THE UNIVERSITY OF HULL

The Role of Aldehyde Dehydrogenases in Acetyl-CoA Production by *Saccharomyces cerevisiae*

being a thesis submitted for the Degree of
Doctor of Philosophy
in the University of Hull

by

WAYNE DANNY TESSIER, BSc (Wales)

April 1998

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisors at Hull; Drs. F. M. Dickinson and M. Midgley. Their guidance, patience, advice and amiableness throughout my three years has been inestimable. I would also like to thank them for the rapid reading of this thesis. I must also express my gratitude to Dr. C. Boulton at Bass for many useful discussions and for his hospitality towards me whilst in Burton-on-Trent.

Sincere thanks is also due to Dr. P. G. Meaden (Heriot-Watt University, Edinburgh) for his expert genetic work and excellent collaboration. I am indebted to the Institute of Brewing and Bass Brewers for receipt of the Henry Mitchell Memorial Scholarship and for additional research funding.

This work could not have taken place without the skilled technical assistance of those at Hull: Mrs. C. Wadforth, Mr. S. Dack and Mr. D. Grantham and those at Bass: Dr. C. Boulton, Mrs. W. Box, Dr. A. Prest, Mr. C. Powell and all in the pilot plant.

Finally, I would like to thank my Mum and Dad for their infinite support and encouragement and last but not least my fiancee, Caroline, for her patience, kindness and constant support throughout this study.

In nature's infinite book of secrecy
A little I can read.

Shakespeare
(Antony and Cleopatra, I.ii)

CONTENTS

MATERIALS AND METHODS

RESULTS

ABBREVIATIONS

The following abbreviations have been used throughout this thesis:

Mg^{2+}-ACDH:	Magnesium activated, NADP linked, cytosolic acetaldehyde dehydrogenase.
K^{+}-ACDH:	Potassium activated, NAD(P) linked, mitochondrial acetaldehyde dehydrogenase.
NAD:	Nicotinamide adenine dinucleotide (oxidised form).
NADP:	Nicotinamide adenine dinucleotide phosphate (oxidised form).
NADH:	Nicotinamide adenine dinucleotide (reduced form).
NADPH:	Nicotinamide adenine dinucleotide phosphate (reduced form).
ATP:	Adenosine triphosphate.
ADP:	Adenosine diphosphate.
CoA:	Coenzyme A.
DTT:	Dithiothreitol.
PDH:	Pyruvate dehydrogenase.
PDC:	Pyruvate decarboxylase.
PYC:	Pyruvate carboxylase.
ACS:	Acetyl-CoA synthetase.
CAT:	Carnitine acetyltransferase.
ADH:	Alcohol dehydrogenase.
SDH:	Succinate dehydrogenase.
ICL:	Isocitrate lyase.
PMS:	Phenazine methosulphate.
DTNB:	Dithionitrobis.
DEAE:	Diethylaminoethyl.

INTRODUCTION

Acetyl-coenzyme A is of fundamental importance in the metabolic and biosynthetic pathways of *Saccharomyces cerevisiae*. It has a central role in major biosynthetic pathways such as formation of fatty acids and generation of mevalonic acid and sterols. A better understanding of acetyl-CoA production in *S. cerevisiae* might allow a better control of the concentrations and utilisation of this molecule. For instance, in brewing yeast, acetyl-CoA is central to the synthesis of esters which are the largest group of flavour-active compounds in beer. Therefore, elucidation of the pathways involved in carbon flux to acetyl-CoA could lead to the development of possible strategies for maximising fermentation efficiency and controlling beer flavour production.

At the outset of this research, the nature and contribution of the central pathways to producing acetyl-CoA were not fully understood. It is against this backdrop that the present work was initiated.

1. Cytosolic acetyl-CoA production during growth on hexoses

Most routes of acetyl-CoA consumption require cytosolic acetyl-CoA. The major exception to this is the citric acid cycle, which uses acetyl-CoA present in the mitochondrion.

In *S. cerevisiae*, cytosolic acetyl-CoA arises directly in the cytosol, or can be made in the mitochondria and then exported. The inner mitochondrial membrane is however impermeable to acetyl-CoA and so export is indirect. When growing on hexoses (such as glucose) a number of potential routes to cytosolic acetyl-CoA have been considered including:

1.1 ATP: citrate lyase (ACL)

This cytosolic enzyme, first discovered in chicken liver (Srere and Lipmann,

1953) catalyses the ATP-dependent cleavage of citrate to oxaloacetate and acetyl-CoA. Citrate is transported from mitochondrion to cytosol via a citrate/malate translocase (Evans *et al*, 1983). In mammalian systems, this enzyme has been assigned a key role in lipogenesis, but it is not present in non-oleaginous yeasts such as *S. cerevisiae* (Boulton and Ratledge, 1981 a, b).

1.2 Carnitine acetyltransferase

Mammals, fungi and yeast possess carnitine acetyltransferase (CAT) which catalyses the interconversion of acetyl-CoA and acetyl-carnitine (Ratledge and Gilbert, 1985; Woeltje *et al*, 1990) (Figure 1). The reaction is as follows:

$$\text{Acetyl-CoA + carnitine} \longleftrightarrow \text{Acetylcarnitine + CoA}$$

Acetyl-carnitine has no known metabolic function and can be transported across the inner mitochondrial membrane by an acyl-carnitine/carnitine antiporter protein (Ramsay and Tubbs, 1976). Kohlaw and Tan-Wilson (1977) stated that CAT was the most likely candidate for acetyl group transfer out of the mitochondrion which is consistent with production of acetyl-CoA in the mitochondrion by the PDH complex and subsequent transport to the cytosol by CAT. Kawamoto *et al* (1978) demonstrated that CAT may also be found in peroxisomes. This is of particular relevance during growth on fatty-acids, when acetyl-CoA is formed directly in these organelles by β-oxidation (Atomi *et al*, 1993) (see Section 3.2.2).

1.3 The PDH bypass

This pathway is perhaps the major candidate for cytosolic acetyl-CoA production in *S. cerevisiae* (Figure 2). Pyruvate produced by glycolysis can undergo decarboxylation in the cytosol by the action of pyruvate decarboxylase (PDC) (Neuberg and Karczag, 1911). Acetaldehyde is formed which can then be reduced to ethanol by alcohol dehydrogenase I (ADH I) or converted to acetate by a Mg^{2+}-activated

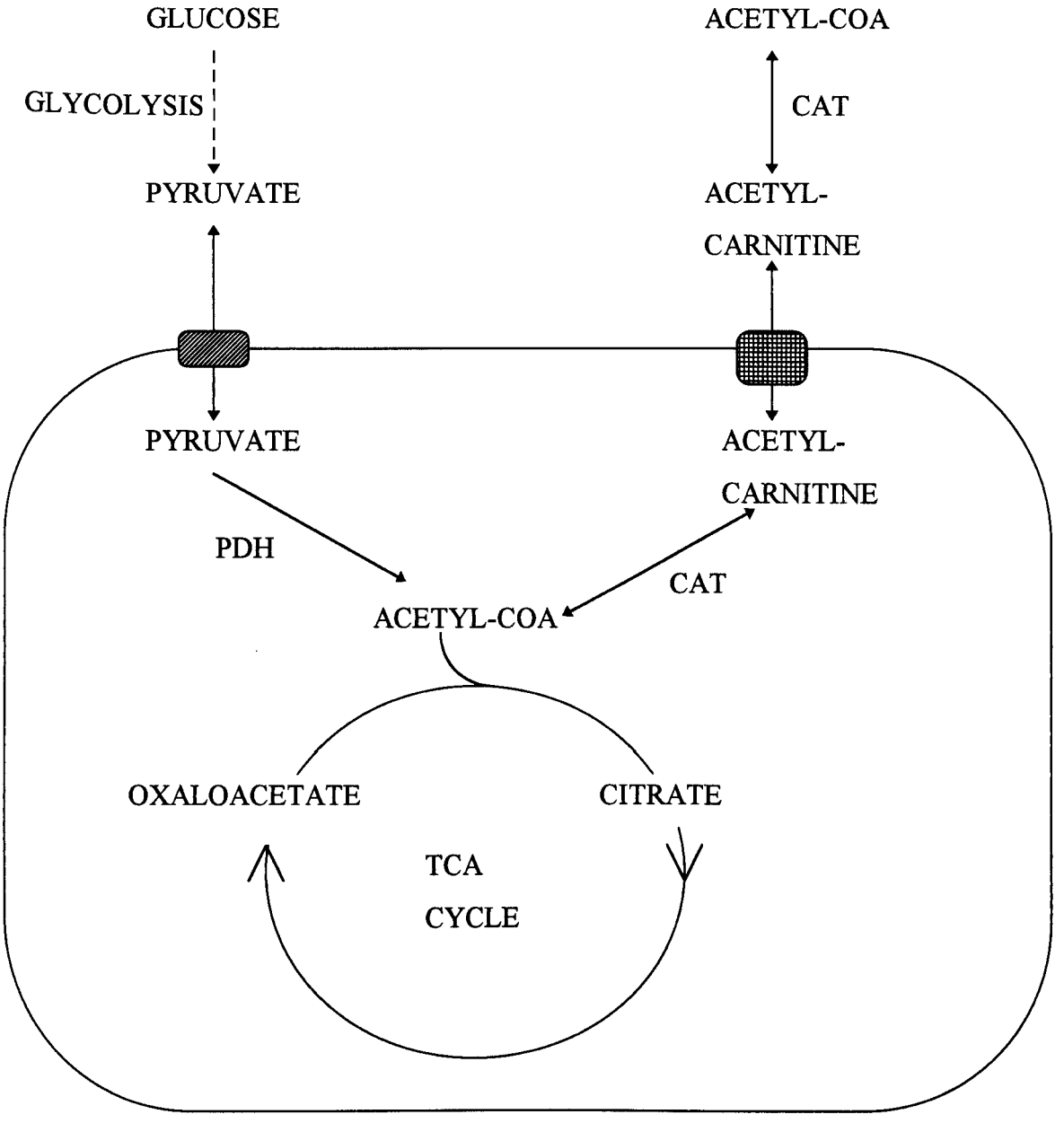

Figure 1: Production of cytosolic acetyl-CoA involving carnitine acetyltransferase (CAT). Acetyl-CoA is produced in the mitochondrion by the pyruvate dehydrogenase (PDH) complex and then exported via a transportable intermediate acetyl carnitine (ACN) and by the action of CAT.

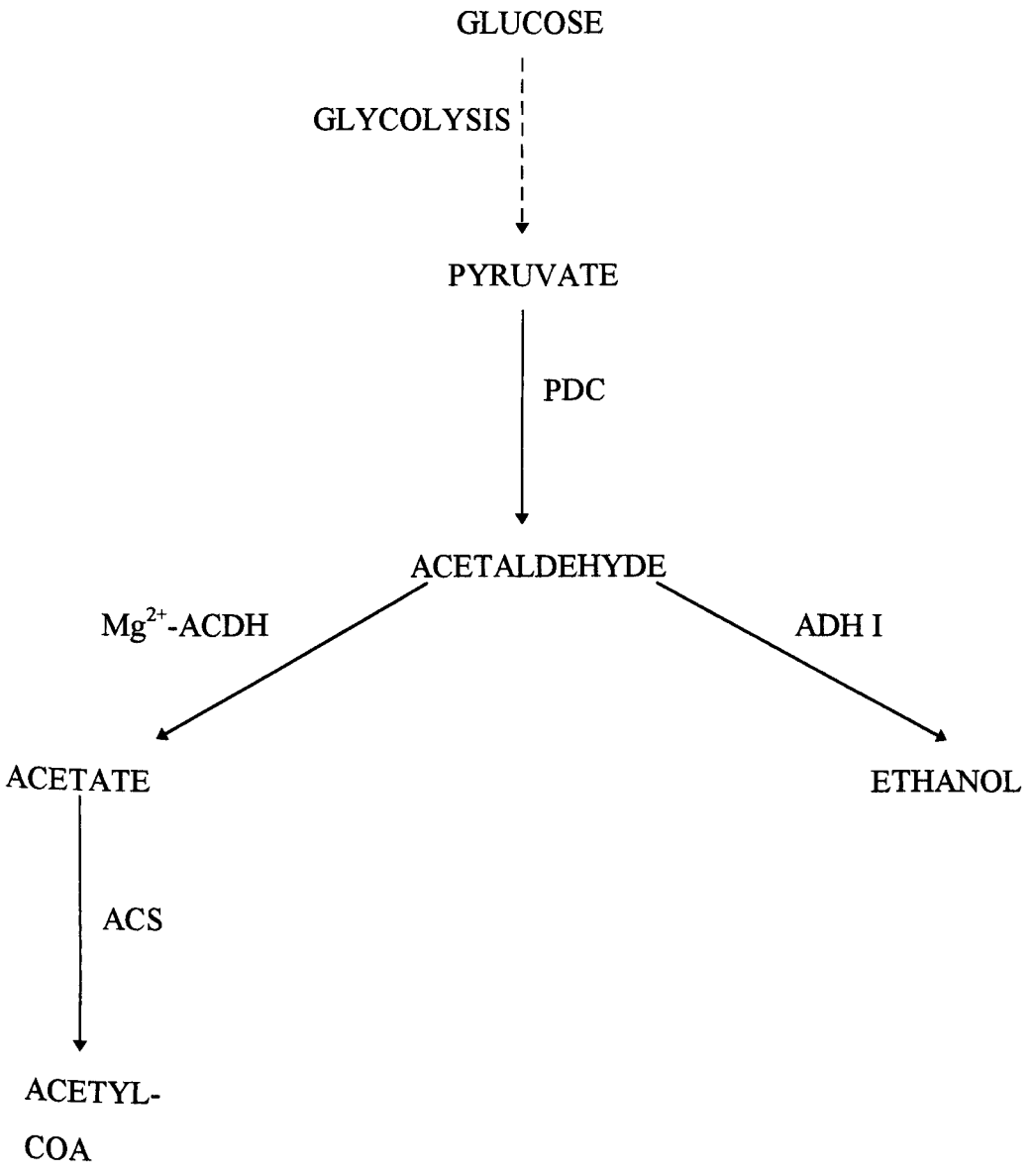

Figure 2: Production of cytosolic acetyl-CoA by the pyruvate dehydrogenase (PDH) bypass. Acetyl-CoA is produced in the cytosol by the concerted action of pyruvate decarboxylase (PDC), Mg^{2+}-acetaldehyde dehydrogenase (Mg^{2+}-ACDH) and acetyl-CoA synthetase (ACS).

acetaldehyde dehydrogenase (ACDH) (Seegmiller, 1953). Acetyl-CoA can then be formed from acetate by an ATP-driven reaction that is catalysed by acetyl-CoA synthetase (ACS) (Klein and Jahnke, 1968).

This pathway has been named the PDH bypass, because it does not involve the mitochondrial pyruvate dehydrogenase (PDH) complex and is an entirely cytosolic process. During growth of *S. cerevisiae* under certain conditions though, the PDH complex may also function at the same time (Figure 3).

2. Evidence for operation of the PDH bypass

During growth on glucose, pyruvate is generated by the enzymes of glycolysis and so is present in the cytosol. Until the recent work of Flikweert *et al* (1996) and van den Berg & Steensma (1995) on PDC and ACS, it was thought that pyruvate is channelled through PDC only under certain conditions. These conditions which are detailed below are (i) anaerobic growth on glucose or (ii) batch aerobic growth on a concentration of glucose exceeding 0.8% (w/v) or aerobic chemostat growth on excess glucose. Under all other conditions, it was assumed that pyruvate is converted to acetyl-CoA solely by the PDH complex.

2.1 Anaerobic growth on glucose

S. cerevisiae is a fermentative yeast and so can ferment glucose to ultimately produce ethanol. The PDH complex is known to be active during fermentative growth (Wenzel *et al*, 1993) but citric acid cycle and respiratory chain enzyme synthesis is limited (Chapman & Bartley, 1968; Wales *et al*, 1980). There has been disagreement over the presence/absence of mitochondria under anaerobiosis although recent work has shown that mitochondria of *S. cerevisiae* fulfil a vital role in anaerobic sugar metabolism (Visser *et al*, 1995). However, under anaerobic growth conditions, acetyl-CoA can be formed by the PDH bypass.

