concentration on yeast cell growth was investigated. Shake flasks (250 ml Erlenmeyers) containing 50 ml of medium D and concentrations of benzaldehyde ranging from 0 to 3 g/l were inoculated with 10^6 yeast cells per ml of medium and incubated as in Section 2.2.4 for 10 h. Total viable counts (Section 2.2.6), culture optical densities (Section 2.2.5) and PAC levels (Section 2.3.1) were monitored. The results are presented in Table 3.24 and Figure 3.22.

Table 3.24 Phenylacetyl carbinol production during growth of yeast in different benzaldehyde concentrations. Medium D, containing 0-3 g/l benzaldehyde was inoculated with 10^6 yeast cells/ml medium

Benzaldehyde Concentration (g/l)	Phenylacetyl carbinol (g/l) produced after 1h	4h	7h	10h
0.00	0.00	0.00	0.00	0.00
0.50	0.01	0.04	0.08	0.15
1.00	0.01	0.03	0.05	0.05
2.00	0.01	0.03	0.04	0.05
3.00	0.01	0.02	0.02	0.02

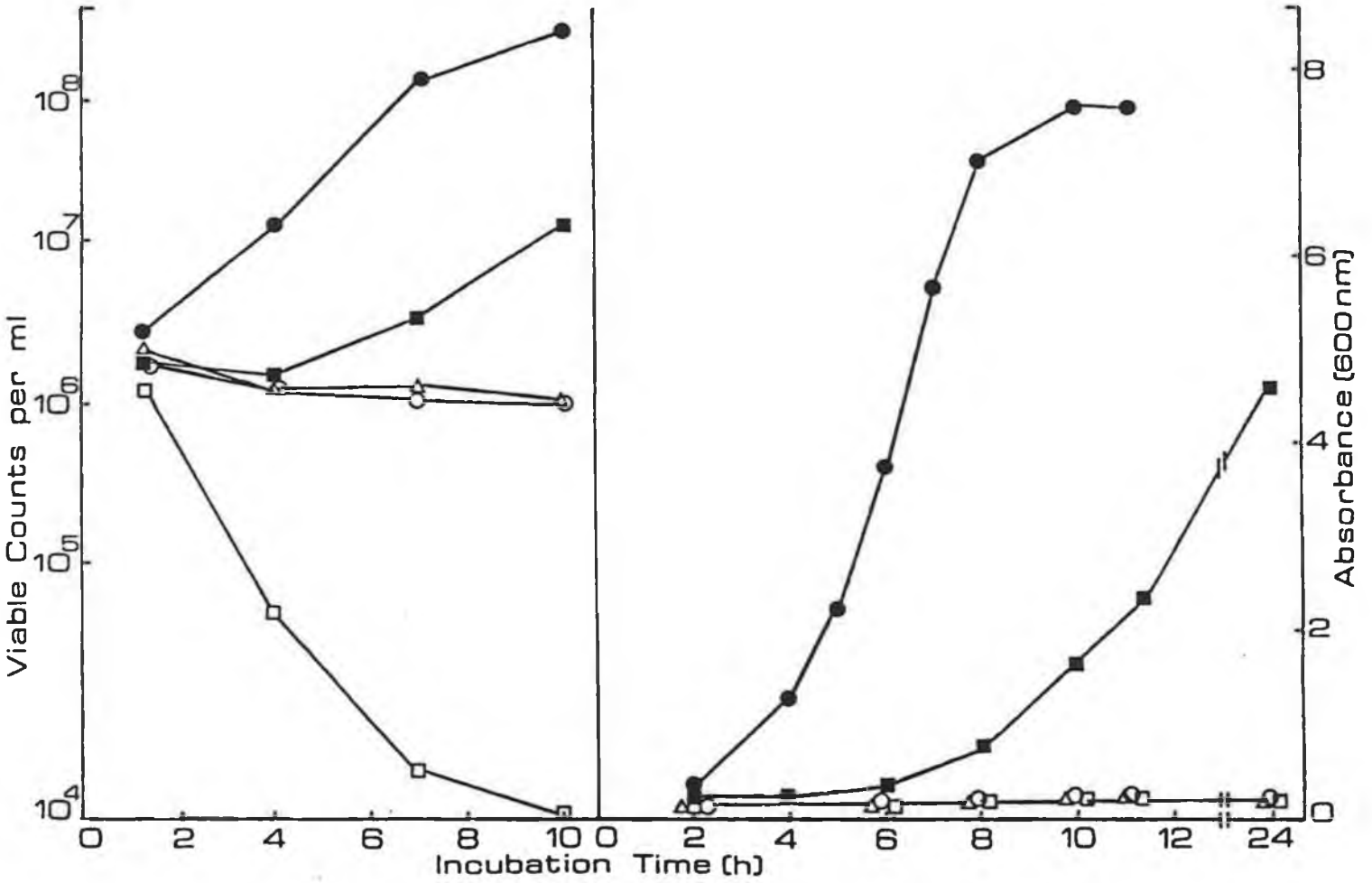


Figure 3.22. Total viable counts and culture absorbance of cells growing in media containing 0-3 g/l benzaldehyde. Incubation conditions are described in Section 3.8. Benzaldehyde concentration, g/l: ●, 0 ; ■, 0.5 ; △, 1.0 ; ○, 2.0 ; □, 3.0.

Levels of PAC produced were extremely low in all cultures with highest values observed at the lowest banzaldehyde concentration.

Retarded growth was observed at a concentration of 0.5 g/l benzaldehyde. At a concentration of 3.0 g/l benzaldehyde a reduction in viable counts was observed over the incubation period. At intermediate benzaldehyde levels, up to a concentration of 2.0 g/l, growth was inhibited although the starting viable count was maintained. This result might appear to conflict with Figure 3.14, where yeast growth is observed when a starting benzaldehyde concentration of 2 g/l is used. However, comparison of the initial viable counts in Figure 3.22 and Figure 3.14 indicates that the yeast concentration is one hundred times higher in Figure 3.14 This higher yeast count results in rapid metabolism of the benzaldehyde, enabling growth to occur. The lower count in Figure 3.22 was chosen in order that initial growth patterns could be observed without major fluctuations in benzaldehyde concentration.

Cells exposed to 2.0 g/l benzaldehyde for 7 h were inoculated into the yeast growth medium (1 ml containing 10^6 cells per 100 ml medium) and their growth monitored over a 24 h incubation. A control, (cells not exposed to benzaldehyde) was run in parallel. The growth curves are illustrated in Figure 3.23. The cells exposed to 2.0 g/l benzaldehyde manifested a very long lag period (about 17 h) as compared to the control which had a lag phase of about 3 h.