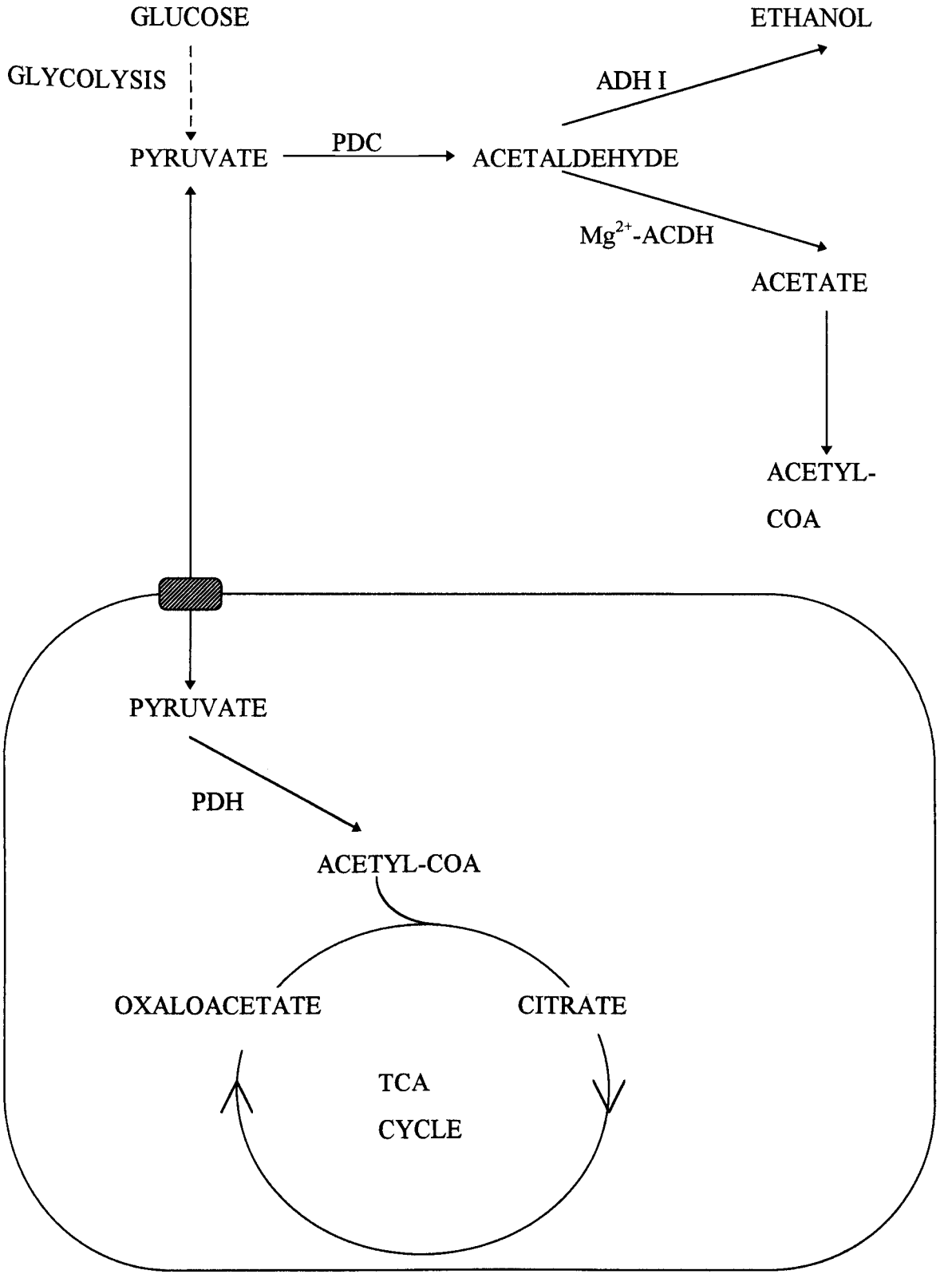

Figure 3: Concomitant production of acetyl-CoA by the pyruvate dehydrogenase (PDH) bypass and PDH complex. Acetyl-CoA is produced in the mitochondrion by the pyruvate dehydrogenase (PDH) complex. Alternatively or concomitantly, acetyl-CoA can be produced in the cytosol by the PDH bypass.

2.2 Batch aerobic growth on glucose

Batch aerobic growth on concentrations of glucose exceeding 0.8% (w/v) or chemostat growth on glucose excess results in what has been called the 'Crabtree effect'. This aerobic alcoholic fermentation is caused by insufficient capacity of respiratory routes of pyruvate dissimilation (van Urk *et al*, 1990). Under these conditions, ACS and the Mg^{2+}-ACDH are present, indicating that cytosolic acetyl-CoA can be made under such conditions (Pronk *et al*, 1996).

However, recent work (Flikweert *et al*, 1996) has shown that even when *S. cerevisiae* is growing under conditions of glucose limitation, pyruvate is channelled through PDC. It was previously thought that when aerobic chemostat growth takes place under conditions of glucose limitation, the PDH complex is solely responsible for acetyl-CoA production (Pronk *et al*, 1994). However, *pdc*$^-$ strains have been constructed which in defined medium do not grow on glucose (Flikweert *et al*, 1996). This is consistent with the suggestion by Wenzel *et al* (1992), that the PDH complex is not essential for growth on glucose. This suggests that the PDH bypass plays a crucial role in cytosolic acetyl-CoA production and that this role cannot be fulfilled by the PDH complex.

2.3 Control of metabolic flux at the pyruvate branch-point

What factor(s) determine whether pyruvate is channelled through the PDH complex or the PDH bypass?

Holzer (1961) first proposed that the intracellular concentration of pyruvate might be an important parameter in the regulation of fermentative and respiratory assimilation in *S. cerevisiae*. This was based on the observation that the K_m of the PDH complex is an order of magnitude lower than that of PDC. According to Holzer's model, PDC is largely by-passed at low intracellular concentrations of pyruvate, thus

enabling respiratory assimilation of pyruvate via the PDH complex. In contrast, high intracellular concentrations of pyruvate will involve PDC in its assimilation. Thereafter, acetaldehyde can be assimilated via ADH to form alcohol or via the Mg^{2+}-ACDH to form acetate and ultimately acetyl-CoA. It is unclear at present how in glucose-limited cultures enough pyruvate is diverted into the PDH bypass to form acetyl-CoA but without the onset of alcoholic fermentation. Some workers have proposed that alcoholic fermentation will only occur if the synthesis of the Mg^{2+}-ACDH and ACS is limited (Postma *et al*, 1989).

High concentrations of pyruvate are assimilated via PDC because the respiratory capacity of PDH is limited. In experiments where respiratory capacity is saturated by adding excess glucose to respiring, glucose-limited cultures, only a small increase of the respiration rate is observed directly after glucose addition (Petrik *et al*, 1983; van Urk *et al*, 1990).

Much of the literature to date has focused on the enzymes PDC and PDH, but has neglected the role of pyruvate carboxylase (PYC). This catalyses the anaplerotic synthesis of oxaloacetate from pyruvate in the cytosol. Its activity is increased in the presence of acetyl-CoA, presumably because this will lead to a replenishment of the acceptor molecule oxaloacetate (Pronk *et al*, 1996). The enzyme seems to be constitutive since its activity has never been reported to increase more than two-fold (de Jong-Gubbels *et al*, 1995). PYC may well assimilate pyruvate along with PDC when intracellular levels of pyruvate are high, thus delaying the onset of alcoholic fermentation.

3. Proposed roles for carnitine acetyltransferase

Three forms of CAT have been identified in *S. cerevisiae* and these are encoded by two genes. The *YCAT* gene encodes the peroxisomal and mitochondrial forms of the enzyme and differential targeting is achieved by an internal peroxisomal targeting

sequence (Elgersma *et al*, 1995). Targeting is dependent on the growth conditions. In acetate-grown cells, the product of *YCAT* is predominantly mitochondrial, whereas during fatty-acid growth, the product is peroxisomal. Cells lacking only the mitochondrial CAT enzyme were reported to grow as the wild-type except that they dissimilated pyruvate into acetate and lactate rather than into alanine and TCA cycle intermediates (like the wild-type). This the authors suggested was a result of diminished flux through the PDH complex (Kispal *et al*, 1993).

The second CAT gene of *S. cerevisiae* is known as *YAT1*. It is ethanol-inducible and has been shown to code for the cytosolic form of the enzyme (Schmalix and Bandlow, 1993). Disruption of this gene does not result in a detectable phenotype, which suggests that the isozymes might be able to compensate for each other.

CAT activity has been detected during growth of *S. cerevisiae* on glycerol, ethanol, glucose and oleic acid (Kohlaw and Tan-Wilson, 1977; Claus *et al*, 1983; Kispal *et al*, 1991; Atomi *et al*, 1993). Atomi *et al* (1993) detected activity in mitochondria, peroxisomes and the cytosol, indicating that CAT plays a central role in inter-organelle transport of acetyl-CoA and (indirectly) transport of metabolic intermediates.

3.1 Involvement of CAT during growth on glucose

One might not expect CAT to function during growth on glucose, since the PDH bypass can provide the necessary cytosolic acetyl-CoA. However, Claus *et al* (1983) detected CAT activity during derepressed growth of *S. cerevisiae* on glucose. This implies that acetyl-CoA may be formed (i) by the PDH complex (and then exported to the cytosol by CAT) and also the PDH bypass or that (ii) acetyl-CoA formed by the PDH bypass may be transported to the mitochondrion by CAT. This warrants further work.

During repressed growth on glucose, CAT activity was absent, suggesting that under these conditions, cytosolic acetyl-CoA is formed by the PDH bypass.

3.2 Involvement of CAT during growth on C2 compounds and fatty acids

3.2.1 Growth on C2 compounds

Saccharomyces cerevisiae can grow on C2 compounds such as ethanol and acetate. Ethanol is converted to acetyl-CoA via acetaldehyde and acetate. This conversion can occur in a number of ways (Figure 4). Ethanol can be oxidised to acetaldehyde either by the inducible, cytosolic alcohol dehydrogenase (ADH II) or by the inducible, mitochondrial alcohol dehydrogenase (ADH III). It has been shown that ADH III is dispensable, since a null mutant was still able to grow on ethanol (Young and Pilgrim, 1985). The acetaldehyde that is produced in either compartment can then be metabolised by either the cytosolic, Mg^{2+}-dependent, $NADP^{+}$-linked acetaldehyde dehydrogenase (ACDH) or by the mitochondrial, K^{+}-linked, $NAD(P)^{+}$-linked ACDH. It seems possible that acetaldehyde can be produced in one compartment and then diffuse passively through the inner mitochondrial membrane to the other compartment. Acetaldehyde is a small, uncharged molecule and may well pass through membranes in this way. Acetate generated by the K^{+}-ACDH is unlikely to be metabolised in the mitochondria because there is no known ACS associated with this organelle (De Virgillio *et al*, 1995). However, acetate (being a small molecule) may passively diffuse across the inner mitochondrial membrane (Lowenstein, 1967) and thus be converted to acetyl-CoA by the cytosolic (or peroxisomal) ACS.

Growth of *S. cerevisiae* on acetate (and acetate derived from ethanol) requires that it is first metabolised to acetyl-CoA by ACS. This can occur in the cytosol (as described above) or peroxisomally, since the non-cytosolic form of ACS is thought to be located in the peroxisomes (De Virgillio *et al*, 1995). This location has been assigned on the basis that the predicted ACS gene product possesses a peroxisomal targeting sequence. However, the citric acid cycle (as well as the glyoxylate cycle) must be primed.

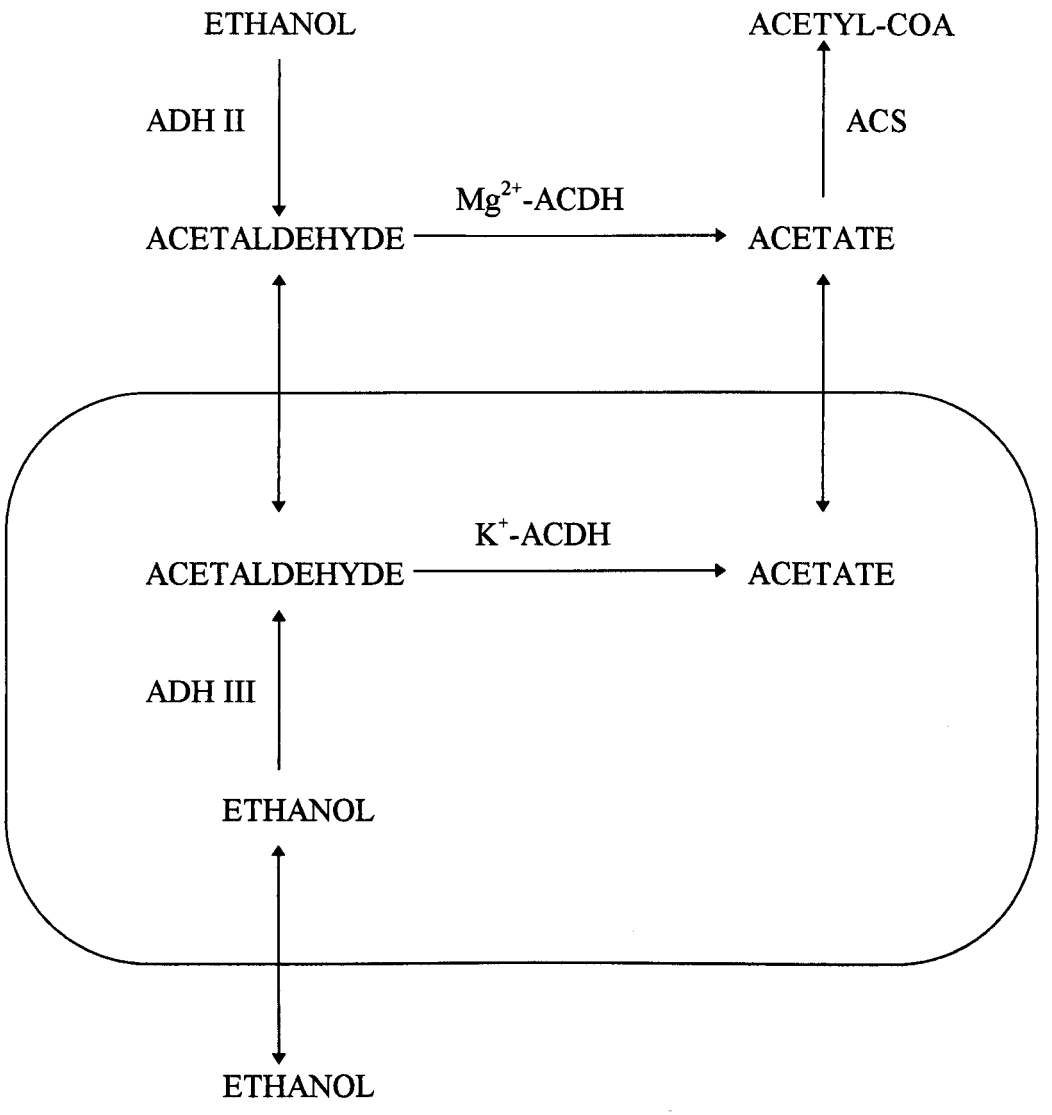

Figure 4: Production of cytosolic acetyl-CoA by the passive diffusion of acetate and acetaldehyde across the inner mitochondrial membrane. Acetaldehyde produced in the cytosol or mitochondrion by alcohol dehydrogenase (ADH). Depending on its location the acetaldehyde can then be oxidised to acetate by the mitochondrial, K^{+}-ACDH or the cytosolic, Mg^{2+}-ACDH. Any mitochondrial acetate can then diffuse to the cytosol for conversion to acetyl-CoA.

This is done by making use of glyoxylate cycle enzymes that are located in peroxisomes as follows (Figure 5):

Cytosolic acetyl-CoA is transported to the peroxisomes as its transportable intermediate acetylcarnitine via the action of cytosolic CAT (Elgersma *et al*, 1995). This is necessary because peroxisomal membranes are impermeable to acetyl-CoA (van Roermund *et al*, 1995). A peroxisomal form of CAT then converts the acetylcarnitine intermediate back into acetyl-CoA which is free to enter the glyoxylate cycle to generate citric acid cycle intermediates (such as citrate, succinate, malate and oxaloacetate) that can then pass to the mitochondria. The glyoxylate cycle bypasses the two decarboxylation steps of the citric acid cycle and allows entry of two acetyl-CoA molecules per turn, compared to the one that is allowed entry to the citric acid cycle.

3.2.2 Growth on fatty-acids

Growth on fatty-acids is essentially the same as for acetate, except that acetyl-CoA is produced directly in the peroxisomes by fatty-acid β-oxidation (McCammon *et al*, 1990). Acetyl-CoA enters the glyoxylate cycle immediately to produce citric acid cycle intermediates (Figure 5) or is transported to the mitochondria (via cytosolic and mitochondrial CAT). It can be seen though, that the role of CAT is mainly concerned with C2 metabolism. For growth on glucose however, the PDH bypass is the more likely candidate for cytosolic acetyl-CoA production.

4. The enzymes and genes of the PDH bypass of *S. cerevisiae*

The PDH bypass consists of PDC, Mg^{2+}-ACDH and ACS. The cytosolic acetyl-CoA that is produced by the concerted action of these three enzymes is then available for biosynthetic reactions within the cell.

4.1 Pyruvate decarboxylase

This enzyme catalyses the TPP- and Mg^{2+}-dependent decarboxylation of pyruvate

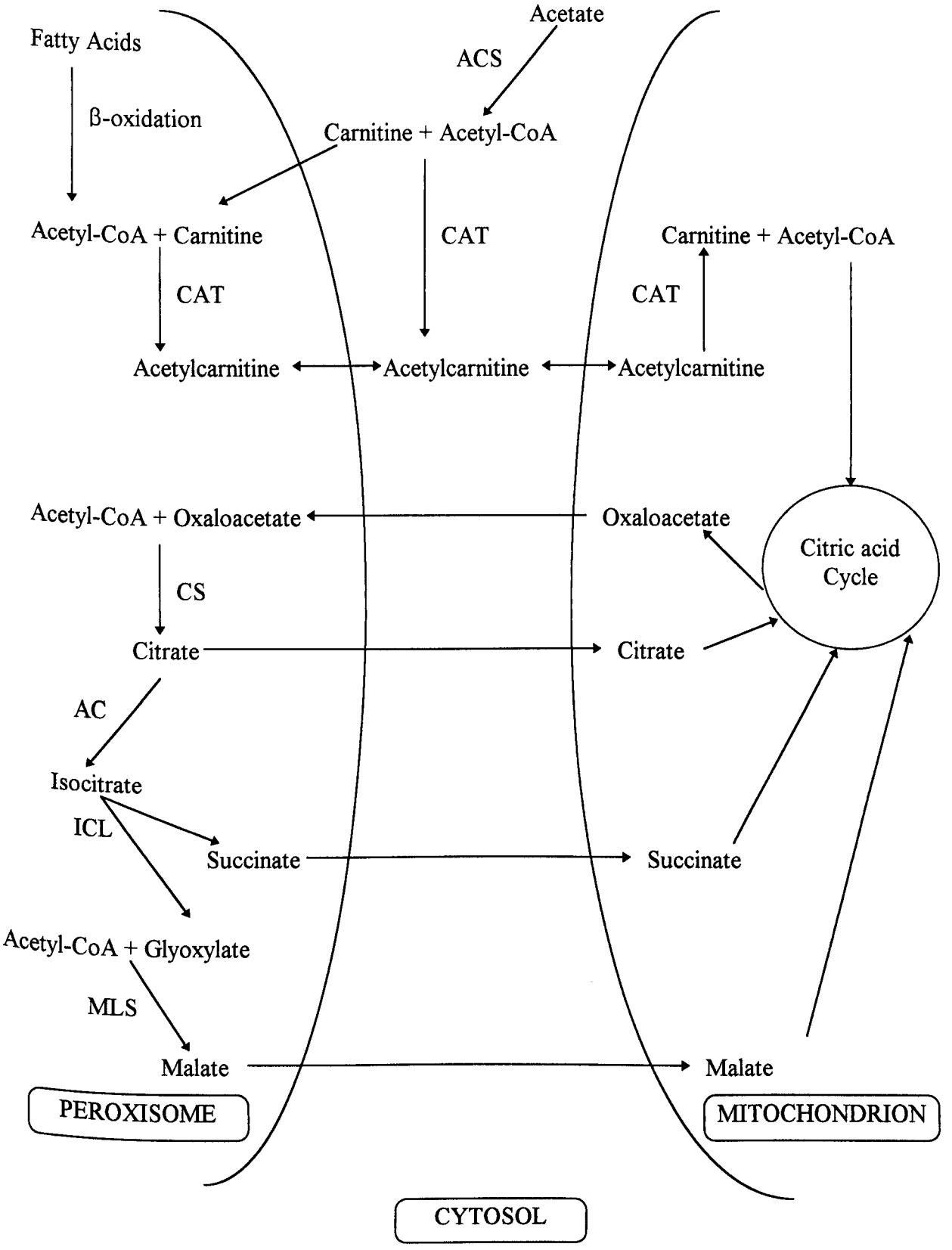

Figure 5: Production of acetyl-CoA by *S. cerevisiae* during growth on acetate and fatty acids. By use of the various shuttles, acetyl-CoA can be directed to cytosol, mitochondria or peroxisomes.

Key to abbreviations: ACS - Acetyl-CoA synthetase, CAT - Carnitine acetyltransferase, CS- Citrate synthase, AC - Aconitase, ICL- Isocitrate lyase, MLS - Malate synthase.

to acetaldehyde and carbon dioxide. The enzyme in *S. cerevisiae* is cytosolic and is a tetramer, composed of four identical or highly related subunits of 62 kd. PDC displays cooperativity with respect to pyruvate and this effect is enhanced by phosphate (Boiteux and Hess, 1970) which is thought to be a physiologically relevant effector in the control of PDC activity *in vivo*.

There are six known genes for PDC in *S. cerevisiae*. Three of these (*PDC1*, *PDC5* and *PDC6*) are structural genes and simultaneous disruption of all three results in no detectable activity for this enzyme (Flikweert *et al*, 1996). *PDC2* is known to be required for full expression of *PDC1* and *PDC5* (Hohmann, 1993), whilst the role of *PDC3* and *PDC4* remains unclear (Wright *et al*, 1989).

The enzyme is never absent and its activity is regulated. Activity increases during growth on glucose and especially in the absence of oxygen (Weusthuis *et al*, 1994). Its two main roles are the first committed step in alcoholic fermentation and the first step in the provision of cytosolic acetyl-CoA (Flikweert *et al*, 1996). A role in glycolytic control has also been proposed for this enzyme (Pronk *et al*, 1996).

4.2 Acetyl-CoA synthetase

Satyanarayana *et al* (1974) described two forms of ACS, an 'aerobic' form and an 'anaerobic' form. The enzymes differ in that kinetic characteristics change under different physiological conditions and are located in different subcellular compartments (Klein and Jahnke, 1968, 1971 and 1979).

De Virgillio *et al* (1992) have since identified the gene *ACS1* which is glucose repressed and from its start codons may code for two different products. Expression of the gene may be induced by ethanol, acetaldehyde or acetate and its product may be targeted to peroxisomes (on the basis of its N-terminal sequence, SVKL). The gene product appears to correspond to the 'aerobic' form of ACS described by Satyanarayana

et al (1974). Disruption of the *ACS1* gene resulted in an inability to grow on acetate. However, mutants were able to grow on ethanol, suggesting that alternative pathways to ethanol exist under these conditions.

Van den Berg and Steensma (1995) have identified the gene encoding the cytosolic or 'anaerobic' form of ACS (*ACS2*). The product of this gene is constitutive and mutants lacking the gene cannot grow on glucose, suggesting that insufficient cytosolic acetyl-CoA is produced in its absence. As the enzyme is part of the PDH bypass, the lack of growth would seem to be consistent with the role of this pathway in generating cytosolic acetyl-CoA.

4.3 Mg^{2+}-acetaldehyde dehydrogenase

Far less attention has been paid to this enzyme than to any other of the PDH bypass. A partial purification procedure has been available for the enzyme for many years (Seegmiller, 1953, 1955), but a lack of homogeneity and quality of the preparation has left the enzyme poorly characterised. This has also hindered genetic studies of the enzyme. The Mg^{2+}-ACDH is activated by the divalent cations Ca^{2+} and Mg^{2+}, is located in the cytosol and is $NADP^+$ -dependent. Under fermentative conditions, the NADPH that is generated by this enzyme is used in the biosynthesis of fatty acids (Llorente and de Castro, 1977). Mauricio and Ortega (1993) suggested that the enzyme is constitutive and reported that it is operative during aerobic and anaerobic growth.

Most physiological studies of the Mg^{2+}-ACDH have worked with wine yeasts grown on grape musts (Millan and Ortega, 1988; Mauricio and Ortega, 1993; Mauricio *et al*, 1997) and have merely confirmed the roles proposed by Llorente and de Castro (1977) i.e. that the enzyme is part of a pathway that offers an alternative route to acetyl-CoA in the cytosol.

The only genetic work on this ACDH has used mutants generated by random

mutagenesis (Kurito and Ito, 1994). However, only mutants partially deficient in ACDH activity were isolated. They showed a lack of ethanol utilisation and a decrease in growth rate on glucose. However, the mutants were poorly characterised and the subcellular localisation of ACDH activity was ambiguous. However, this is the first study to propose a role for the Mg^{2+}-ACDH in ethanol catabolism.

More recently, a purification procedure has been developed for the enzyme in this laboratory (Dickinson, 1996). This has allowed a more accurate kinetic analysis, which has shown that there is no absolute requirement for divalent cations if a high concentration of $NADP^+$ is used. The analysis has also demonstrated that disulfiram (a common aldehyde dehydrogenase inhibitor) inhibits Mg^{2+}-ACDH activity. N-terminal sequencing of the protein has also enabled identification of the gene encoding the Mg^{2+}-ACDH (this study).

4.4 K^+-acetaldehyde dehydrogenase

4.4.1 Traditional role of the K^+-ACDH

Far more attention has been paid to this enzyme, which was first described by Black (1955). It is active with NAD^+ (and to a lesser extent $NADP^+$) and is dependent on monovalent cations such as Na^+ and K^+. Jacobson and Bernofsky (1974) reported its location as mitochondrial and proposed that the enzyme plays a role in the oxidative metabolism of ethanol in mitochondria. A purification procedure was reported by Steinman and Jakoby (1967) but the preparation was subject to proteolysis. The use of PMSF (a protease inhibitor) by Bostian and Betts (1978) and fast processing of extracts enabled a completely homogenous preparation of the enzyme.

Physiological studies have shown that the enzyme is strongly repressed by hypoxia and its activity is regulated by *de novo* synthesis (Llorente and de Castro, 1977). In addition, the K^+-ACDH is repressed by high concentrations of hexose, particularly

glucose (Llorente and de Castro, 1977).

Unlike the Mg^{2+}-ACDH, the gene for this enzyme has supposedly been randomly mutated and identified in the same study (Saigal *et al*, 1991). This group identified a gene named *ALD1* through genetic complementation by restoring the ability of mutants lacking the K^+-ACDH to grow on ethanol. Biochemical and immunoblotting studies were carried out by Saigal *et al.* in support of their identification of Ald1p as the mitochondrial, K^+-ACDH. However, no genetic analysis of the ACDH-negative mutants isolated by these workers was reported and systematic gene disruption or deletion experiments were not performed. This gene has not since been identified in the systematic sequencing of the yeast genome or by any other workers.

Another study has identified the gene encoding the K^+-ACDH of *S. cerevisiae*, but has not attempted to disrupt the gene. The study of the K^+-ACDH was by Larsson *et al* (1997) who published a method for separating proteins by 2-dimensional electrophoresis to then enable micro-sequencing of the separated products. To validate this method, they separated several proteins from *S. cerevisiae* and from the amino acid sequences of the separated products were able to identify the corresponding genes. One protein identified in the study was the K^+-ACDH which they deduced was encoded by the gene YOR374w. However, no gene disruption was performed in this study. This group has since registered the gene with the *Saccharomyces* Genome Database and it has been assigned the functional name *ALD7*.

A third group determined the N-terminal sequence of the commercially available K^+-ACDH but did not identify the gene (Chalmers *et al*, 1991). This sequence is consistent with the predicted product of the gene YOR374w.

4.4.2 Another role for the K^+-ACDH

Some workers have attributed a key role to the K^+-ACDH in the response of

yeast to osmotic stress. When a micro-organism encounters a medium of high osmotic pressure (low water activity) dehydration of the organism and inhibition of growth can result. Yeasts can overcome this by synthesising and accumulating compatible solutes (such as polyols) in the cytoplasm. This lowers the water activity of the cytoplasm compared to the environment. While some yeasts osmoregulate by accumulating more than one type of polyol, *S. cerevisiae* exclusively uses glycerol (Brown, 1978).

In producing glycerol, the enzymes of the anabolic pathway responsible for glycerol production are noticeably more active e.g glycerol-3-phosphate dehydrogenase (Blomberg and Adler, 1989). As a result of increased glycerol synthesis, large amounts of the cellular NADH reserve is used. So, to redress the NAD:NADH ratio, other changes take place. For instance, alcohol dehydrogenase (which utilises NADH in ethanol production) is repressed, whilst the activity of the K^+-ACDH (which regenerates NADH) is increased (Blomberg and Adler, 1989). As a result of increased K^+-ACDH activity, acetate is secreted into the external medium. A cytosolic enzyme might seem more likely to function in this role, since the NADH generated by the K^+-ACDH is mitochondrial. The formation of NADPH by the Mg^{2+}-ACDH is of no use, because no transhydrogenase has been detected in *S. cerevisiae*.

Interestingly, a gene named *ALD2* in *S. cerevisiae* has been proposed to encode a cytoplasmic aldehyde dehydrogenase (Miralles and Serrano, 1995). This was identified in a study of genes up-regulated by osmotic stress, in which *ALD2* exhibited maximum induction with 0.3M NaCl. This gene does not encode Mg^{2+}-ACDH.

5. Existence of the PDH bypass in brewing yeast

To the authors knowledge, no study has yet examined the role of the two ACDHs or the PDH bypass in a commercial brewing strain of yeast. Certainly no genetic investigations of this pathway have been undertaken because of the known

polyploid state of brewing yeast and the difficulty of working with yeast of this nature. One would expect the PDH complex to be repressed in yeast growing in a brewery wort because of (i) the absence of oxygen approximately 10 to 20 hours after pitching and (Figure 6) (ii) the repression of mitochondrial enzyme synthesis caused by the high concentrations of maltose (Figure 6). If the PDH complex is repressed then cytosolic acetyl-CoA must be formed by an alternative pathway such as the PDH bypass.

Cytosolic acetyl-CoA is important in brewing for two reasons (i) formation of sterols and fatty acids and (ii) assimilation of fatty acids into esters (Nordstrom, 1965). Fatty acid synthesis takes place throughout a fermentation but unsaturated fatty acids are only produced in the presence of oxygen (Sumper, 1974). Esters are the most flavour-active compounds present in beer and have a tremendous effect on beer taste and quality (Rose and Harrison, 1993). Thus, any factors governing production and consumption of acetyl-CoA will have an effect on beer quality.

The intracellular concentration of acetyl-CoA is known to increase late on in a fermentation (Quain, 1988). This correlates well with the increased rate of ester synthesis. Ester synthesis has been proposed to fine-tune the acetyl charge within the cell i.e. a way of controlling the relative concentrations of acetyl-CoA and 'free' CoA (Hampsey and Kohlaw, 1981).

To date, the role of the acetaldehyde dehydrogenases and the PDH bypass in producing cytosolic acetyl-CoA in brewing yeast is unknown.