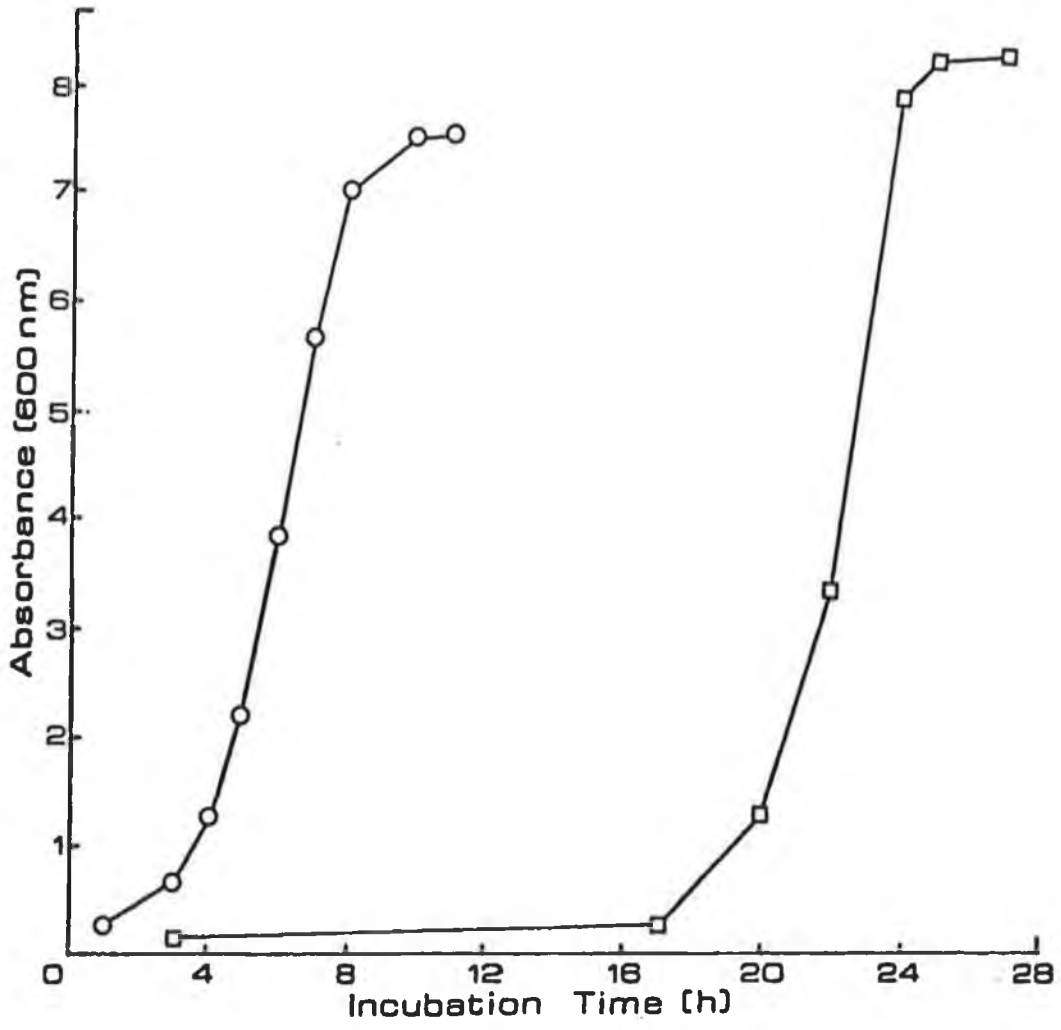


Figure 3.23. Growth curve of cells taken from a medium which contained 2 g/l benzaldehyde and inoculated into medium D (□) compared to a growth curve of a control (○). i.e. cells not exposed to benzaldehyde.

3.8.1 Appraisal of Results

Yeast cells produce low levels of PAC in growth phase even at low (0.5 g/l) benzaldehyde concentrations. When benzaldehyde is present in the growth medium, yeast growth is inhibited but viable counts do not decrease up to a benzaldehyde concentration of 2 g/l. This inhibition is reversible as the cells grow normally when inoculated into a medum which does not contain benzaldehyde (except that there is a longer lag period compared to the control).

3.9 BIOTRANSFORMATION OF BENZALDEHYDE BY VARIOUS YEAST AND BACTERIAL SPECIES

The production of PAC by bakers' yeast, Saccharomyces cerevisiae, has been studied in detail. Others have looked at PAC, but not benzyl alcohol production in other yeast species (Section 1.2). In the following experiments PAC and benzyl alcohol production was studied in a number of yeast species, comparing sucrose and sodium pyruvate as co-substrates. In bakers' yeast, pyruvate decarboxylase mediates the production of PAC in the presence of benzaldehyde substrate (Section 3.3.3). It is not a common enzyme in bacteria (Section 1.4.5), however some of the bacteria known to possess this enzyme were screened for their ability to produce PAC.

3.9.1 Production of L-Phenylacetyl Carbinol and Benzyl Alcohol by Various Yeast Species

The production of PAC and benzyl alcohol by various species of the genus Saccharomyces and 1 species of the genus Candida was investigated. For each yeast species, the production of PAC and benzyl alcohol in 2 different media was compared: Medium A, which contains sucrose and Medium B, which contains sodium pyruvate.

Yeast cultures were maintained and grown as described in Sections 2.2.2 and 2.2.4 respectively. Cells from a 24 h culture were recovered by centrifugation and resuspended in medium A or B to a density of 8-10 mg/ml dry weight. Benzaldehyde was added at a rate of 2 $gl^{-1}h^{-1}$ for 4 h. The amount of PAC and benzyl alcohol produced after 1 h and 6 h was determined (Sections 2.3.1 and 2.4.2). The results are presented in Tables 3.25 and 3.26.

PAC and benzyl alcohol were produced by all strains tested. In general, higher levels of PAC were produced in medium B than in medium A, even though there was a higher initial rate of PAC production in medium A. The highest titre of PAC produced in medium B was 8.29 g/l, produced by S. diastaticus L#1353. This strain also produced the highest level of PAC in medium A which was 4.21 g/l.

The results show that a relatively high initial rate of PAC production does not necessarily indicate that a given strain will produce large amounts of this compound. For example, in medium A, S. carlsbergensis L#27 produced 1.15 g/l PAC in 1 h. This only increased to 1.97 g/l after 6 h. In the same medium, S. diastaticus L#1353 produced 1.10 g/l PAC in 1 h and 4.21 g/l in 6 h The collection of yeast strains tested was not extensive enough to make conclusions concerning the relationship between the yeast classification and PAC production, however, there were substantial differences between the titres produced by different species. S. cerevisiae L#1271 produced 3.36 g/l PAC in a 6 h incubation as compared to the 8.29 g/l produced by S. diastaticus L#1353 in the same period.

Table 3.25 Production of L-phenylacetyl carbinol and benzyl alcohol by various yeast species in a medium containing sucrose (medium A). Benzaldehyde was added at a rate of 2 gl^{-1} h^{-1} for 4 h. Reaction conditions are described in Section 3.9.1.

Yeast Species	Collection Number	L-Phenylacetyl carbinol (g/l) 1 h	6 h	Benzyl alcohol (g/l) 1 h	6 h
Saccharomyces carlsbergensis	L# 27	1.15	1.97	0.44	0.99
Saccharomyces carlsbergensis	L# 15	1.02	2.63	0.51	1.16
Saccharomyces cerevisiae	L# 1271	0.28	1.26	0.65	1.02
Saccharomyces cerevisiae	L# 1421	0.70	2.91	0.89	2.75
Saccharomyces cerevisiae	–	0.97	2.71	0.82	1.66
Saccharomyces diastaticus	L# 1353	1.10	4.21	0.78	2.01
Saccharomyces bayanus	L# 227	0.78	3.88	0.91	2.09
Candida utilis	L# 1597	0.72	2.51	0.93	1.94

Table 3.26 Production of L-phenylacetyl carbinol and benzyl alcohol by various yeast species in a medium containing sodium pyruvate (medium B). Benzaldehyde was added at a rate of 2 gl^{-1} h^{-1} for 4 h. Reaction conditions are described in Section 3.9.1.