6. Summary and aims of the investigation

At the outset of this investigation, the contribution to acetyl-CoA production and nature of the PDH bypass was poorly understood. It was thought that only under certain growth conditions (e.g. fermentative), this pathway might be responsible for cytosolic acetyl-CoA production in *S. cerevisiae*. The initial aim of this work was to therefore

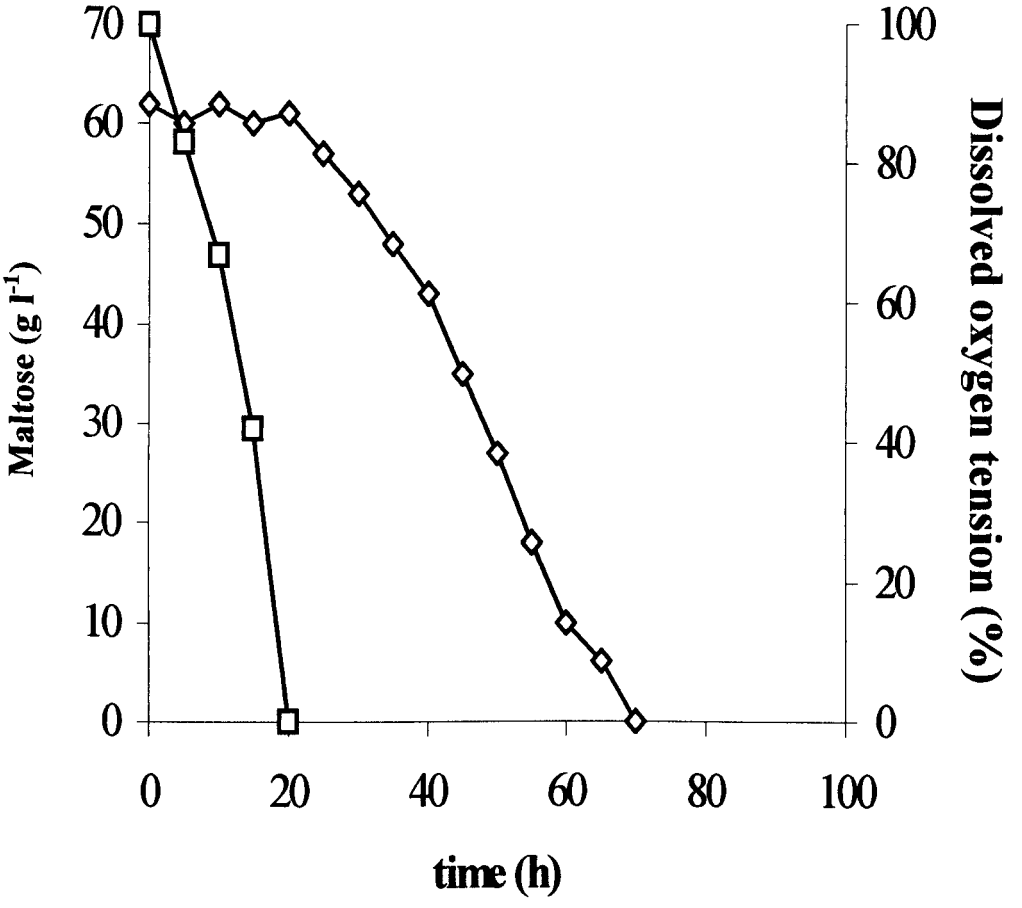

Figure 6: Disappearance of dissolved oxygen (-□-) and maltose (-✧-) during fermentation of an ale wort of original gravity 1.040. Dissolved oxygen relates to air saturation. Reproduced from a review by Boulton (1991).

study the enzymes that constitute the PDH bypass under a range of growth conditions. This work was to be performed in batch fermenters, using defined medium where possible and using a range of *S. cerevisiae* strains. These included laboratory strains and commercial brewing strains.

The contribution of CAT to cytosolic acetyl-CoA production was also to be investigated during growth under defined conditions. This enzyme is mainly expressed during growth on C2 compounds, but it was intended that its role during growth on hexoses could also be investigated. This may well confirm the work of Claus *et al* (1983).

Of all the components of the PDH bypass, the Mg^{2+}-ACDH has received the least attention. Hence the second aim of this work was to characterise the role of this enzyme by constructing mutant strains lacking the gene. Identification of the gene was to be made possible by working out the DNA sequence from the 15 N-terminal amino acid sequence. This was determined analytically from a preparation of the enzyme purified in this laboratory (Dickinson, 1996).

In contrast to the Mg^{2+}-activated enzyme, the K^{+}-ACDH was seemingly well characterised. Its main role of ethanol catabolism has been established by several workers and the gene has been identified as *ALD1* or *ALD7*. However, the origin and identity of the gene for this protein remained unclear and necessitated further investigation. This would allow a functional characterisation of the two ACDHs.

Finally, it was envisaged that the conclusions derived from a study of the two ACDHs could be applied to the metabolism of yeast grown on a conventional brewery wort. This would form the basis for two areas of investigation (i) the role of the PDH bypass in brewing yeast and (ii) the response of mutants altered in their central metabolism to growth in a conventional brewery wort.

MATERIALS AND METHODS

1. Organisms, media and growth conditions

1.1 Organisms

Saccharomyces cerevisiae D22 and CBS 8066 were obtained from laboratory stocks. *Saccharomyces cerevisiae* BB1, BB3, BB7, BB9, BB10, BB11, BB16 and BB18 were kindly provided by Bass Brewers Ltd, Burton-on-Trent, UK. *Saccharomyces cerevisiae* Y41 was obtained from the American Type Culture Collection (ATCC, Rockville, USA). *Saccharomyces cerevisiae* YPH499 was provided by Dr. P.G. Meaden, Heriot Watt University, Riccarton, Edinburgh, UK. Mutant strains RY124 to RY127 and RY270 to RY275 were derived from the diploid yeast strain YPH501 as described in Appendix 1. All strains were stored in 20% glycerol (w/v) stocks at - 80°C and used from working stocks on YEPD (with adenine added at 100 mg l^{-1} for YPH499 and mutant strains) agar plates stored at 4°C.

1.2 Media

1.2.1 Preparation of Media

Growth of yeast strains was either on YEPD or YNBD. YEPD consisted of 1% (w/v) yeast extract, 2% (w/v) proteose peptone and 2% (w/v) glucose. YNBD consisted of YNB (0.67% (w/v) yeast nitrogen base (Difco, Detroit, MI)) and 2% (w/v) glucose. For strain YPH499 and mutant strains, the nutritional supplements adenine, histidine, leucine, lysine, tryptophan and uridine were added to YNB at 100 mg l^{-1}. For solid media, bacteriological agar was added at 1.5% (w/v).

Where growth on a carbon source other than glucose was required, the glucose in YEPD or YNBD was replaced by the required carbon source. Where growth on basal YEP or YNB was required, the glucose or other carbon source was omitted. Unless

stated otherwise, shake flask cultures were grown on a gyrorotary shaker (145 rpm).

1.2.2 Sterilisation of media

Medium was sterilised by autoclaving at 121°C for 15 min in a bench top autoclave or under the same conditions but with 5 min 'free steaming' in a larger free standing autoclave. Volatile substances were not autoclaved. Ethanol was added directly to media as a 70% (v/v) stock solution and acetaldehyde was first filter sterilised using a disposable 0.22 μm micropore filter (Acrodisk, Gelman, MI).

1.3 Growth

1.3.1 Growth trials

Growth trials for all strains were conducted in 250 ml shake flasks containing 50 ml of YEP or YNB with the required carbon source. Trial cultures were grown for 48 h at 30°C by introducing a 1% (v/v) inoculum of starter culture. Starter cultures were grown on YEP with 0.5% (w/v) glucose for 36 h at 30°C .

1.3.2 Batch fermentation

Batch fermenter cultures were grown at 30°C in 1 litre Biolab minifermenters (B. Braun Biotech, Melsungen, Germany) with 800 ml working volume. The stirrer speed was maintained at 500 rpm and the dissolved O_2 tension kept above 50% of air saturation (monitored with an autoclavable galvanic probe). Air was delivered at 300 ml min^{-1} through a sterile air filter. pH was maintained at 5.0 $\pm$0.1 by automatic titration of 1 M KOH. Foaming was controlled by addition of polyglycol P2000 antifoam (Bevaloid Ltd, Beverley) as and when required.

Where anaerobic conditions were needed, air was replaced by nitrogen (containing less than 5 p.p.m O_2). Prior to inoculation, the fermenter was flushed with nitrogen at a flow rate of 1.2 litres min^{-1} for 30 min and a flow of N_2 was maintained at 0.15 litres min^{-1} thereafter. To minimise diffusion of O_2 into the system from the air,

neoprene tubing was used. Sampling from the fermenter was enabled by allowing the positive pressure inside the vessel to forcibly eject the required volume of culture into a sterile universal bottle.

1.3.3 Growth curves

Growth curves, were performed either in 1 litre shake flasks containing 200 ml medium or in batch fermenters as described (1.3.2). Starter cultures were grown as in section 1.3.1 and were harvested aseptically at room temperature by centrifugation, in bottles which had been sterilised by standing them for 24 h filled with 70% (v/v) ethanol. After being washed once aseptically with sterile deionised water, cells were then transferred to a sterile universal bottle. The optical density (at 540 nm) of this cell suspension was measured. An appropriate volume of the suspension was then aseptically transferred to the flask or fermenter so as to arrive at a starting optical density of 0.1 (unless otherwise indicated).

Growth was monitored by removing a suitable volume of culture under aseptic conditions at 1.5h intervals. Maximum specific growth rates (μ_{max}) were determined by plotting the natural logarithm of optical density against time and obtaining the gradient of the best straight line.

1.3.4 Brewery fermentations

The following work was carried out at the Bass brewery, Burton-on-Trent, UK. BB1 was grown in a stainless steel cylindroconical fermenter (working volume 850 litres) as part of a standard pilot scale commercial lager fermentation. Yeast cells for the fermentation were taken from yeast collection vessels in the main brewery and were acid washed (by addition of phosphoric acid) prior to pitching into the vessel. The wort was prepared according to Bass Brewery, Burton-on-Trent, pilot plant standard practices so as to arrive at a starting gravity of 1060°. Prior to pitching, the wort was oxygenated to

27 ppm (1.7 mM). The yeast used were 8th generation and were pitched into the vessel to arrive at 1.3 x 10^7 viable cells ml^{-1}. The vessel temperature was linearly increased during the fermentation from 12°C to 15°C.

Samples were removed by 'dipping' into the vessel from above. After centrifugation at 5,000 *g*, cells were processed as described in section 3.1 and supernatants were frozen at - 20°C for further analysis.

1.3.5 Incubation experiments

These can be divided into three different types of experiment.

1.3.5.1 Short term ethanol incubation experiments

A starter culture consisting of 50 ml YNB + 0.5% (w/v) glucose in a 250 ml flask was grown for 36 h at 30°C. The 50 ml culture was then transferred to a 3 litre flask containing 450 ml YNB + 0.5% (w/v) glucose and grown for 16 h under the same conditions. This culture was then harvested aseptically and washed as described in section 1.3.3.

A 500 ml shake flask containing 100 ml YNB + 1% (v/v) ethanol and one containing 100 ml YNB + 0.5% (w/v) glucose were then inoculated separately with the above starter culture to arrive a starting optical density of 2.0. The cultures were then incubated for 2 h at 30°C and at suitable time intervals, a 2 ml culture sample was removed from each flask. This was centrifuged at 13,000 rpm for 5 min in a Micro Centaur bench top microfuge and the supernatant retained and frozen at - 20°C for analysis of acetate and acetaldehyde.

1.3.5.2 Long-term incubation experiments

An inoculum was prepared from a starter culture as described in section 1.3.5.1. A 1 litre shake flask containing 200 ml YNB + 1% (v/v) ethanol was then inoculated to arrive at a starting optical density of 1.0. A cell-free extract was prepared from the

remaining inoculum as described in section 3.1 to enable enzyme assays to be conducted. The inoculated culture was incubated overnight at 30°C and harvested after 16 h by centrifugation, after which a cell-free extract was prepared. An optical density measurement was also obtained for the incubated culture to measure the extent of any growth. Where cells were incubated in medium containing a carbon source other than ethanol, the same protocol was adopted.

1.3.5.3 NaCl conditioning experiments

These experiments were performed essentially as Blomberg *et al* (1989). A starter culture was grown as described in section 1.3.5.1 and was harvested by centrifugation at room temperature. Two 1 litre shake flasks were then inoculated with the cell suspension to a starting optical density of 0.5. One flask contained 200 ml YNB + 0.5% (w/v) glucose + 0.7 M NaCl and the control flask contained 200 ml YNB + 0.5% (w/v) glucose. The two flasks were then incubated at 30°C for 1 h. Samples (1 ml) were withdrawn at 20 min intervals. These samples were centrifuged at 13,000 rpm for 5 min in a Micro Centaur bench top microfuge and the supernatant retained and frozen at - 20°C for analysis of acetate. Cell-free extracts were prepared (as described in section 3.1) from the remaining inoculum and from incubated cells.

1.3.6 Cell viability measurements

A starter culture was grown and a sterile inoculum prepared as described in section 1.3.5.1 (but with the change that all starter volumes were scaled down to 10% of that previously described). Two 100 ml shake flasks were then inoculated with the cell suspension to a starting optical density of 1.0. One flask contained 20 ml YNB + 1.0% (v/v) ethanol and the control flask contained 20 ml YNB basal medium. The two flasks were then incubated at 30°C for 20 h, and 100 µl samples were withdrawn at 0, 4 and 20 h. Samples were diluted appropriately in sterile deionised water and plated onto

YEPD agar containing adenine at 100 mg l^{-1}. Agar plates were incubated for 2 to 4 days at 30°C and the number of colony forming units (CFU's) was counted.

1.3.7 Monitoring of growth

Growth was monitored by reading the optical density at 540 nm of a suitably diluted culture sample so that a reading of between 0.1 and 0.4 was obtained. Readings were done on a Pharmacia Novaspec II spectrophotometer in disposable plastic cuvettes. The machine was always first set to zero with water. When required, the cell biomass was then calculated from a calibration curve of optical density vs cell dry weight (mg ml^{-1}). For the strains used in this work, the dry weights equivalent to an optical density of 1.0 fell within the range of 0.42 to 0.63 mg ml^{-1} .

The calibration curve was established by measuring the turbidity of a series of dilutions of yeast in deionised water. A known volume of known optical density was then centrifuged at 5,000 *g*. The supernatant was carefully decanted and the sides of the tube dried and the pellet resuspended in deionised water to the initial volume. Portions of the cell suspension were quantitatively transferred to tared glass vials previously dried to constant weight. The vials containing the cell suspension were returned to an oven at 108° and dried until constant weight was once more achieved.

2. Analyses

2.1 Acetaldehyde

Acetaldehyde concentrations were determined spectrophotometrically by measuring the total change in absorbance at 340 nm using an enzymic method (Lundquist (in Bergmeyer, 1974)) involving NAD^+ and acetaldehyde dehydrogenase (ACDH). The assay consisted of 1 ml standard / sample (containing between 1 and 10 µg acetaldehyde); 0.5 ml NAD^+-buffer mixture and 75 mU ACDH. The NAD^+-buffer mixture consisted of 0.57 M Tris / HCl pH 8.0; 4.75 mM EDTA, 0.28 M KCl and 0.72

mM NAD^+.

2.2 Acetic acid

Acetic acid concentrations were determined using a Boehringer Manheim enzymic test kit.

2.3 Carbohydrate

Total carbohydrate was determined using the method of Dubois *et al* (1956). To a 1 ml sample containing 10 to 80 μg aldose, 1 ml 0.53 M phenol was added in acid washed test tubes. To this solution, 5 ml concentrated sulphuric acid was added, left for 10 min and subsequently transferred to a water bath (25°C to 30°C) for 20 min. The hexoses were then measured at 490 nm against a reagent blank. Results were compared to a standard curve constructed using glucose (0-100 μg).

2.4 Ethanol

Ethanol concentrations were determined using a spectrophotometric assay which included commercially available alcohol dehydrogenase (ADH) and NAD^+ (Dickinson & Dalziel, 1967). The assay consisted of 0.9 M Tris buffer pH 10.3; 1.5 mM NAD^+; 100 to 500 units ml^{-1} ADH and 130 μM ethanol / sample (containing between 0.1 mM and 1 mM ethanol). Using an ATI Unicam 8625 UV / VIS spectrophotometer (set to zero with water) the total change in absorbance at 340 nm was monitored on addition of ethanol. The reaction typically took 10 min for completion.

The concentration of ethanol in a sample was determined by using the total change in A_{340} and the extinction coefficient for NADH at 340 nm of 6,220 M^{-1} cm^{-1} to calculate the concentration of NADH produced. This was equal to the concentration of ethanol in the assay.

2.5 Pyruvic acid

Pyruvic acid concentrations were determined using a Boehringer Manheim

enzymic test kit.

2.6 Samples from brewery fermentations

Concentrations of the following compounds were determined by the Analytical Services Laboratory, Bass Technical Centre, Cross St, Burton-on-Trent, UK using gas liquid chromatography: diacetyl, ethyl acetate, iso-amyl acetate, iso-butyl acetate, ethyl butyrate and ethyl hexanoate.

3. Treatment of cells

3.1 Harvesting of cells and preparation of cell-free extracts

Prior to harvesting, all cultures were microscopically examined for purity. Cells were usually harvested during active growth (unless otherwise stated) by centrifugation in an MSE 6L centrifuge. Cells were spun at 5,000 *g* and 4°C for 10 min and washed in 50 mM Tris / HCl buffer pH 7.5, before final resuspension in 5 to 25 ml of a buffer of the same composition (cells from brewery fermentations were washed twice in a buffer consisting of in 50 mM Tris / HCl buffer pH 7.5 with 5% (w/v) glycerol). Cells were then disrupted by two passages through a pre-cooled French pressure cell (35 MPa). The crude extract was then clarified by centrifugation at 8,000 *g* for 15 min and was used directly for enzyme assay.

Cells from brewery fermentations were frozen at - 20°C after resuspension in buffer and stored until thawed for passage through the French press.