Yeast Species	Collection Number	L-Phenylacetyl carbinol (g/l) 1 h	6 h	Benzyl alcohol (g/l) 1 h	6 h
Saccharomyces carlsbergensis	L# 27	0.42	7.40	0.43	1.02
Saccharomyces carlsbergensis	L# 15	0.35	7.00	0.27	0.61
Saccharomyces cerevisiae	L# 1271	0.26	3.36	0.60	1.06
Saccharomyces cerevisiae	L# 1421	0.34	3.94	0.92	1.36
Saccharomyces cerevisiae	–	0.36	7.76	1.14	1.34
Saccharomyces diastaticus	L# 1353	0.29	8.29	0.72	1.77
Saccharomyces bayanus	L# 227	0.24	7.34	0.63	1.10
Candida utilis	L# 1597	0.43	8.05	0.52	1.20

Note: L = Labatts culture collection

The carbon source present in the medium did not greatly effect the amount of benzyl alcohol produced. On average, 1-2 g/l benzyl alcohol was produced by the genera tested. The highest titre, 2.75 g/l, was produced by S. cerevisiae L 1421 in Medium A.

3.9.2 Preliminary Studies on the Biotransformation of Benzaldehyde by Bacterial Cultures

The ability of 4 bacteria to produce PAC in the presence of benzaldehyde was investigated. Lactobacillus plantarum and Streptococcus faecalis both produce acetoin while L. plantarum and Erwinia amylovora are known to contain pyruvate decarboxylase (Section 1.4.5). Certain strains of Enterobacter aerogenes have been shown to be capable of producing acyloin compounds similar to PAC from aldehyde substrates (Section 1.4.1). Each bacterium was grown in the appropriate medium (Section 2.2.3) and harvested at stationary phase. Cells were resuspended in medium A or B to a density of 0.5-1.0 mg/ml dry weight. Benzaldehyde was added at a rate of 2 $gl^{-1}h^{-1}$ for 4 h. PAC produced after 5 h was determined (Section 2.3.1). The results are presented in Table 3.27.

Table 3.27 Biotransformation of benzaldehyde by bacteria. Bacteria were grown in the appropriate medium (Section 2.2.3), cells were then resuspended in medium A or B and benzaldehyde was added at a rate of 2 $gl^{-1}h^{-1}$ for 4 h. PAC was determined after 5 h.

Bacterium	Strain	L-Phenylacetyl Carbinol (+/) Medium A	Medium B
Lactobacillus plantarum	ATCC 8014	-	-
Erwinia amylovora	NRRL B-127	-	-
Enterobacter aerogenes	NRRL B-494	-	-
Streptococcus faecalis	ATCC 19433	-	-

Note: ATCC = American type culture collection
NRRL = Northern regional research laboratories

None of the bacteria screened produced PAC in either medium A or B.

3.9.3 Appraisal of Results

PAC and benzyl alcohol were produced by all yeast species tested. In general, higher levels of PAC were produced in medium B. The different levels of PAC produced by different species or strains of the same species may be due to a number of factors including differences in metabolism and/or permeability of the yeast cell membrane to the substrate(s). Failure of all the bacteria tested to produce PAC could be due to differences in the metabolism of sucrose and pyruvate, differences in the enzyme structures or in the permeability of the bacterial cell to the substrates.

4. DISCUSSION

The advantages of using biological systems in the formation of chemical intermediates has been known for a number of years. These include the ability of such systems to form only one isomer of an optically active compound which may be of use commercially. The conversion of benzaldehyde to the optically active L-phenylacetyl carbinol (PAC) by the yeast Saccharomyces cerevisiae is an example of such a reaction. This biotransformation and the formation of other acyloin compounds has been the major topic of interest during the course of this work.

Preliminary studies on the biotransformation indicated that a medium containing bacteriological peptone and sucrose buffered at pH 4.5 using sodium citrate, was optimum for PAC formation over a 5 h fermentation. Different workers have found various pH values ranging from 5.0 (Gupta et al., 1979) to 6.3 (Vojtisek and Netrval, 1982) to be optimal for the biotransformation. A method to purify PAC from a fermentation broth was developed (Section 3.2.2). The procedure involves several steps and would be laborious to carry out on a large scale. Complete transformation of the benzaldehyde substrate and the elimination of aromatic alcohol by-product formation would be required to simplify the purification. The pure PAC was used to validate the use of acetyl benzoyl as standard in the colorimetric assay of Groger and Erge (1965) in routine determination of PAC (Section 3.2.3). The finding that acetyl benzoyl absorbs more strongly than PAC at 580 nm (Section 3.2.3) was not in agreement with that of Groger and Erge (1965) who found the opposite to be the case. The observed differences may be due

to differences in the quality of the reagents used or in the experimental conditions. The concentration range used in Section 3.2.3 and that used by Groger and Erge are substantially different as are comparable absorbance values.

Even recently authors have referred to the enzyme which catalyses the formation of PAC from benzaldehyde as carboligase, which has yet to be purified (Agarwal et al., 1987) even though Hanc and Kakac (1956) showed using a preparation of pyruvate decarboxylase that this enzyme catalyses the reaction. This was confirmed in Section 3.3.3. However, it does not eliminate the possibility that there are other enzymes present in yeast that can catalyse acyloin formation. Morimoto et al. (1988) describe a "new" acyloin-forming enzyme in Saccharomyces cerevisiae which catalyses the synthesis of an aliphatic acyloin compound from 2 molecules of the aliphatic aldehyde without α-keto acid. From the results presented in Section 3.3.4, it is unlikely that this enzyme plays a major role, if any, in PAC formation. Its presence was not confirmed in yeast cell-free extracts, therefore its activity must be negligible in comparison to that of pyruvate decarboxylase.

L-acetyl aromatic carbinols and aromatic alcohols were produced from substituted aromatic aldehydes containing a - CH_3, - CF_3, -OCH_3, or - Cl in the ortho, meta and para ring position, illustrating the broad substrate specificity of the system. A small number of L-acetyl aromatic carbinols have previously been isolated including L-2-Chlorophenylacetyl carbinol (Neuberg and Liebermann, 1921). The size of the substituent group does not seem to be a major factor in L-acetyl aromatic carbinol production. For

example, the chloro group (which has a Van der Waals radius of 1.8Å, (Klinman 1976)), when substituted in the meta position (m-chloro-benzaldehyde) gave rise to lower titres of L-acetyl aromatic carbinol than the corresponding methyl and methoxy substituted aldehydes which have radii of 2.0 and 3.0 Å respectively. Aldehydes substituted in the ortho position were poor substrates for carbinol production while with the exception of the OCH_3 substituent, aldehydes substituted in the para position produced highest carbinol titres. While steric properties and the position of the substituent group on the substrate may be rate determining in biotransformations using pure enzymes, this is not necessarily the case for whole cell systems where the permeability of the cell to the substrate and the toxicity and solubility properties of the substrate are major factors which must be considered.

It has been shown that a wide range of aldehydes are substrates for the biotransformation. It is also known that a number of α-keto acids, apart from pyruvate are substrates for pyruvate decarboxylase (Juni, 1961). This implies a potential for the formation of acyloin compounds with a larger aliphatic side-chain with or without a substituted aromatic ring using yeast cells. The ability to form such compounds is interesting both from an academic point of view and may also yield compounds of commercial interest. No studies have been published on the ability of other TPP requiring decarboxylases such as phenylglyoxylate decarboxylase and phenylpyruvate decarboxylase to form acyloin compounds (Section 1.4.2). However, very recent work in our laboratory (Bourgalt, 1989, unpublished) indicates that these other TPP linked

decarboxylases can in fact form acyloin compounds. Further characterisation of such enzymes would enable the appropriate enzyme to be selected based on the nature of the oxoacid co-substrate. Recent developments in genetic manipulation offers potential to engineer existing enzymes in a manner which would modify the substrate specificity. Through such methods it may be possible to alter the specificity of these decarboxylases so that they can be specific for selected unnatural substrates.

Initial rate studies were carried out to more accurately determine parameters related to biotransformation activity. Optimum conditions for the initial rate of PAC production were determined (Section 3.5) which were as close as possible to optimum conditions for enzyme activity considering we were dealing with whole cells and not pure enzymes. A concentration of 6 g/l benzaldehyde (which gave maximum rate of product formation in 1 h) would not be viable for a prolonged fermentation due to toxicity problems (i.e. enzyme denaturation, cell permeabilisation and membrane disruption), but initial rate studies (as would be used in enzyme kinetic studies) allow the most accurate estimation of the effect of various parameters such as pH, temperature, and substrate and product concentration on the rate of an enzyme reaction. In prolonged 24 h reactions optimal benzaldehyde concentration for PAC production was reported to be 1.8 g/l with complete inhibition of the process at 2.2 g/l benzaldehyde (Agarwal et al., 1987). The rate of PAC production was negligible at benzaldehyde concentrations above 6 g/l. Increasing product (PAC and benzyl alcohol) concentrations decreases the rate of PAC formation (Section 3.5.6). This is a critical observation as overall PAC titres cannot be improved in

this system unless there is efficient and continuous removal of the products. In a complex cellular system it is difficult to conclude how the products interfere with PAC production. It may be an equilibrium effect or inhibition (reversible or non-reversible) of key enzymes by substrate and/or products. Overall inhibition of cellular metabolism due to toxicity effects of substrate and/or products which may denature enzymes or permeabilise membranes leading to disruption of membrane-bound enzymes or the release of essential cofactors is also a possibility

Investigation of reaction rates using selected substituted aromatic aldehydes confirmed the broad substrate specificity of the carbinol forming system and illustrated that the reaction rate depends on the nature of the substrate. In general, the rate of L-acetyl aromatic carbinol production was greater when pyruvate was used rather than sucrose as a co-substrate. For more prolonged biotransformations, however, pyruvate should not be used as the carbon source. As it is expensive, it would not be a viable alternative for large scale fermentations. More importantly, the synthesis of pyruvate decarboxylase is induced by glucose (Entian et al., 1984) and the enzyme decays rapidly in its absence (Polakis and Bartley, 1965). As pyruvate decarboxylase is a key enzyme in the biotransformation (Section 3.3.3) the reaction could only be prolonged in the presence of a fermentable substrate. The higher rate of aromatic alcohol production in sucrose/aromatic aldehyde reactions is probably due to the presence of NADH produced by glyceraldehyde-3-phosphate dehydrogenase and the necessary reoxidation of the NADH to maintain glycolysis. NADH is not

generated in the pyruvate/aromatic aldehyde biotransformation and the low levels of aromatic alcohol observed probably reflect the endogenous NADH level.

One of the major problems of cellular biotransformations is the tendancy to produce unwanted by-products. A major by-product in the conversion of benzaldehyde to PAC is the aromatic alcohol, benzyl alcohol. The role of yeast alcohol dehydrogenase in the conversion of benzaldehyde to benzyl alcohol was confirmed (Section 3.4). The enzyme can also convert a wide range of substituted aromatic aldehydes to the corresponding aromatic alcohols illustrating the broad substrate specificity of the enzyme (Section 3.4.2). The results obtained using benzaldehyde, p-anisaldehyde and m-tolualdehyde as substrates are at variance with the results of Bowen et al. (1986) who state that these aldehydes are not substrates for the enzyme. The elimination of this side reaction is a difficult problem as the alcohol dehydrogenase reaction regenerates the NAD required for glycolytic conversion of hexoses to pyruvate, the key pathway in the transformation (Caubet et al., 1988; Wiesenfeld et al., 1975). The use of inhibitors such as N-alkyl nicotinamide derivatives (Heitz and Anderson, 1968), 1,10-phenanthroline (Hock and Vallee, 1956) and pyrazole (Coleman and Weiner, 1973), mutants which do not have alcohol dehydrogenase or use of pure enzymes (which may be easily denatured/inhibited by substrate and products) would eliminate by-product formation but would also upset this co-enzyme balance. Yeasts which do not have alcohol dehydrogenase may have alternative mechanisms to regenerate NAD, for example, mitochondria from Candida parapsilosis, can reoxidise cytoplasmic NADH through a mitochondrial pathway under

oxidative conditions (Caubet et al., 1988). This alternative chain starting from a NADH- and NADPH-dehydrogenase-transhydrogenase complex, located on the external face of the inner membrane (Guerin et al., 1982), merges into the main respiratory chain at the cytochrome C level (Guerin and Camougrand, 1986). Such an enzyme/pathway present under fermentative conditions, which would not interfere with the biotransformation would eliminate the problem of by-product formation. Acetaldehyde decreases the ratio of benzyl alcohol to PAC produced in a fermentation (Ose and Hironaka, 1957; Becvarova et al., 1963). This phenomenon was illustrated in Section 3.1.6 where the ratio of alcohol to PAC was reduced from 3:1 (in the absence of acetaldehyde), to 1:1. Acetaldehyde is not the ideal solution as it does not completely eliminate aromatic alcohol nor does it increase the PAC titre (Section 3.1.6). Successful elimination of by-product formation should not only increase the amount of product formed but it would also make the recovery of the product from the fermentation broth (Section 3.2.2) a lot easier.

The existence of 3 major isozymes of ADH has been well documented (Lutstorf and Megnet, 1968; Ebisuzaki and Barron, 1957; Fowler et al., 1972; Wenger and Bernofsky, 1971) and a multiplicity of enzyme forms has been reported for each isozyme (Sugar et al., 1970; Sugar et al., 1971). The isozymes of yeast alcohol dehydrogenase were separated on non-denaturing polyacrylamide gels and their reactivity towards benzyl alcohol and ethanol compared (Section 3.4.3). The zymograms obtained with each substrate displayed a number of bands. Differences in intensities of the bands illustrate differences in substrate specificities of the isozymes

for the two substrates. Bands 1, 3 and 5 (benzyl alcohol) were all of equal intensity and were more reactive with the substrate than bands 2 and 6. Band 5 (ethanol) was by far the most reactive with the ethanol substrate. Even though the three isozymes are very similar (Section 1.5.2), the substrate specificities of the three isozymes are different (Ganzhorn et al., 1987; Wills and Jornvall, 1979; Singh and Kunkee, 1977) and have been used to distinguish isozymes (Eltayeb and Berry, 1982). It is difficult to compare the patterns obtained to those in the literature as it has been found that different yeast species give different banding patterns (Heich et al., 1969), additional bands can be obtained upon storage of the preparation (Heich et al., 1969) and different types of gels give different resolution/banding patterns. For example, mitochondrial ADH gives 5 bands on starch gel electrophoresis which represent 5 different combinations of two distinct monomers (Sugar et al., 1970). When using an in vitro detection method such as the tetrazolium reaction, which is driving the enzymic reactions in a non-physiological direction, the possibility that some of the bands are artifacts and the reactions do not occur in vivo must be considered.