3.2 Determination of protein concentration

3.2.1 Biuret method

For determining the protein concentration of extracts prepared from fermenter-grown and brewery-grown cells, the biuret method was used. To a 1 ml solution containing 1 to 10 mg ml^{-1} of protein, 4 ml of biuret reagent was added, mixed and allowed to stand at room temperature for 30 min. The extinction of the mixture was

then read at 550 nm against a blank prepared using water instead of protein-containing solution. The protein concentration of the sample was then obtained by reference to a calibration curve prepared using standard solutions of bovine serum albumin (BSA).

Biuret reagent was prepared as follows: 1.5 g and 6.0 g of $CuSO_4.5H_20$ and sodium potassium tartrate respectively were dissolved in 500 ml water. To this was added 300 ml 10% (w/v) NaOH with constant swirling, before diluting the entire mixture to 1 litre and storing in a polythene bottle.

3.2.2 Bradford method

For determining the protein concentration of most extracts and for the use in purification studies the dye binding method of Bradford (1976) was adopted. A novel step was included which aimed to solubilise membrane associated protein. The step involved adding 150 µl 0.2M NaOH to a 150 µl sample (containing less than 30 µg protein). 3 ml Bradford reagent was then added to this mixture and the A_{595} read within 2 to 3 min of mixing. A blank containing water instead of protein sample was used as a reference. The protein concentration of the sample was then obtained by reference to a calibration curve prepared using standard solutions of BSA. Such standards contained 5 to 30 µg protein per 150 µl sample.

Bradford reagent was prepared as follows: 100 mg brilliant blue G250 was dissolved in 50 ml 95% (v/v) ethanol, 100 ml 85% (w/v) phosphoric acid was added and the mixture was made up to 1 litre with deionised water.

4. Enzyme assays

Reaction rates were linearly proportional to the amount of extract added unless stated to the contrary.

4.1 Aldehyde dehydrogenase assays

The aldehyde dehydrogenases were assayed fluorimetrically at 25°C using a filter

fluorimeter, as designed by Dalziel (1962), set to give full-scale deflection with 2 μM-NAD(P)H. Excitation wavelength was 366nm; emission wavelength was 460nm.

4.1.1 Mg^{2+}-ALDH [EC 1.2.1.4; Mg^{2+}-activated, $NADP^+$ linked, cytosolic aldehyde dehydrogenase] was assayed under the following conditions: 50 mM Na-Hepes buffer pH 7.5, 16.25 μM $NADP^+$, 3.75 mM $MgCl_2$ and 125 μM acetaldehyde. Reactions were initiated by the addition of acetaldehyde.

4.1.2 K^+-ALDH [EC 1.2.1.5; K^+-activated, $NAD(P)^+$ linked, mitochondrial aldehyde dehydrogenase] was assayed under the following conditions: 62.5 mM Tris / HCl buffer pH 8.0, 375 μM NAD^+, 0.75 mM dithiothreitol (DTT), 62.5 mM KCl, 1 mM pyrazole, 5 mM NaN_3 and 1.5 mM acetaldehyde. Reactions were initiated by the addition of acetaldehyde.

4.2 Pyruvate decarboxylase [EC 4.1.1.1; pyruvate decarboxylase] was assayed by the procedure of Holzer and Goedde (1957) under the following conditions: 10 mM citrate buffer pH 7.5, commercial ADH (100 units ml^{-1}), 5 mM $MgCl_2$, 300 μM NADH, 200 μM TPP (thiamine pyrophosphate), 5 mM pyruvate. Assays were performed at 30°C using a spectrophotometer set to zero with assay buffer. Reactions were initiated by the addition of pyruvate and the rate of oxidation of NADH monitored at 340 nm.

4.3 Alcohol dehydrogenase [EC 1.1.1.1; alcohol oxidoreductase]. This was measured by two different methods. In extracts prepared from cells that were grown in a brewery fermentation, an attempt was made to measure the ADH I isozyme activity by the method of Dickinson (personal communication). This assay consisted of 10 mM phosphate buffer pH 7.0, 5 mM NaN_3, 150 μM NADH and 5 mM acetaldehyde. Assays were performed at 30°C using a spectrophotometer set to zero with assay buffer. Reactions were initiated by the addition of acetaldehyde and the rate of oxidation of NADH was monitored at 340 nm.

All other alcohol dehydrogenase assays were performed as described by Lutstorf and Megnet (1968): 30 mM pyrophosphate buffer {containing 1 mM EDTA (ethylenediaminetetra-acetic acid)} pH 8.8, 0.8 mM NAD^+, 1 M or 5 mM ethanol. Reactions were assayed fluorimetrically at 25°C using a filter fluorimeter, as designed by Dalziel (1962), set to give full-scale deflection with 2 μM-NAD(P)H.

Total alcohol dehydrogenase was also determined by way of activity gel electrophoresis. This was performed essentially as described in section 6.3 with the following modifications. SDS was excluded from all steps. Untreated cell-free extracts were loaded onto gels instead of pure proteins or marker proteins. After removal from between the glass plates, gels were submerged in 21 ml activity stain consisting of (i) 20 ml 30 mM pyrophosphate buffer {containing 1 mM EDTA), (ii) NAD^+ (20 mg), (iii) 500 μl nitro blue tetrazolium (NBT), containing 50 mg NBT dissolved in 70% dimethyl formamide (DMF), (iv) 200 μl 1:10 dilution phenazine methosulphate (PMS) solution (1 mg ml^{-1}), (v) 200 μl absolute or 81 mM ethanol.

4.4 Acetyl-CoA synthetase [EC 6.2.1.1; acetyl-CoA synthetase] was assayed by the procedure of Berg (1956). The discontinuous assay consisted of 60 μl 100 mM ATP pH 7.0, 60 μl 100 mM $MgCl_2$, 60 μl 100 mM K^+-acetate, 60 μl 1 mM CoA, 60 μl 2 M hydroxylamine pH 7.4 (prepared fresh weekly), 120 μl 200 mM K^+-phosphate buffer pH 7.4 and 0 to 180 μl enzyme or cell extract to a final volume of 0.6 ml. The reaction (performed in a 1.5 ml Eppendorf tube) was initiated by the addition of enzyme or cell extract and incubated at 30°C for 10 to 40 min. The reaction was terminated by the addition of 0.9 ml acid ferric chloride {10% $FeCl_3$ + 3.3% trichloroacetic acid (TCA) in 0.66 M HCl}. The mixture was centrifuged (Micro-Centaur) to pellet any precipitated protein and the A_{540} of the resulting supernatant measured against a blank of water (0.6 ml) and ferric chloride (0.9 ml).

Berg (1956) quotes a ΔA_{540} of 0.325 for 1 μmole of acetyl-hydroxamate, the final product of the reaction sequence. This is equivalent to an extinction coefficient of 812.5 M^{-1} cm^{-1}.

4.5 Carnitine acetyl transferase [EC 2.3.1.7; carnitine acetyltransferase] was assayed by a modified method of Ramsey & Tubbs (1975) under the following conditions: 25 mM K^+-phosphate buffer pH 7.8, 100 μM acetyl-CoA (prepared as described in section 5.0), 120 μM dithiodipyridine (DTP), 0.1 M KCl, and 2 mM L-carnitine. Assays were conducted at 30°C and monitored using spectrophotometer set to zero with assay buffer. Reactions were initiated by addition of L-carnitine and the increase in absorbance at 324 nm monitored.

4.6 ATP: citrate lyase [EC 4.1.3.8; ATP: citrate oxaloacetate lyase] was assayed at 30°C by the coupled procedure of Srere (1963) in which the oxaloacetate product is reduced to malate by the action of malate dehydrogenase, with the concomitant decrease in A_{340} due to the oxidation of NADH.

The assay consisted of 10 mM Tris / HCl pH 8.6, 1 mM DTT, 10 mM $MgCl_2$, 20 mM K^+-citrate, 14 mM coenzyme A, 5 mM ATP pH 7.0, malate dehydrogenase (5 units ml^{-1}), 3 mM NADH and 10 mM NaN_3. Reactions were initiated by the addition of CoA.

4.7 Glycerol-3-phosphate dehydrogenase [EC 1.1.1.8; glycerol-3-phosphate oxidoreductase] was assayed at 30°C as described by Blomberg & Adler (1989). The assay consisted of 20 mM imidazole / HCl buffer pH 7.0, 1 mM DTT, 1 mM $MgCl_2$, 45 μM NADH, 5 μM NaN_3 and 670 μM dihydroxyacetone phosphate (DHAP). The reaction was initiated with DHAP and the increase in NADH oxidation was monitored at 340 nm.

4.8 Citrate synthase [EC. 4.1.3.7; citrate oxaloacetate-lyase (CoA acetylating) was assayed by the method of M.Midgley (personal communication). The assay was

conducted at 30°C and consisted of 50 mM Tris /HCl pH 8.0, 250 μM 5' 5'dithionitrobis (prepared in 20 mM Tris / HCl pH 8.0), 100 μM oxaloacetic acid (prepared in 100 mM Tris / HCl pH 8.0), 100 mM acetyl-CoA (prepared as in section 5.0) and 10 mM NaN_3. The reaction was initiated by the addition of oxaloacetic acid and the increase in A_{412} measured.

4.9 Succinate dehydrogenase [EC 1.3.99.1; succinate oxidoreductase] was assayed by a discontinuous method modified from the procedure of Ackrell *et al*, (1978). The assay contained in a 1 ml volume: 40 mM K^+-phosphate buffer pH 7.4, 1 mg iodonitrotetrazolium violet (INT), 50 mM Na^+-succinate and 0.22 mg phenazine methosulphate (PMS). The reaction was initiated by addition of cell-extract or enzyme and incubated in glass stoppered tubes at 30°C in the dark. After a fixed time period (10 to 30 min), 1 ml of 10% (w/v) TCA was added, followed by 4 ml of ethyl acetate. The formazan salt (the product of the reaction) was then extracted into the ethyl acetate by vortexing the tubes for 2 x 10 sec. The absorbance of the mixture was then read at 490 nm against an ethyl acetate blank, using silica glass cuvettes and compared to an assay that did not contain succinate. The activity of the dehydrogenase was calculated using an extinction coefficient of 17,400 M^{-1} cm^{-1}.

4.10 Isocitrate lyase [EC 4.1.3.1; threo-D_s-isocitrate glyoxylate-lyase] was assayed by the procedure based on Kornberg (1965) (in Armitt *et al* (1976)). The reaction mixture consisted of 25 mM imidazole buffer pH 6.8, 5 mM $MgCl_2$, 1 mM EDTA, 4 mM phenylhydrazine HCl and 2 mM isocitrate. The reaction was initiated by the addition of isocitrate and the isocitrate-dependent change in A_{324} monitored at 30°C.

5. Preparation of acetyl-CoA

The following procedure produces acetyl-CoA at a concentration of approximately 12 mM. This solution can be diluted to its required concentration in

50 mM Hepes buffer pH 8.0. The protocol is as follows: in a screw-top Eppendorf tube, 5 mg coenzyme A, 125 µl 0.4 M potassium hydrogen carbonate, 375 µl deionised water and 7 µl acetic anhydride were mixed. This solution was left to stand in an ice-bucket for 10 to 15 min and then tested for the presence of free thiol groups by the addition of a small amount of 10 mM DTNB (prepared in 0.1 M Tris pH 8.0) to a sample of the preparation. If a yellow colour was observed, the reaction was not complete and more acetic anhydride was added.

6. Purification studies of the aldehyde dehydrogenases

6.1 Purification of the cytosolic, Mg^{2+}-ACDH

The cytosolic ACDH was purified from commercial bakers' yeast as described by Dickinson (1996) and partially sequenced from the N-terminus by Alta Bioscience, University of Birmingham, Birmingham, UK.

6.2 Purification of the mitochondrial K^{+}-ACDH

The mitochondrial ACDH was purified from *S. cerevisiae* strains YPH499, RY124, BB1, BB11 and from a commercial bakers' yeast by the method of Bostian and Betts (1978) except for the following modifications. Cells were inoculated from a stock plate into 200 ml YEP + 0.5% (w/v) glucose in a 1 litre flask and grown for 48 h at 30°C. Cells were then harvested and a cell-free extract prepared as described in section 3.1 except that the harvesting buffer was 10 mM potassium phosphate pH 7.5 containing 0.5 mM DTT and 5% (v/v) glycerol. The temperature used in the heat step for each strain differed slightly but fell within the range 50°C to 54°C (as determined by pilot experiments). The procedure used here did not include the second heat step, the acid precipitation step or the ammonium sulphate step. The DEAE step used in this work differed in that a 200 ml column was used instead of a batch adsorption process and the protein was eluted from the column stepwise by the addition of 10 mM

potassium phosphate pH 7.5 containing 0.5 mM DTT, 5% (v/v) glycerol and KCl. First one column volume of 50 mM KCl was used to elute inactive protein. This was followed by 350 mM KCl, which eluted the enzyme. The final affinity chromatography step was also omitted and instead replaced by an hydroxyapatite step. This was performed using a 5 ml mini-column attached to a Pharmacia Biotech FPLC system. The protein was eluted with a linear gradient of 10-400 mM potassium phosphate, pH 7.5 containing 0.5 mM DTT and 5% (v/v) glycerol. A total of 30 ml was collected at a flow rate of 1 ml min^{-1}.

This purification was suitable for small volumes of extract and was considerably faster than any previously published procedure, normally requiring 10 hours for completion. The speed of the process and the use of phenylmethanesulphonyl fluoride (PMSF) should serve to minimise any proteolysis of the enzyme since this is a well-known problem with K^+-ACDH (Clark & Jakoby, 1970). At the final stage, the purity of the protein was determined by sodium dodecyl sulphate (SDS) gel electrophoresis.

The purified K^+-ACDH was partially sequenced from the N-terminus by the Microchemical Facility, The Babraham Institute, Cambridge.

6.3 Gel electrophoresis

Gel electrophoresis was performed as follows: approximately 20 ml resolving gel was prepared consisting of 10 ml Protogel (containing 30% w/v acrylamide and 0.8% w/v *bis* acrylamide), 3.75 ml resolving buffer (3 M Tris / HCl pH 8.8), 14.5 ml deionised water, 0.3 ml SDS (10% w/v), 1.5 ml ammonium persulphate (1.5% w/v) and 15 µl temed (N,N,N`,N` - tetramethyl-ethylenediamine). The resolving gel was then injected in between two clamped, clear glass plates and allowed to set at room temperature. A comb was inserted into the top of the plates and the stacking gel {consisting of 3.5 ml Protogel, 5 ml stacking buffer (0.5 M Tris / HCl pH 6.8), 10.3 ml deionised water, 0.2

ml SDS (10% w/v), 1 ml ammonium persulphate (1.5% w/v) and 15 µl Temed)} was then injected and left to set to allow the formation of loading wells. The gel was then inserted into a vertical gel electrophoresis tank (with a lower and upper reservoir) and both reservoirs filled with buffer (25 mM Tris, 0.19 M glycine and 0.1% (w/v) SDS).

Purified protein samples were mixed 10:1 (v/v) with a solution of diluent (consisting of 10 ml stacking buffer, 1 ml glycerol, 0.2 g SDS, 0.5 ml β-mercapto ethanol and 0.2 mg bromophenol blue dye) and 100:1 with undiluted β-mercapto-ethanol (βME), before boiling for 2 min. A solution (2 mg ml^{-1}) of molecular weight markers (including myosin, β -galactosidase, phosphorylase b, BSA, ovalbumin and carbonic anhydrase) was also mixed 10:1 with diluent and boiled for 2 min. Standards were loaded at 15 µl per well and purified samples were loaded at 10 to 50 µg protein (as determined by the Bradford method; section 3.2.1) per well. Gels were then run at 20 mA constant current and at 4°C until samples could be seen to approach the bottom of the resolving gel.

After removal from between the glass plates, gels were then stained overnight in Coomassie blue brilliant dye (prepared with 2.5% w/v dye in destain, consisting of 40% v/v methanol and 7% v/v acetic acid) and subsequently destained for 24 h. By this time, any protein bands were fully visible.

6.4 Separation of the two acetaldehyde dehydrogenases

A method was developed which enabled separation of the two ACDHs from *S. cerevisiae*. Cells were grown by inoculating from a stock plate into 200 ml YEP + 0.5% (w/v) glucose in a 1 litre flask and incubated at 30°C until the stationary phase of growth. Cells were then harvested and a cell-free extract prepared as described in section 3.1 except that the harvesting and buffer was 10 mM sodium phosphate pH 7.5 containing 0.5 mM DTT and 5% (v/v) glycerol. Extracts were dialysed against

extraction buffer and ultra-centrifuged for 1 h at 45,000 g (using a Beckman L8-M preparative ultra-centrifuge). Fast protein liquid chromatography (FPLC) of the resulting extract was carried out on a Pharmacia Biotech system using a hydroxyapatite mini-column (5 ml; Bio-Rad, Hercules, CA). Proteins were eluted using a two-stage gradient of sodium phosphate buffer (10 to 400 mM pH 7.0) containing 1 mM DTT and 5% (w/v) glycerol. The first 30 ml of the gradient was passed through at a flow rate of 1 ml min^{-1} (10 to 300 mM). In the second phase of the gradient, (300 to 400 mM) 4.5 ml was collected at the same flow rate.

6.5 Sequence analysis and software

Homology searches were conducted using FASTA and TFASTA (Pearson and Lipman, 1988). A TFASTA search of the Genbank database (release 93.0) was made through the SEQNET facility (Daresbury, UK). FASTA was used to search databases of translations of all standard *S. cerevisiae* open reading frames (ORFs) maintained by the *Saccharomyces* Genome Database (SGD; http://genome-.www.stanford.edu/ Saccharomyces/).

The following analysis was carried out by Dr P.G.Meaden (Heriot-Watt University, Riccarton, Edinburgh, UK). CLUSTAL W (Thompson et al; 1994) was used to align amino acid sequences, and to produce a data file for the construction of a phylogenetic tree with TreeView version 1.3 (Page, 1996; from R. Page, Institute of Biomedical and Life Sciences, University of Glasgow, UK; http://taxonomy.zoology. gla.ac.uk/rod/treeview.html). Prediction of mitochondrial targeting of proteins (Nakai and Kanehisa,1992) was carried out using PSORT (http://psort.nibb.ac.jp/).

7. Respiration studies

Rates of O_2 consumption were measured using a 3 ml perspex reaction vessel, maintained at 30°C by means of a water jacket, and fitted with a Clark-type oxygen

electrode (Model 777, Beckman Instruments) attached to a potentiometric recorder (Servoscribe).

Reaction mixtures consisted of 2.9 ml 20 mM 2-(N-morpholino) ethane sulfonic acid (MES) pH 6.0 and 30 µl pyruvate, acetate, ethanol, glucose or acetaldehyde at the following concentrations; 1 M, 0.1 M or 0.01 M. 0 to 100 µl yeast cell suspension (in 20 mM MES buffer) was also added to the reaction vessel.

Cells were harvested during active growth by centrifugation and washed in MES buffer before final resuspension to a volume of approximately 1 ml. The optical density of the suspension at 540 nm was then measured so that the equivalent dry weight (mg ml^{-1}) of the suspension could be calculated from the value obtained in section 1.3.7. Thus it was possible to calculate the dry weight of cells being introduced to the reaction mixture. After the electrode drift (that is the rate obtained without any yeast or substrate) was established as sufficiently low (< 1% min^{-1}), the yeast cells were added using a micro-syringe and after a steady endogenous rate had been reached, the substrate was introduced to the reaction chamber. The reaction chamber was washed thoroughly with deionised water between assays to avoid carry-over of substrate or yeast.

Respiration rates were measured in terms of O_2 consumed min^{-1} (mg dry weight cells)$^{-1}$. A value of 0.24 mM was used for the solubility of O_2 in MES buffer at 30°C.

8. Mitochondrial preparation

8.1 Growth and harvesting of cells

A method was used which was based on that of Bruinenberg *et al* (1985). Cells were grown as described in section 1.3.5.1, harvested by centrifugation and washed in Buffer A (consisting of 0.1 M Tris buffer pH 9.0 and 2.5 mM DTT). After washing, cells were resuspended in Buffer A at 5 ml / g cells before being incubated at 28°C for 20

min.

8.2 Sphaeroplast formation (digestion of cell walls)

Cells were then centrifuged at 12, 000 *g* for 10 min and washed in Buffer B {consisting of 5 mM 3-[N-morpholino] propanesulphonic acid (MOPS) buffer pH 7.2, containing 2 M sorbitol and 1 mM EDTA}. After a second spin at 12,000 *g*, cells were resuspended in Buffer B (to 2 ml / g cells), containing 1% (v/v) βME and lyticase (1 mg ml^{-1}).

Cells were then incubated in a shaking water bath and samples removed at 10 min intervals. Samples were diluted 100 fold (i) in Buffer B and (ii) in deionised water and the optical density determined. As sphaeroplast formation progressed, cells lysed upon exposure to water and so the optical density decreased. Monitoring those cells diluted in Buffer A, which contained sorbitol (and so stabilised osmolarity) confirmed the extent of spontaneous lysing. At approximately 60% sphaeroplast formation, spontaneous lysing increased and so incubation with lyticase was terminated.

8.3 Disruption of the plasma membrane by dialysis

Following incubation with lyticase enzyme, cells were centrifuged at 3,000 *g* for 10 min and washed in Buffer B. This separated sphaeroplasts from cells. The step was repeated with the supernatant (which contained the sphaeroplasts) and the final resuspension of sphaeroplasts was made up to 20 ml. This suspension was poured into a dialysis bag and dialysed against 5 litres Buffer B (without sorbitol) at room temperature. A gradual reduction in sorbitol concentration by dialysis was favoured over stepwise reduction by Bruinenberg *et al* (1985). This was because, using the gradual reduction, larger amounts of mitochondrial marker enzymes were found in the mitochondrial fraction.

Disruption of sphaeroplasts was monitored by removing 0.5 ml samples at 10 min

intervals from the dialysis bag and centrifuging for 3 min at 6,500 rpm in a Micro-Centaur bench-top microfuge. The concentration of sorbitol remaining in the dialysed cell suspension was then determined by comparing the value obtained by using a refractometer to a calibration curve. Dialysis was allowed to continue until the sorbitol concentration in the dialysis bag had fallen to 0.9 M. This value was pre-determined by analysing release of pyruvate decarboxylase ((PDC); a known cytosolic enzyme (Perlman & Mahler, 1974)) from such cells. At this pre-determined point, the release of PDC reached a plateau and so it was decided to minimise organelle disruption (which may occur by prolonged dialysis) by terminating dialysis.

8.4 Differential centrifugation

The contents of the dialysis bag were centrifuged at 1,930 *g* for 15 min and the pellet was discarded. 1 ml of supernatant was retained to represent the 'total' fraction and the remaining supernatant was then centrifuged first at 12,000 *g* for 10 min to generate pellet 1 (P1), and then at 45,000 *g* for 20 min to generate pellet 2 (P2) and a final soluble fraction (S). Both pellets were then resuspended in a small volume of Buffer B.

8.5 Assessing the quality of mitochondrial preparations

The criterion used for assessment of quality was the respiratory control index (RCI) i.e. demonstration of respiratory control using the oxygen electrode. This was achieved by introducing 2.9 ml of Lambowitz (1972) buffer (consisting of 0.3 M sucrose, 8 mM NaH_2PO_4, 8 mM Tris, 5 mM $MgCl_2$ and 0.7 mM EDTA) pH 7.2 to the electrode chamber and allowing a basal rate to be established. Approximately 50 µg P1 or P2 was then introduced to the vessel and again the endogenous rate allowed to settle. 30 µl 1 M succinate was then added and after obtaining a steady rate, 30 µl 30 mM ADP also. The RCI was calculated by determining the ratio of activity after addition of

ADP to the activity prior to addition.

8.6 Location of acetaldehyde dehydrogenases

The activity of both the K^+-ACDH and Mg^{2+}-ACDH enzymes was measured in the subcellular fractions P1, P2, S and the 'total' fraction. The fractions were all disrupted by two passages through the French Press and then the activities determined. The activity of two marker enzymes was also measured as a control. These were citrate synthase (a 95% mitochondrially located enzyme (Chapman and Bartley, 1968)) and pyruvate decarboxylase (Perlman & Mahler, 1974).

RESULTS

1. Studies of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisae*

At the beginning of this work, neither the route of cytosolic acetyl-CoA production, nor the contribution to growth by the pyruvate dehydrogenase (PDH) bypass under various physiological conditions were fully understood. This section describes experiments carried out to measure some of the activities of the enzymes of this pathway, using batch cultures of *S. cerevisae*, grown under a range of different physiological conditions. Two different types of yeast were used; a laboratory strain and commercial brewing strains.

1.1 Assessment of the growth of laboratory and brewing strains on a range of carbon sources

Preliminary experiments were concerned with investigating which carbon sources the organisms could grow on. Table 1 shows the results obtained from this assessment using the procedures outlined in Materials and Methods section 1.3.1. Growth was investigated on YEP media.

Strain CBS 8066 grew poorly on glycerol and acetate, as did the brewing strains. Since growth of all the brewing strains was similar on the compounds tested, it was decided to choose two for further work. These were the ale yeast and lager yeast, BB1 and BB11 respectively.

1.2 Investigation of selected enzymes involved in the PDH bypass

It was decided to measure the activities of the enzymes involved in this route during batch growth on several carbon sources. Two different concentrations of glucose were used. 0.5% (w/v) glucose was chosen in order to minimise on the catabolite repressive effects on respiratory enzymes that glucose is noted for (Gancedo, 1992).

Table 1: Growth of laboratory and brewing *S. cerevisiae* strains on various carbon sources

Carbon source	CBS 8066	BB1	BB3	BB7	BB9	BB10	BB11	BB16	BB18
Acetate	+	+	+	+	+	+	+	+	+
Glucose (0.5%)	+++	+++	+++	+++	+++	+++	+++	+++	+++
Glucose (5%)	+++	+++	+++	+++	+++	+++	+++	+++	+++
Fructose	+++	+++	+++	+++	+++	+++	+++	+++	+++
Maltose	+++	+++	+++	+++	+++	+++	+++	+++	+++
Sucrose	+++	+++	+++	+++	+++	+++	+++	+++	+++
Galactose	+++	+++	+++	+++	+++	+++	+++	+++	+++
Glycerol	+	+	+	+	+	+	+	+	+
Ethanol (1%)	++	++	++	++	++	++	++	++	++

+ Poor growth, corresponding to a minimum dry weight of 0.35 mg ml^{-1} and a maximum of 1.0 mg ml^{-1}.

++ Good growth, corresponding to a maximum dry weight of 2.0 mg ml^{-1}.

+++ Excellent growth, corresponding to a maximum dry weight of 5.0 mg ml^{-1}.

All carbon sources present at 2% (w/v) unless otherwise stated.

Additionally, 5% (w/v) glucose was used to study the effect that catabolite repression could have on the operation of the PDH bypass route. The inclusion of 5% (w/v) glucose also offered a study of fermentative growth in the presence of oxygen (so called respiro-fermentative growth). True fermentative growth was made possible by anaerobic growth on 0.5% (w/v) glucose. Galactose (2% w/v) was utilised in an attempt to eradicate any effects of catabolite repression that glucose may have exerted. It was also interesting to investigate growth on a C2 compound and since growth of all strains was poor on acetate (Table 1), growth on ethanol was used.

CBS 8066 was grown in batch culture in defined medium (YNB) with the indicated carbon sources addded and harvested during the active phase of growth (Table 2). Although, YEP media was used for the growth trials (Table 1) cells also grew well on YNB media and gave rise to higher specific activities for the enzymes. Anaerobically grown cells were cultured on complex medium (YEP). This provided a supply of sterols and unsaturated fatty acids, since yeast require these when grown anaerobically (Andreasen and Stier, 1953, 1954). The cells were processed as described in Materials and Methods section 3.1 and the activities of the enzymes shown were measured in cell-free extracts. If acetyl-CoA is synthesised in the cytosol, it may be transported into mitochondria via the action of carnitine acetyltransferase (CAT) (Schmalix and Bandlow, 1993). Hence, the activity of this enzyme was measured. To test whether catabolite repression of respiratory enzymes was occurring under certain growth conditions, the activities of two mitochondrial enzymes were determined. One was an enzyme present in the TCA cycle; namely succinate dehydrogenase (SDH). The other was the K^+-ACDH.

One interesting observation was that of the absence of measurable activity for CAT in cells grown on 5% glucose. Under these conditions, the activities of the two

Table 2. Specific activities of selected enzymes in *S. cerevisiae* CBS 8066

Growth substrate	Specific activity [nmoles min^{-1} (mg protein)$^{-1}$]			
	Mg^{2+}-ACDH	CAT	K^{+}-ACDH	SDH
2% galactose	25	9	42	0.67
0.5% glucose	55	99	74	1.3
0.5% glucose *	70	01	17	0.24
5% glucose	27	0	24	0.12
1% ethanol	22	550	184	2.23

* Anaerobically grown

mitochondrial enzymes were also very low in comparison to the activities during growth on other substrates (especially ethanol). This would suggest that a cytosolic pool of acetyl-CoA cannot be formed by first being synthesised by PDH and then transported out of the mitochondrion by the action of CAT. Thus under these conditions, the PDH bypass may play a crucial role in the supply of cytosolic acetyl-CoA.

No firm conclusion regarding this can be made from the levels of CAT activity during growth on galactose or 0.5% glucose (aerobically or anaerobically). Certainly, one cannot be certain which CAT isozyme is active at any one time because the enzyme assay measures total activity. However, activities are considerably lower than that observed during growth on ethanol. The operation of the PDH bypass under these conditions is feasible because of the presence of the Mg^{2+}-ACDH.

The mitochondrial marker enzymes provided a good index for the degree of respiratory repression. This was apparent for SDH and the K^{+}-ACDH, from the difference in levels of enzyme activity between ethanol grown and 5% glucose grown cells. The results for brewing strains BB1 and BB11 are not shown but were similar to those observed for CBS 8066.

2. Identification and disruption of the *ALD6* gene of *Saccharomyces cerevisiae*

The three enzymes pyruvate decarboxylase (PDC), the Mg^{2+}-activated acetaldehyde dehydrogenase (Mg^{2+}-ACDH) and acetyl-CoA synthetase (ACS), together, catalyse the reactions of the PDH bypass in *S. cerevisiae*. This pathway has been implicated as the main route for production of cytosolic acetyl-CoA (Flikweert et al, 1996). Work on PDC and ACS (van den Berg & Steensma, 1995) mutants has confirmed this proposal. However, little or no work of a genetic nature has focused on the Mg^{2+}-ACDH. This has largely been due to the lack of a procedure to purify this enzyme to homogeneity (Seegmiller, 1953, 1955). It was thought that the cloning of the gene for this enzyme would provide valuable insight into the role of the gene product in cytosolic acetyl-CoA production.

The following experiments describe the identification and disruption of the gene from a wild-type, haploid strain (YPH499) and examine the nature of mutants lacking the gene.

2.1 Identification of the *ALD6* gene

Identification was made possible by using an N-terminal protein sequence consisting of 15 amino acids. This was obtained from enzyme purified in this laboratory from commercial bakers' yeast by Dickinson (1996). The N-terminal sequence was found to be TKLHFDTAEPVKITL and a TFASTA search of Genbank (release 93.0) found a perfect match between this sequence and amino acids 2 to 16 of the deduced translation product of an open reading frame (ORF) on the left arm of *S. cerevisiae* chromosome XVI. This gene has the systematic name of YPL061w and its encoded product shows homology to other members of the *S. cerevisiae* ALDH family (Bussey, 1997) (discussed in Results, section 3). The gene has now been registered with the *Saccharomyces* Genome Database (SGD).

2.2 Deletion of the *ALD6* gene

The method used to disrupt *ALD6* is described in Appendix 1. Verification that the gene had been deleted in transformants is described in Appendix 2. The deletion was carried out in the haploid host strain YPH499 and yielded four disruptants: RY124 to RY127.

2.3 Confirmation for the loss of the Mg^{2+}-ACDH

As part of the characterisation of the disruption mutants they were checked for activity of the Mg^{2+}-ACDH. Table 3 shows the activity for this enzyme and for the other ACDH (the K^+-activated mitochondrial ACDH) in crude extracts prepared from wild-type and mutant cells.

In addition to checking enzyme activity in crude extracts, the two ACDHs were separated. This ensured that (a) loss of Mg^{2+}-ACDH activity in the mutants was not due to the presence of an inhibitor or (b) that any measured K^+-ACDH activity was not masking small amounts of the Mg^{2+}-ACDH enzyme (since the K^+-ACDH is also active with $NADP^+$). Separation was made possible by using fast protein liquid chromatography (FPLC) with an hydroxyapatite mini-column as described in Materials and Methods (Section 6.4). Figure 7 shows an elution profile from the column. There is one activity peak for each of the ACDHs from wild-type cells but only that of the K^+-ACDH in mutant cells.