Due to difficulties in comparing the zymograms, caused by differences in degrees of reactivities with the substrates (which led to apparently different banding patterns), the bands were characterised by molecular weight analysis (Section 3.4.5). The results of the molecular weight analysis appear very complex and are difficult to explain. Only band 1,2 in the case of ethanol and bands 1 and 2 in the case of benzyl alcohol were shown to have a molecular weight of 150,000, the published molecular weight for

yeast alcohol dehydrogenase, (Branden et al., 1975). One would expect band 5 (ethanol) which corresponded to commercial ADH, which in turn corresponded to band 6 for benzyl alcohol to have a molecular weight of 150,000. Bands 3, 4 and 5 (benzyl alcohol) had molecular weights of 55,000, 45,000 and 45,000 respectively, while bands 3, 4 and 5 (ethanol) had molecular weights of 110,000, 98,000 and 105,000 respectively. While the first three bands have molecular weights which are not multiples of 37,000 (the subunit molecular weight, (Branden et al., 1975) and therefore cannot be active aggregates of 2 or more subunits, 2 of the latter 3 proteins reacting with ethanol (bands 3 and 5) could theoretically be a combination of 3 monomers (each of 37,000 molecular weight). The multiple bands could be due to different combinations of subunits (this may also explain the closeness of bands 1 and 2 (benzyl alcohol) which are probably the same isozyme). However, there is no evidence in the literature of the existence of independently active subunits to support this theory. Fowler et al. (1972) while presenting electrophoretograms of similar gels, suggested that a certain peak was due to a "fragmentation product of ethanol oxidase" (ADH II), but they produce no evidence to support this statement. Heich et al., (1969) found that when an enzyme preparation was left standing at $4^{o}C$, additional bands appeared on the gel which they claim represent an alteration of the original enzyme - presumably fragmentation. These fragments, which must be active (due to the method of detection) could have different substrate specificities which would explain the different bands obtained from the two substrates. Sugar et al. (1970), who characterised ADH I, II and III by heat inactivation, report the presence of one group of 2 and one of 3 weak bands between ADH II

and ADH III fractions on starch gel electrophoresis which they designate as isozymes which could not be characterised by heat inactivation. They did not further characterise these bands. On the evidence available it would be most reasonable to assume that the proteins of various molecular weights reacting with the two substrates are active fragments of one or more of the three major isozymes of alcohol dehydrogenase. Further purification/ characterisation of the bands would be necessary to conclude the presence of new enzymes catalysing the aldehyde to alcohol reaction.

Yeast cells could grow, albeit at a retarded rate, in the presence of 0.5 g/l benzaldehyde. At concentrations of 1-2 g/l benzaldehyde, growth was totally inhibited but yeast viability was maintained whereas at 3 g/l benzaldehyde viability was reduced (Section 3.8). These results indicate that it is important to keep benzaldehyde concentrations low in order to maintain yeast cell viability. Even at low benzaldehyde concentrations (0.5 g/l) the amount of PAC produced by the yeast cells was low (when compared to that produced by a similar number of cells in another fermentation, Section 3.6.1) which suggests a relationship between cell age and PAC producing ability. Agarwal et al. (1987), when investigating the effect of cell age on the biotransformation, found that maximum conversion rates were obtained with 15 to 18 h old cells and that the rate of PAC formation gradually decreased when the age of the cells was more than 18-24 h (Section 1.2). The inhibition of growth by the benzaldehyde substrate and the requirement for 15-18 h old cells makes it difficult to maintain conditions ideal for the biotransformation.

A selection of yeast strains were screened for their bio-transformation ability (Section 3.9.1). PAC was produced in all strains tested although it is known that all yeast do not have the capacity to produce PAC (Netrval and Vojtisek, 1982). Highest titres of PAC were observed in a medium containing pyruvate even though the initial rate of PAC production was lower in this medium than in one containing sucrose. (This seems to contradict the findings of the initial rate studies, but it may depend on the particular yeast strain and also the cultivation and storage conditions. These cells were grown in the laboratory while those used in initial rate studies were obtained commercially in the form of a compressed cake.) The lower rate may be due to a slow influx of pyruvate molecules through the yeast cell membrane. However, once pyruvate is in the cell it is a direct substrate for the pyruvate decarboxylase enzyme leading to the formation of PAC in the presence of benzaldehyde. The glycolytic pathway and any problems which may be associated with it (i.e. denaturation of enzymes, etc.) is by-passed. As the same amount of cells was used in each case, the results reflect definite differences between yeast strains in their ability to produce PAC. However, as we are dealing with whole cells we cannot make a statement about differences in enzymes in the various strains, although Becvarova and Hanc (1963) made a correlation between pyruvate decarboxylase activity and PAC producing ability in a range of yeast species. They found that yeast with low pyruvate decarboxylase activity formed small amounts of PAC and vice versa. The differences in levels of PAC produced may simply be due to differences in the

ability of yeast strains to tolerate extracellular benzaldehyde and PAC which have been shown to effect the rate of PAC production in Saccharomyces cerevisiae (Section 3.5).

A small selection of bacteria known to contain pyruvate decarboxylase (Lactobacillus plantarum and Erwinia amylovora) or to produce acetoin (L. plantarum and Streptococcus faecalis) or capable of producing acyloin compounds apart from acetoin (Enterobacter aerogenes) were screened for their ability to produce PAC from benzaldehyde and a sucrose or pyruvate co-substrate (Section 3.9.2). None of these bacteria synthesised PAC. A strain of E. aerogenes has previously been shown to make acyloin compounds similar to PAC from aldehydes of the general formula R_1CHO (US Patent No. L118, 293, 1968) where R_1 is a 3,4 disubstituted phenyl radical. The strain used in this case could not use benzaldehyde as a substrate but perhaps if a number of strains of E. aerogenes were screened, one would be found with PAC producing ability. E. aerogenes, S. faecalis and L. plantarum, unlike yeast, do not use free acetaldehyde in the formation of acetoin but synthesise it via α-acetolactate (Juni, 1950; Dolin and Gonsalus, 1951; Hickey et al., 1983). However, L. plantarum does have pyruvate decarboxylase (Hickey et al., 1983) as does E. amylovora (Haq, 1984). Like the yeast enzyme, both require TPP and Mg^{2+} for activity. Because of this requirement for TPP and Mg^{2+}, Haq (1984) calls the E. amylovora a 'yeast-type' pyruvate decarboxylase, while Hickey et al. (1983) state that the L. plantarum enzyme is not yeast-like as it does not utilise acetaldehyde in the formation of significant amounts of acetate. From these conflicting statements the criterion for a yeast-type pyruvate decarboxylase is not clear.

Apart from the cofactor requirements these enzymes may differ significantly in structure and mechanism from the yeast enzyme explaining why they do not seem to function in acyloin formation in these organisms. On the other hand, the inability of these bacteria to produce PAC may not be due to the nature of the pyruvate decarboxylase enzyme, but to other barriers such as the impermeability of the cells to the substrates. This could be confirmed by carrying out biotransformation studies using cell free extracts or purified pyruvate decarboxylase.

The comparison of three model fermentations, F1, F2 and F3 which differed only in yeast concentration and rate of benzaldehyde addition (Section 3.6.1), illustrated the relationship between substrate and product concentration and yeast cell viability. Increased benzaldehyde concentration resulted in decreased yeast cell viability, a cessation in PAC production and reduced sucrose metabolism. The higher initial rate of formation of PAC in F3 was consistent with the findings in initial rate studies which showed that the initial biotransformation rate is optimal at a benzaldehyde concentration of 6 g/l. Even though PAC is a product of the glycolytic pathway (in the presence of benzaldehyde), an increase in PAC concentration was not concomitant with a decrease in sucrose concentration. In the case of F1 and F2, sucrose metabolism increased as benzaldehyde concentration approached zero. In F3, sucrose concentration did not decrease following the equilibration period. As sucrose is converted to glucose and fructose (Section 1.3.1) and glucose concentration is a major regulator of yeast metabolism (levels above 50-100 mg/l must be maintained for fermentative metabolism, (Oura, 1974; Caubet *et al*.,

1988)), it may be necessary to make supplementary sucrose additions to maintain a source of co-substrate in extended biotransformations. If invertase, the enzyme which converts sucrose to glucose and fructose is inactivated/denatured by the substrate or products, the further additions of sucrose would be futile. This is a possible rate limiting step in the biotransformation.

By recovery of yeast cells which had lost viability and reinoculation into fresh biotransformation media, it was confirmed that when cell viability is diminished, the cells are no longer capable of producing significant amounts of PAC and benzyl alcohol (Section 3.6.2). Comparison of biotransformation abilities of whole and homogenised cells indicate that whole cells are not necessary for PAC production. However, the homogenised cells require supplementation with TPP and Mg^{2+}, cofactors of pyruvate decarboxylase for the reaction to occur efficiently (Section 3.6.9). It has previously been demonstrated that acetone powders of brewers yeast can synthesise PAC (Smith and Hendlin, 1953). In this case, the addition of TPP and Mg^{2+} was also necessary. The fermentative activity of the living yeast cell is apparently associated with the structural entity of the cell and is located in the periphery of the cell, several reports indicate that the enzymes may be membrane bound (Rothstein et al., 1959; Green et al., 1965; Atkinson, 1969; Sols and Marco, 1970; Beavan et al., 1982). The enzymes responsible for fermentation are readily solubilised by homogenisation (Rothstein et al., 1959). Dilution of the enzymes and cofactors required for the biotransformation would therefore result in a decrease in rate of product formation.

Modification of cell permeability by benzaldehyde and/or benzyl alcohol (which has been shown to affect membrane fluidity in studies using erythrocytes (Hubbell et al., 1970)), leading to a reduction in cofactor concentration may be an important contributing factor to the low biotransformation ability of cells incubated with high product concentrations.

Comparisons of intracellular and extracellular concentrations of substrates and products of biotransformation indicate that the cell membrane maintains a permeability barrier to benzaldehyde, resulting in reduced intracellular benzaldehyde levels. At higher concentrations of benzaldehyde, this barrier appears to be damaged and intracellular components are exposed to substantially higher benzaldehyde levels (Section 3.6.3). As previously mentioned, benzyl alcohol has been shown to directly affect membrane lipids in erythrocytes (Hubbell et al., 1970) and also in bateria (Ingram, 1976). In yeast, the effect of alcohols, especially ethanol, on cell viability and metabolism has attracted a lot of interest due to its commercial value in brewing, baking and also in production of ethanol for use as a fuel. Hayashida et al. (1974) and Hayashida and Hongo (1976) reported that sake yeasts (strains of S. cerevisiae) acquired enhanced tolerance to ethanol when grown in the presence of a fraction from the envelope of Aspergillus oryzae containing unsaturated fatty-acyl residues. Populations of yeast cells suspended anaerobically in buffered ethanol remained viable to a greater extent when their plasma membranes were enriched in linoleyl (C18:2) rather than oleyl (C18:1) residues (Thomas et al., 1978). This is possibly an adaptation on the part of the organism to high concentrations of ethanol on both sides of the plasma

membrane. A stress of a high concentration of an amphipathic compound on both sides of the membrane could cause the monolayers to part, and an increased synthesis of C18:2 fatty acyl residues might be viewed as an adaptation to maintain membrane integrity (Beavan et al., 1982). (Benzaldehyde, benzyl alcohol and PAC may also be classed as amphipathic molecules). The toxic effect of ethanol results from inhibition and denaturation of intracellular enzymes (Thomas et al., 1978; Millar et al., 1982). As the presence of multiply unsaturated fatty-acyl residues increases, the stability of membrane bound enzymes (Thomas et al., 1978), growth of the yeast used for the biotransformation of benzaldehyde in the presence of linoleyl residues and a sterol with a unsaturated alkyl chain (which also creates a barrier to entry of ethanol into yeast cells, (Thomas et al. (1978)) may enhance the tolerance of the yeast cells to the observed concentrations of benzaldehyde and benzyl alcohol. The actual values of intracellular substrates and products (Table 3.16) must be interpreted with caution because they are dependent on the accuracy of estimates of intracellular volume and are influenced by laboratory techniques (Saez and Lagunas, 1976; Dasari et al., 1983; Guijarro and Lagunas, 1984; Dasari et al., 1985; D'amore et al., 1988). In addition, overall intracellular levels ignore internal distribution between cell wall, membrane and cytoplasmic phases.

Extracellular ethanol concentrations were determined for F1, F2 and F3 (Section 3.6.10), levels did not exceed 13 g/l. As ethanol is toxic to the cell at levels ranging between 80 and 180 g/l (depending on the strain of yeast and the metabolic state of the culture (Stewart et al., 1984)), and significant denaturation of

glycolytic enzymes does not occur below 120 g/l ethanol (Millar et al., 1982), the amount of ethanol produced in the presence of benzaldehyde is not a significant factor in yeast cellular toxicity. A decrease in yeast cell viability was not observed in the presence of an initial concentration of 15 g/l ethanol (Section 3.6.12).

Studies on the effect of benzaldehyde and biotransformation products on stability of the key biotransforming enzymes indicate that pyruvate decarboxylase is substantially resistant to denaturation by benzaldehyde at concentrations up to 7 g/l. Alcohol dehydrogenase is more susceptible to benzaldehyde denaturation. Both enzymes appear fairly resistant to denaturation by transformation products. Loss of biotransformation activity in vivo may however be due to reversible inhibition of these enzymes by the substrate and/or products. The enzymes were diluted more than 100-fold in the assay which would leave negligible concentrations of benzaldehyde and products. Another disadvantage of in vitro assays is that enzymes may show differences in kinetic behaviour depending on the concentration of protein in the assay (Banuelos and Gancedo, 1978). These workers showed, however, that in the case of glycolytic enzymes, values obtained by conventional methods do not differ from those obtained at physiological protein concentration. Each enzyme was relatively more resistant to denaturation by its own reaction product, thus illustrating that enzymes are more stable in the presence of their reactants (Citri, 1973; Schmid, 1979; Godfrey, 1983). Cessation of PAC production by Saccharomyces carlsbergensis, from sucrose and benzaldehyde, can be restored with initial velocity by addition of pyruvate (Vojtisek

and Netrval, 1982). This suggests that one or more glycolytic enzymes between sucrose and pyruvate are inactivated or become rate limiting. Prakash and Holzer (1985) studied the effect of m-Cl-peroxy benzoic acid on glycolysis. Assays of the activities of 9 different enzymes of the glycolytic pathway as well as analysis of steady state concentrations of metabolites suggest that glyceraldehyde-3-phosphate dehydrogenase is the most sensitive enzyme of glucose fermentation. Phosphofructokinase and alcohol dehydrogenase are slightly less sensitive.