2.4 Assessment of growth rate in *ALD6* disruption mutants

The growth rate of the disruption mutants on glucose was compared to that of wild-type cells. Table 4A shows a significant reduction in maximum specific growth rate (μ_{max}) in shake flask for two haploid yeast strains deleted in *ALD6*. To verify that the results were not biased by the conditions encountered during shake flask growth, the same experiments were performed in aerated 1 litre fermenters (Table 4B) but only for

Table 3. ACDH levels in YPH499 (*ALD6*) and *ald6* yeast strains

Yeast strain	Specifc Activity [nmoles min^{-1} (mg protein)$^{-1}$]	
	Mg^{2+}-ACDH	K^{+}-ACDH
YPH499 (*ALD6*)	74 ±2	93 ±14
RY124 (*ald6*)	0	41
RY125 (*ald6*)	0	49
RY126 (*ald6*)	0	49
RY127 (*ald6*)	0	45

All cells were grown in complex medium containing 0.5% (w/v) glucose.

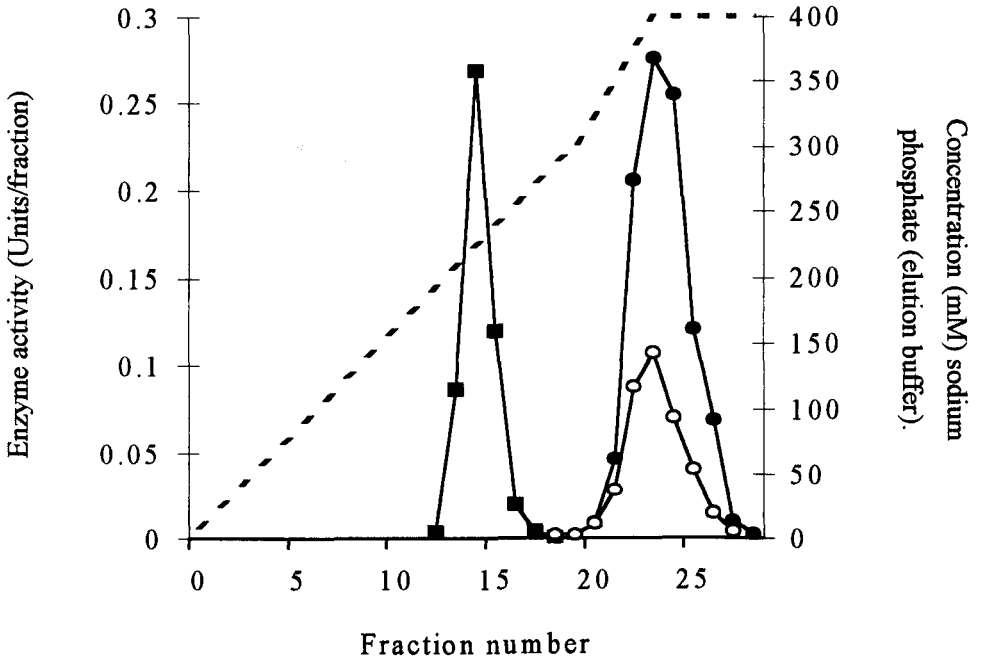

Figure 7: Separation of mitochondrial (K^+-activated) and cytosolic (Mg^{2+}-activated) acetaldehyde dehydrogenases (ACDHs) from *S. cerevisiae* using an hydroxyapatite column at pH 7.5. Key: –●– K^+-activated, mitochondrial ACDH from YPH499 (*ALD7*); –■– Mg^{2+}-activated, cytosolic ACDH from YPH499 (*ALD7*); –O– K^+-activated, mitochondrial ACDH from RY124 *(ald7)*; dashed line, concentration profile (mM) of sodium phosphate in the elution buffer. Fraction volume was 1.5 ml.

Table 4: A. Growth rates (μ_{max} h^{-1}) for shake-flask grown cultures of *S. cerevisiae* wild-type and mutants

Culture conditions	Maximum specific growth rate (h^{-1})		
	YPH499 (*ALD6*)	RY124 (*ald6*)	RY125 (*ald6*)
YNB 0.5% Glucose	0.29	0.16	0.17
5.0% Glucose	0.29	0.14	0.16
YEP 0.5% Glucose	0.32	0.12	NT
5.0% Glucose	0.30	0.10	NT

NT: Not tested

B. Growth rates (μ_{max} h^{-1}) for batch-fermenter grown cultures of *S. cerevisiae* YPH499 (*ALD6*) and RY124 (*ald6*)

Culture conditions	Maximum specific growth rate (h^{-1})	
	YPH499 (*ALD6*)	RY124 (*ald6*)
YNB 0.5% Glucose	0.29	0.15
5.0% Glucose	0.43	0.19
YEP 0.5% Glucose	0.32	0.18
5.0% Glucose	0.35 (0.31)	0.12 (0.11)

Values in parentheses are for cells grown anaerobically.

one of the mutant strains (RY124) and the wild type. The patterns observed were very similar. Growth under anaerobic conditions in the same fermenters was also assessed for mutant and wild-type cells (Materials and methods, Section 1.3.2). It is clear from Tables 4A and 4B that the growth of *ALD6* mutants on glucose was severely impaired. It was also apparent from this, that the Mg^{2+}-ACDH plays an important role in cytosolic acetyl-CoA production. From the work of Flikweert *et al* (1996), it would be expected that affecting the production of cytosolic acetyl-CoA would have a deleterious effect on the yeast cell. This became especially apparent on exposing a strain lacking the Mg^{2+}-ACDH to anaerobic conditions i.e. purely fermentative metabolism (see Table 4A).

Nonetheless, that mutants could utilise glucose aerobically or anaerobically without the Mg^{2+}-ACDH prompted an in-depth investigation into the physiology and enzymology of these mutants.

2.5 Assessment of the growth of wild-type and mutant strains on a range of carbon sources

Since growth of the disruption mutants on glucose was poor compared to the parental strain, it was considered useful to examine growth on other carbohydrates and also on some C2 compounds such as ethanol and acetate. Table 5 shows the degree of growth exhibited by wild-type cells and two disruption mutants on various carbon sources over a 48 h period.

Table 5 shows that cells lacking the Mg^{2+}-ACDH also grow poorly on sucrose. In addition final mutant cell densities were somewhat lower. On average, mutant cell densities attained 50% of the levels observed with parental cultures.

Mutants failed to use ethanol as a carbon source, both in defined medium (Table 5) and complex medium (results not shown). Growth on ethanol is the role more traditionally associated with the K^{+}-ACDH (Jacobson and Bernofsky, 1974). An

Table 5: Growth of wild type (*ALD6*) and mutant (*ald6*) cells on various carbon sources in minimal medium.

Culture conditions	YPH499 (*ALD6*)	RY124 (*ald6*)	RY125 (*ald6*)
Basal medium only	+	+	+
0.5% glucose	+++	++	++
5.0% glucose	+++	++	++
2% sucrose	+++	++	++
1% acetate	+	+	+
2% glycerol	+	+	+
1% ethanol	+++	+	+

+ Poor or no growth, corresponding to a cell density of < 0.01 mg (dry weight) ml^{-1}

++ Average growth, corresponding to a cell density of between 0.5 and 0.8 mg (dry weight) ml^{-1}

+++ Good growth, corresponding to a cell density of between 1.2 and 1.5 mg (dry weight) ml^{-1}

Growth was carried out as in Materials and Methods, Section 1.3.1.

investigation into the role of the Mg^{2+}-ACDH in ethanol catabolism is discussed in Results, section 2.8 onwards.

2.6 Growth of wild-type and mutant cultures on glucose with supplementation

Flikweert *et al* (1996) showed that mutants of pyruvate decarboxylase (the first enzyme in the PDH bypass) failed to grow on glucose in batch or continuous culture. However, they demonstrated that growth was possible in glucose-limited continuous culture by supplementing the minimal medium with small amounts of acetate. This was not the case in batch culture. They argued that the differences were possibly due to the repressive effects of glucose seen in batch culture that are not seen in glucose-limited continuous culture.

With this work in mind, I attempted to grow the wild-type and two mutants in batch culture on glucose in minimal medium, with the inclusion of varying concentrations of acetate. To minimise the possibility of glucose repression, 0.5% (w/v) glucose was used. If acetate is produced by the action of the Mg^{2+}-ACDH, then providing this compound directly might alleviate the need for the enzyme. In short, the presence of acetate might restore the rate and extent of mutant growth to wild-type levels.

Only a very slight increase in growth rate was observed when mutant cultures were supplemented with acetate (Table 6A). This increase may have been within the experimental error and did not restore growth rates to wild-type levels. In addition, neither the rate nor extent of wild-type growth increased in the presence of acetate (Table 6A). To avoid the possibility that glucose repression was occurring, cultures were grown on sucrose. Unlike glucose, sucrose has not been associated with the ability to repress respiratory enzymes or regulatory processes. Table 6B shows that acetate added in the presence of sucrose, did not improve growth of the mutants. The failure to

Table 6: A. Supplementation of *S. cerevisiae* glucose cultures with acetate

YNB + 0.5% glucose with:	Maximum specific growth rate (h^{-1})		
	YPH499 (*ALD6*)	RY124 (*ald6*)	RY125 (*ald6*)
No carbon source	0.33	0.20	0.18
0.1% (w/v) acetate	0.35	0.23	0.22
0.5% (w/v) acetate	0.33	0.23	0.24
1.0% (w/v) acetate	0.32	0.23	0.24
2.0% (w/v) acetate	0.33	0.22	0.21

B. Supplementation of *S. cerevisiae* sucrose cultures with acetate

YNB + 2% sucrose with:	Maximum specific growth rate (h^{-1})		
	YPH499 (*ALD6*)	RY124 (*ald6*)	RY125 (*ald6*)
No carbon source	0.37	0.22	0.16
0.1% (w/v) acetate	0.35	0.23	0.23
0.5% (w/v) acetate	0.33	0.22	0.23
1.0% (w/v) acetate	0.33	0.23	0.23
2.0% (w/v) acetate	0.25	0.20	0.22

restore growth in the presence of glucose or sucrose may have been due to an inability to utilise acetate and consistent with this is that wild-type cells did not use acetate as a carbon source (Table 5). The reason for the failure to utilise acetate is unknown. It is unlikely that permeability of cell membranes to this substrate is a problem, because work in this laboratory has shown that wild-type cells that have been growing on glucose (0.5% or 5%), will assimilate radiolabelled acetate (data not shown).

The Mg^{2+}-ACDH is an essential component of the metabolic pathway for cytosolic acetyl-CoA production. It seemed possible that in providing an intermediate or end-product of the pathway, it would be possible to alleviate inhibition of growth. Supplementation with acetate for either a typical (RY125) or atypical (RY124) *ald6* mutant had, in part, tested and apparently disproved this theory. However, providing an indirect source of acetyl-CoA would further test this theory. Cells of RY125 and YPH499 were thus grown in minimal medium containing 0.5% (w/v) glucose with and without oleic acid and Tween 80 (a water soluble source of oleic acid).

Providing a source of acetyl-CoA did not significantly improve *ald6* mutant growth (and certainly did not restore it to wild-type levels). The results presented in Table 7 reflect this.

2.7 Elucidation of the pathway for cytosolic acetyl-CoA production in Mg^{2+}-ACDH mutants

Given that the Mg^{2+}-ACDH is part of an essential pathway for cytosolic acetyl-CoA production in yeast and that mutants which lack this enzyme can grow on glucose, it was reasonable to suggest that some other pathway was operating to perform this function. Alternative possible routes were investigated.

Table 7: Supplementation of *S. cerevisiae* cultures with sources of oleic acid

	Maximum specific growth rate (h^{-1})	
Culture conditions	YPH499 (*ALD6*)	RY125 (*ald6*)
0.5% (w/v) glucose	0.33	0.18
0.5% (w/v) glucose + 0.1% (w/v) oleic acid	0.33	0.22
0.5% (w/v) glucose + 0.1% (w/v) Tween 80	0.33	0.18

2.7.1 Formation of cytosolic acetyl-CoA by the action of carnitine acetyltransferase

In the *ald6* mutant strains, the only known reaction that can result in production of acetyl-CoA is that of pyruvate dehydrogenase. This would produce a mitochondrial pool of acetyl-CoA that must then reach the cytosol to be used for biosynthesis. While it is widely accepted that CAT can import acetyl-CoA into mitochondria (Kohlaw and Tan-Wilson, 1977), it is not known if the reverse can occur. It was conceivable that the reverse could be taking place in the *ald6* mutants.

Mutant (RY124) and wild-type cells were cultured on 0.5% (w/v) and 5% (w/v) glucose in both complex and defined media and harvested during the period of active growth. Two concentrations of glucose were chosen so as to exert contrasting levels of repression on mitochondrial enzymes (including the PDH complex). The activities of PDC and ACS (the other components of the PDH bypass) were then measured, as were the activities of CAT and the Mg^{2+}- and K^{+}-ACDH. Table 8A shows the results obtained for those cultures grown in complex medium and similar findings were obtained for cells grown in defined medium (Table 8B). During growth on both concentrations of glucose, PDC, ACS and the Mg^{2+}-ACDH were present in wild-type cells showing that the PDH bypass was operative. However, in both strains grown on 0.5% (w/v) or 5% (w/v) glucose, CAT activity was negligible and so did not offer an alternative means for generating cytosolic acetyl-CoA. This was also true for other mutant strains (Tables 16A and 16B, Results, section 2.13). To test that CAT could be expressed under some conditions, the same enzyme activities were determined in the wild-type grown on ethanol. CAT was indeed present and displayed a specific activity of 194 nmoles min^{-1} $(mg\ protein)^{-1}$. This was not investigated in the *ald6* mutant because this strain would not grow on ethanol (Table 5).

Table 8A: Elucidation of a novel pathway for cytosolic acetyl-CoA production in shake flask cultures of *S. cerevisiae* grown in complex medium

Strain	Culture Conditions	Specific Activity [nmoles min^{-1} (mg protein)$^{-1}$]				
		PDC	Mg^{2+}-ACDH	ACS	CAT	K^+-ACDH
YPH499	0.5% glucose	97	54	43	0	29
	5.0% glucose	364	62	38	0	3
RY124	0.5% glucose	55	0	20	0	28
	5.0% glucose	124	0	24	0	21

Table 8B: Elucidation of a novel pathway for cytosolic acetyl-CoA production in shake flask cultures of *S. cerevisiae* grown in minimal medium

Strain	Culture Conditions	Specific Activity [nmoles min^{-1} (mg protein)$^{-1}$]				
		PDC	Mg^{2+}-ACDH	ACS	CAT	K^+-ACDH
YPH499	0.5% glucose	181	43	40	1	97
	5.0% glucose	376	35	32	0	10
RY124	0.5% glucose	92	0	27	6	55
	5.0% glucose	171	0	27	2	45

A pathway that has been proposed to function in oleaginous yeast to produce cytosolic acetyl-CoA involves the enzyme ATP:citrate lyase (Ratledge and Evans, 1989). Although this enzyme has been reported to be absent from *S. cerevisiae* (Boulton and Ratledge, 1981 a, b), the activity was checked for in wild-type and mutant cells. As expected, no activity was detected.

2.7.2 Formation of cytosolic acetyl-CoA via the action of the K^+-ACDH

The results regarding the activity of the K^+-ACDH were unexpected. In the wild-type, growth on 5% (w/v) glucose repressed the activity of this enzyme (Table 8A and 8B). This was as expected (Llorente and de Castro, 1977). However, when cells of RY124 were grown under identical conditions in complex or defined medium, the degree of repression for this enzyme was considerably less than expected (Table 8A and 8B). In the wild-type grown on complex medium, 89% repression of activity was observed, whereas only 25% was observed in the mutant grown under the same conditions. This same phenomenon was seen with other mutant cells (Tables 17A and 17B, Results, section 2.13).