The initial and final yeast dry weight, protein, carbohydrate and lipid determinations indicate that during the biotransformation process, yeast biomass content, especially the cell protein and lipid components are reduced. Reductions in culture absorbance values suggest that 30-40% of the yeast cells are lysed during the 10 h biotransformation. Smaller observed decreases in biomass and protein, lipid and carbohydrate content may be due to insoluble lysed cell components being recovered with the cells during centrifugation.

Preliminary studies on the immobilisation of the yeast cells in calcium alginate were carried out to see if such measures would protect the yeast cells from substrate and product and thus prolong the biotransformation (Section 3.7.1). Results show that cells in the immobilised system produced PAC at a slower rate resulting in lower overall titres than an equivalent amount of free cells. Immobilisation did not enhance the maintenance of yeast cell viability. Immobilised cells may be used to produce PAC in a continuous system (Section 3.7.2), an advantage of such a system is

that the substrate concentration can be tightly regulated and product would be continuously removed from the system. However, results show that in such a system the amount of product formed per litre of medium would be low, making product recovery difficult and expensive.

From the information gained during the course of this work it would seem that a biphasic system such as that described by Antonini et al. (1981) and Lilly (1982) would eliminate many of the problems encountered in the biotransformation. The properties of the substrate and products would be ideal for such a system as they are soluble in organic solvents. The system would allow the maintenance of a constant concentration of benzaldehyde substrate in the medium, regulating the concentration of the substrate to which cells are exposed, while at the same time the product would be constantly removed into the solvent (making product recovery a lot easier). This would greatly reduce toxicity problems, yeast cell viability could be maintained and the problem of inhibition of PAC formation by high concentrations of substrate and products as observed in Section 3.5.6 would be overcome. As benzaldehyde inhibits yeast growth (Section 3.8), the maintenance of low benzaldehyde concentrations would allow the generation of new cells, critical for PAC production (Agarwal et al., 1987). Rezessy-Szabo et al., (1987) have looked at a number of organic solvents for use in biphasic systems and their effect on growth of many bacterial species. Some of these solvents such as dibutyl phtalate may be useful in the yeast system as it is not toxic to most bacteria and it is cheap and easy to handle.

If pure enzymes rather than whole cells were to be used in the biotransformation, the use of organic solvents instead of an aqueous medium (as distinct from a biphasic system) could be suitable for PAC production. In such a system only a minimum amount of water is required (Klibanov, 1977) which is tightly bound to the enzyme molecules (Kazandjian et al., 1986). The advantages include increased solubility of substrate, easier recovery of product and the observation that enzymes may be more stable in organic solvents than they are in water (Klibanov, 1986). The use of 2 substrates in PAC formation (one of which may not be soluble in all organic solvents) and the cofactor requirements of the enzyme (pyruvate decarboxylase) may present difficulties in putting such a system into practice.

The ability of a wide range of yeasts to synthesise PAC provides scope to improve product titres and perhaps eliminate by-product formation once optimum conditions have been determined. With the broad substrate specificity of the system the bioconversion may be applied to the production of modified carbinols for use as precursors to synthesis of new ephedrine-like compounds with potential pharmacological activity.

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APPENDIX A

The following buffers were used during the course of this work.

Sodium Citrate - Citric Acid Buffer

Citrate buffers were prepared by titrating a solution containing the appropriate concentration of citric acid with sodium hydroxide until the required pH was attained. Citric acid solutions were not made up to volume until the pH had been adjusted. pH was checked again following adjustment. The preparation of sodium citrate - citric acid buffers used is described in Table A.1.

Table A.1. Preparation of sodium citrate-citric acid buffers (final volume 100 ml). Citric acid solution (95 ml), was titrated with 10 M NaOH to appropriate pH, volume adjusted to 100 ml and pH rechecked.

Buffer pH	Buffer Concentration	Citric Acid g/100ml
6.0	0.20	4.20
7.5	0.10	2.10
6.0	0.05	1.05

Sodium Pyrophosphate Buffer

Sodium pyrophosphate buffers were prepared by a titration method as above. A sodium pyrophosphate solution of appropriate concentration was adjusted to the desired pH using HCl. The preparation of sodium pyrophosphate buffers used is described in Table A.2.

Table A.2. Preparation of sodium pyrophosphate buffers (final volume 100 ml). Sodium pyrophosphate solution (95 ml) was adjusted to appropriate pH with 5 M HCl, volume adjusted to 100 ml and pH rechecked.

Buffer pH	Buffer Concentration	Sodium Pyrophosphate (g/100 ml)
9	0.1 M	4.46
8.8	60.0 mM	2.68

0.1M Sodium phosphate buffer pH 7.5

A 0.1M solution of NaH_2PO_4 was prepared by dissolving 1.21 g in water and diluting to almost 100 ml. The pH was adjusted to 7.5 with 10 M NaOH and the volume made up to 100 ml.

0.1M Potassium phosphate buffer pH 7.0

A 0.1M solution of KH_2PO_4 was prepared by dissolving 1.36 g in water and diluting to almost 100 ml. The pH was adjusted to 7.0 with 10 M KOH and the volume made up to 100 ml.

Citrate-Phosphate buffer pH 6.8

The citrate-phosphate buffer used in Section 2.7.3 was prepared by dissolving Na_2HPO_4 and citric acid to a final concentration of 0.10 M and 0.02 M respectively, giving a pH of 6.8. $CaCO_3$, $MgSO_4$, thiamine pyrophosphate and $MnSO_4$ were added in concentrations shown in Table A.3.

Table A.3. Components (amounts and final concentrations) used to prepare Citrate-phosphate buffer pH 6.8.

Buffer Component	Amount Added (g/100 ml)	Final Concentration
Na_2HPO_4	1.430	0.1 M
Citric acid	0.380	0.02 M
$CaCO_3$	0.010	1.0 mM
$MgSO_4$	0.004	0.33 mM
TPP	0.005	0.1 mM
$MnSO_4$	0.026	1.7 mM

APPENDIX B

CHROMATOGRAMS

Sample chromatograms are presented. These include a gas chromatogram obtained in the determination of benzyl alcohol and benzaldehyde in a fermentation broth, and a chromatogram obtained in the analysis of a mixture of standard fatty acids by G.C. A chromatogram obtained in the analysis of aromatic aldehydes and alcohols by H.P.L.C. is also presented.

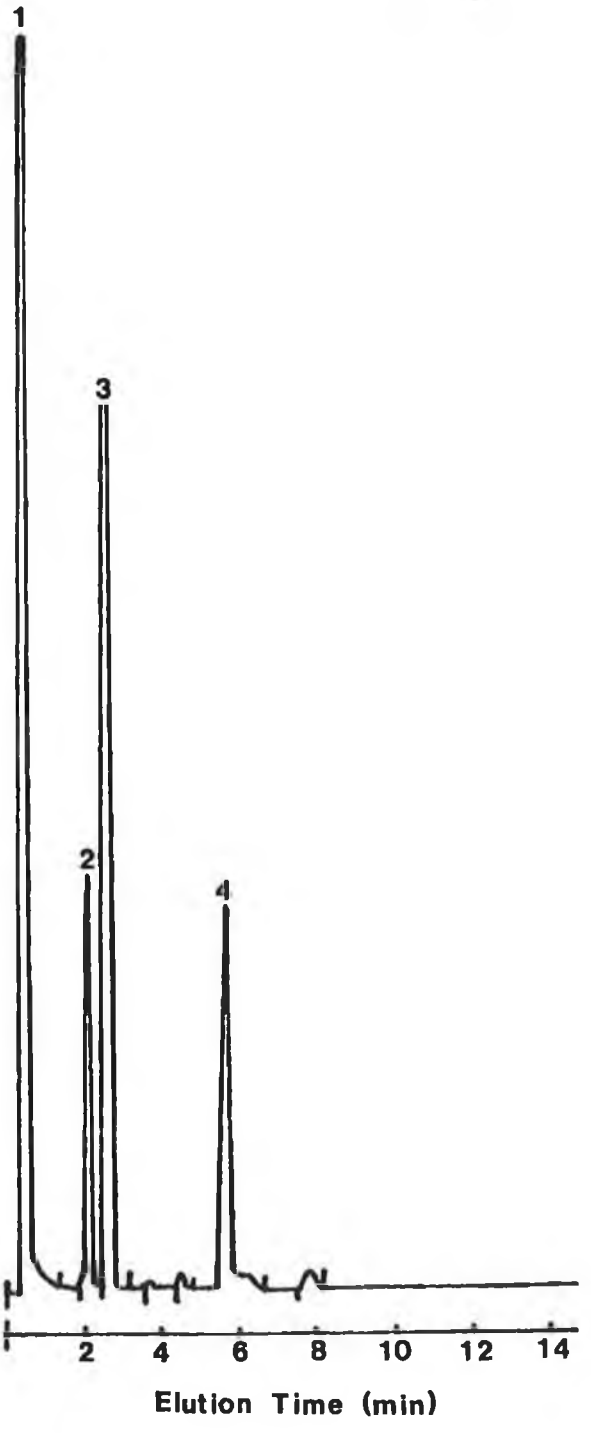

Figure B.1. GC chromatogram obtained in the determination of benzyl alcohol and benzaldehyde in fermentation broths (Section 2.4.2). Peaks labelled 1 to 4 are: 1, solvent (diethyl ether); 2, benzaldehyde; 3, benzyl alcohol and 4, PAC.(Hewlet Packard 5830A GC).

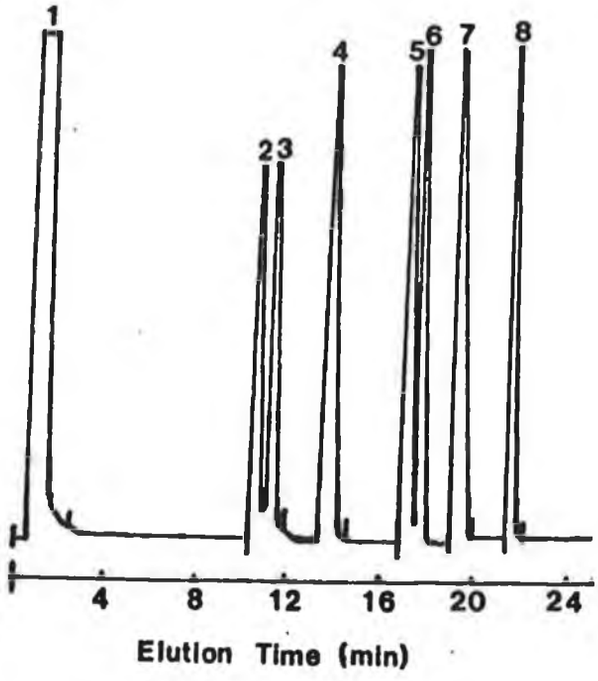

Figure B.2. Chromatogram obtained in the analysis of a mixture of standard fatty acids by GC (Section 2.4.3). The peaks labelled 1 to 8 are: 1, solvent (chloroform); 2, palmitic acid (16:0); 3, palmitoleic acid (16:1); 4, heptadecanoic acid (17:0) (internal standard); 5, stearic acid (18:0); 6, oleic acid (18:1); 7, linoleic acid (18:2) and 8, linolenic acid (18:3).

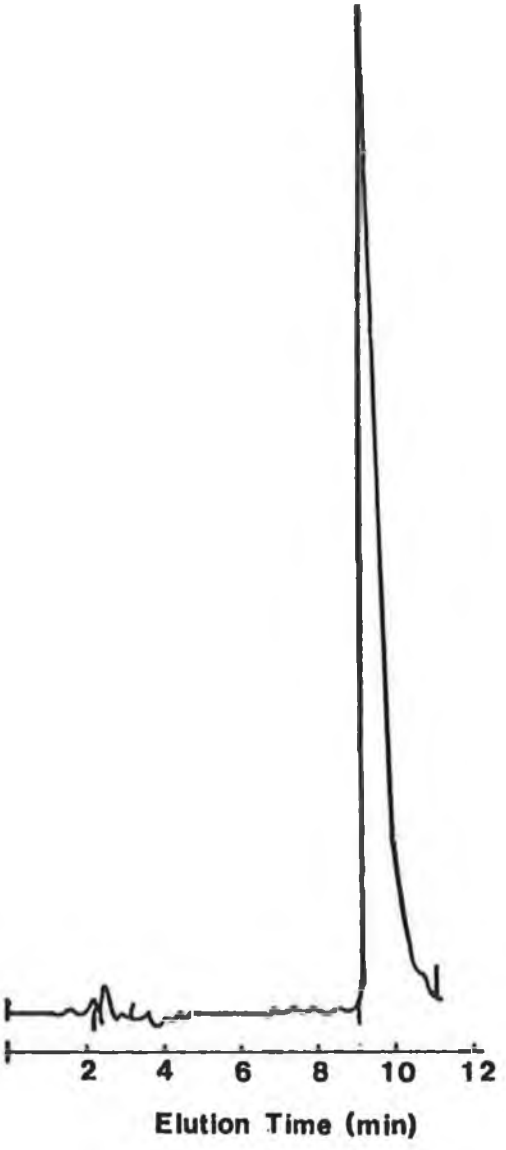

Figure B.3. Sample chromatogram obtained in the determination of aromatic alcohols and aldehydes by H.P.L.C. (Section 2.5). The peak shown is that of 2-chlorobenzyl alcohol.

APPENDIX C

Research Communications

Meetings

"Biotransformation of aromatic aldehydes by yeast: Investigation of reaction rates".

Poster presented at the 1988 annual Society for Industrial Microbiology meeting, Chicago, Illinois, U.S.A. 7-11 August 1988.

"Conversion of Benzaldehyde to L-Phenylacetyl Carbinol and Benzyl alcohol by Saccharomyces cerevisiae".

Oral presentation at the 113th meeting of the Society for General Microbiology, University of Cambridge, England. 4-7 April 1989.

Publications

Long, A., James, P. & Ward, O.P. (1989). Aromatic aldehydes as substrates for yeast and yeast alcohol dehydrogenase. Biotechnology and Bioengineering 33, 657-660.

Long, A. & Ward, O.P. (1989). Biotransformation of aromatic aldehydes by Saccharomyces cerevisiae: investigation of reaction rates. Journal of Industrial Microbiology 4, 49-53.

Long, A. & Ward, O.P. (1989). Biotransformation of benzaldehyde by Saccharomyces cerevisiae: characterisation of the fermentation and toxicity effects of substrates and products. Biotechnology and Bioengineering (in press).