Bearing in mind the results in Table 8A and 8B, it was proposed that during growth on glucose, the K^+-ACDH could somehow compensate for the loss of the Mg^{2+}-activated enzyme (one might also expect that the same could happen in ethanol catabolism, but apparently it does not, because *ald6* mutants cannot grow on ethanol). Despite the distinct cellular compartmentalisation of these two enzymes, Figure 8 shows that such an alternative scheme for growth on glucose is plausible. There is no recognised fate for mitochondrial acetate due to a lack of ACS in this organelle (Klein & Jahnke, 1979). However, acetate and acetaldehyde are small molecules. Thus it is possible that they can passively diffuse across the selectively permeable inner mitochondrial membrane (they can easily cross the outer mitochondrial membrane via

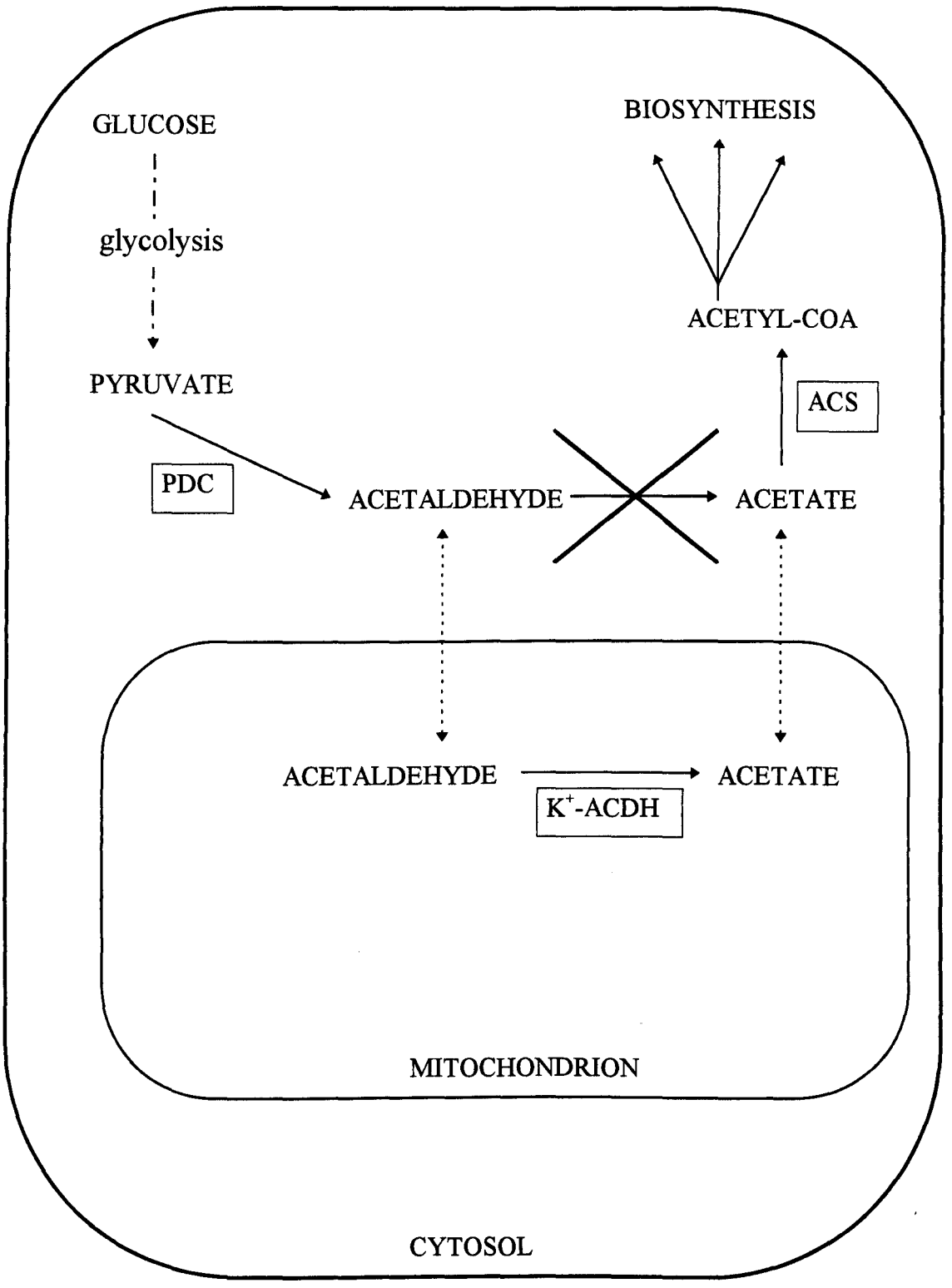

Figure 8: A metabolic scheme suggesting a possible route to cytosolic acetyl-CoA in the absence of the Mg^{2+}-ACDH. This enzyme usually catalyses the conversion of acetaldehyde to acetate. The scheme presupposes that acetaldehyde and acetate (being small molecules) can freely diffuse across the inner and outer mitochondrial membranes.

pores). A similar mechanism (involving passive diffusion) has been proposed for mammalian systems (Lowenstein, 1967). An additional route for acetate may exist; that of mediated transport by the mitochondrial pyruvate carrier. It is feasible that this carrier is not highly specific for pyruvate and therefore may also transport acetate.

To prove or disprove such a hypothesis would require a mutant lacking both the K^+- and Mg^{2+}-ACDH. If such a mutant were then able to grow on glucose, it could be interpreted that a pathway different to the one proposed here produces cytosolic acetyl-CoA in the absence of the Mg^{2+}-ACDH. However, if the double mutation was lethal, one would have to conclude that the K^+-ACDH does in fact compensate for the loss of the Mg^{2+}-activated enzyme. This work is currently underway but has not yet been completed.

If indeed the K^+-ACDH could compensate for the loss of the Mg^{2+}-ACDH, another method by which it could do so exists in addition to the scheme presented as Figure 8. This would be that during synthesis of the K^+-ACDH in the *ald6* mutant, the protein was wrongly targeted and exported from the endoplasmic reticulum to the cytosol (instead of to the mitochondria). This would require the absence of a mitochondrial targeting sequence and so a secondary mutation to bring this about. If the enzyme was incorrectly targeted, cellular fractionation may reveal the true location of the enzyme.

2.7.3 Isolation of mitochondria from wild-type and *ald6* mutant cells

Various methods exist to isolate whole, intact mitochondria. Bruinenberg *et al* (1985) discussed numerous ways of purifying these organelles and numerous criteria by which to judge the quality of the preparation. In the light of their findings, the protocol listed in Materials and Methods, section 8.0 was used.

The aims of this work were (i) to isolate whole mitochondria from wild-type cells

and to study the subcellular localisation of both ACDHs using suitable marker enzymes as controls and (ii) to repeat this with Mg^{2+}-ACDH mutant cells in order to check if the distribution is the same as for the wild-type.

Generally, the quality of mitochondria produced was poor as judged by the respiratory control index (see Materials and Methods, section 8.5). The mitochondrial fraction P1 showed high respiration rates in the absence of exogenous substrate and ADP, suggesting that the mitochondria were not coupled, but likely to be intact. The results in Table 9A and 9B reflect this, since distribution of both the marker enzymes and ACDHs was as expected and with good recovery.

Allowing for some degree of damage to mitochondria during the preparation, the results confirmed that in both wild-type and mutant cells, the K^+-ACDH was located in the mitochondria. Consequently, it was assumed that the scheme presented as Figure 8 is the most likely means by which the K^+-activated enzyme in *ald6* mutants compensates for the loss of the Mg^{2+}-ACDH.

2.8 The response of *ald6* mutants to ethanol

The K^+-ACDH is an enzyme associated with growth on ethanol (Jacobson and Bernofsky, 1974; Llorente and de Castro, 1977) but has never been attributed a role for growth on glucose. It is surprising then, that cells possessing this enzyme but lacking the Mg^{2+}-activated one did not grow on ethanol (Table 5.0).

The growth of YPH499 and RY124 on ethanol was monitored over a 48 h period to establish whether using a larger inoculum size could stimulate mutant growth. To do this, both parental and mutant cells were inoculated into (i) basal minimal growth medium (ii) medium containing 1% ethanol (v/v) and (iii) medium containing 0.5% glucose (w/v). Cells were inoculated to allow progressively higher starting cell densities and growth was monitored.

Table 9A: Activities of Mg^{2+}- and K^+-ACDH and marker enzymes pyruvate decarboxylase and citrate synthase in YPH499 (*ALD6*)

		Activity fraction^{-1} (μmoles min^{-1})			
Fraction	Protein (mg)	PDC	CS	Mg^{2+}-ACDH	K^+-ACDH
'Total'	82	40.2	8.93	3.31	3.05
P1	61	40.5	3.28	2.93	1.48
P2	6.6	0.09	3.15	0.01	0.80
S	4.5	0.09	1.07	0.02	0.26

P1 and P2 are mitochondrial fractions

S is the 'soluble' or cytosolic fraction

Table 9B: Activities of Mg^{2+}- and K^+-ACDH and marker enzymes pyruvate decarboxylase and citrate synthase in RY124 (*ald6*)

		Activity fraction^{-1} (μmoles min^{-1})			
Fraction	Protein (mg)	PDC	CS	Mg^{2+}-ACDH	K^+-ACDH
'Total'	63	43.4	3.66	0	3.62
P1	54	49.4	1.58	0	2.10
P2	2.3	0.41	0.95	0	0.53
S	2.5	0.46	0.61	0	0.51

P1 and P2 are mitochondrial fractions

S is the 'soluble' or cytosolic fraction

Figures 9A and 9B illustrate that whilst mutant cells grew satisfactorily on glucose, negligible biomass was produced by those cells exposed to ethanol. Even using a large amount of inoculum did not stimulate growth. This lack of growth was apparent for all the *ald6* mutants (results not shown) and warranted a deeper investigation into the response to ethanol of a strain lacking the Mg^{2+}-ACDH.

2.9 Respiration studies with *ALD6* and *ald6* strains of *S. cerevisiae*

Cells deleted of the *ALD6* gene cannot grow on ethanol, but may still be able to oxidise this substrate. A sensitive system with which to study this is the oxygen electrode by observing any respiration on provision of an exogenous substrate (ethanol, acetaldehyde etc). Cells were grown in minimal medium until the active phase of growth and then harvested. The results for both parental and mutant cells are given in Tables 10A and 10B.

The rate of O_2 consumption was in general far less for wild-type cells grown on 5% (w/v) glucose (hence repressing respiratory enzymes) than for cells grown under non-repressing conditions (0.5% (w/v) glucose or 0.5% (w/v) glucose + 1% (v/v) ethanol). Repressed cells did not respire when provided with acetaldehyde or acetate but did respire when given glucose, pyruvate or ethanol. The inability to respire with acetate was likely to reflect the repression of mitochondria (and possibly peroxisomes) caused by the growth conditions. This repression could prevent any further metabolism of acetate or its products. The respiratory activity of non-repressed cells was as expected. Neither acetate or acetaldehyde were seen to inhibit oxidation of glucose, pyruvate or ethanol.

Table 10B illustrates that overall, respiratory activity of RY124 was poor compared to that of wild-type cells. Nevertheless, a difference was observed between mutant cells grown on 0.5% (w/v) glucose and 5% (w/v) glucose. The latter would not consume O_2 when given ethanol, acetaldehyde or acetate whilst following growth on

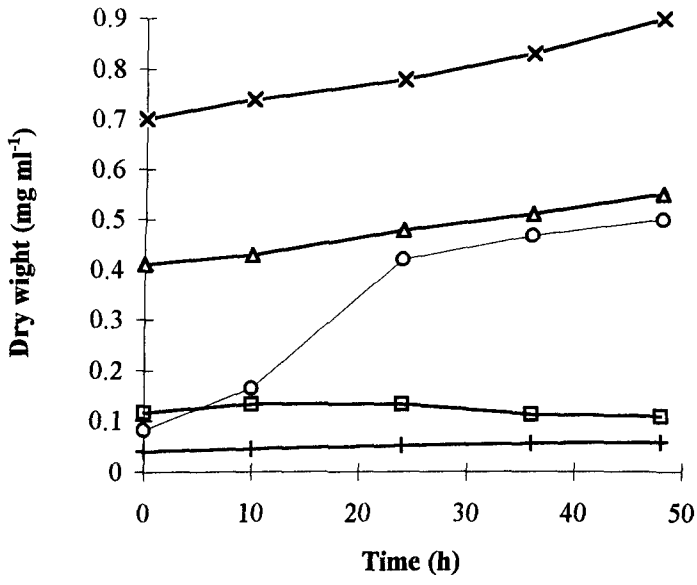

Figure 9A: Growth curves for *S. cerevisiae* RY124 (*ald6*) cultured on minimal medium with the following: 1% (v/v) ethanol (using increasingly higher starting cell densities –□–, –Δ–, –x–); no added carbon source –+–; and 0.5% (w/v) glucose –O–.

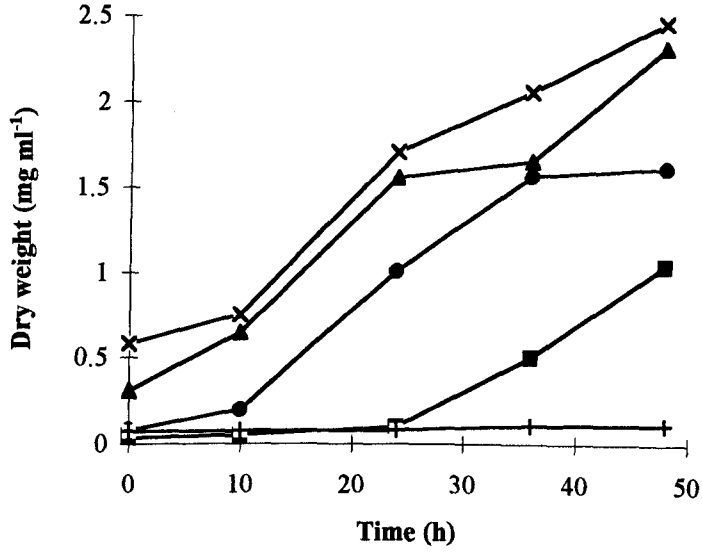

Figure 9B: Growth curves for *S. cerevisiae* YPH499 (*ALD6*) cultured on minimal medium with the following: 1% (v/v) ethanol (using increasingly higher starting cell densities –■–, –x–, –▲–); no added carbon source –+– and 0.5% (w/v) glucose –●–.

Table 10A: Respiration rates for wild-type cells (*ALD6*) of *S. cerevisiae* grown on various carbon sources in minimal medium

		Respiration rate with designated substrate [nmoles min^{-1} $(mg\ cells)^{-1}$]				
Growth conditions	Concentration of substrate in assay (mM)	Glucose	Pyruvate	Ethanol	Acetal-dehyde	Acetate
5.0% (w/v) glucose	10	3.2	0.9	7.1	0	0
	1	0	0	2.6	0	0
	0.1	NT	NT	NT	NT	NT
0.5% (w/v) glucose	10	36	11.1	33.2	24.9	4.2
	1	33.2	8.3	44.3	33.2	4.2
	0.1	NT	NT	33.2	29	NT
0.5% (w/v) glucose + 1% (v/v) ethanol	10	37.9	30.2	39.4	42.9	4.3
	1	32.1	15.1	45.4	62.6	7.6
	0.1	NT	39.4	45.4	56.5	NT

NT: not tested

All values are corrected for the endogenous rate (which was typically 30% of the substrate stimulated rate)

Table 10B: Respiration rates for mutant cells (*ald6*) of *S. cerevisiae* grown on various carbon sources in minimal medium

Growth conditions	Concentration of substrate in assay (mM)	Respiration rate with designated substrate [nmoles min^{-1} $(mg\ cells)^{-1}$]				
		Glucose	Pyruvate	Ethanol	Acetal-dehyde	Acetate
5.0% (w/v) glucose	10	0.7	0.8	0	0	0
	1	0	0	0	0	0
	0.1	NT	NT	NT	NT	NT
0.5% (w/v) glucose	10	15.4	8.7	6.6	12.5	0
	1	19.1	7.3	7.3	19.9	6.3
	0.1	7.3	NT	6.8	11.9	6.3
0.5% (w/v) glucose + 1% (v/v) ethanol	10	3.9	3.4	2.3	1.4	1.3
	1	1.9	0.3	0	0.3	0
	0.1	NT	NT	NT	NT	NT

NT: not tested

All values are corrected for the endogenous rate (which was typically 30% of the substrate stimulated rate)

0.5% (w/v) glucose, cells respired with all substrates tested. It was surprising that cells grown under the conditions tested here, respired when given ethanol. However, respiration was only 5% of wild-type levels. Similar results were found for another mutant strain (RY125).

2.10 Viability of wild-type and mutant cells during exposure to ethanol

It has been suggested that ethanol can be toxic, either (i) directly or (ii) indirectly by the action of acetaldehyde (Jones, 1989).

2.10.1 Direct toxicity by ethanol

This possibility was studied by comparing the viability of glucose-grown mutant cells exposed to 1% (v/v) ethanol to the viability of wild-type cells under identical conditions. Figures 10A and 10B represent the findings from the study. Figure 10B clearly shows that the number of colony forming units (CFUs) did not significantly decrease upon exposure to ethanol for either parental or mutant cells respectively. Indeed, the increase in amount of CFUs for both strains was identical to the increase in cell dry weight (Figure 10A). The results presented as Table 11 show that the other *ald6* mutants were also viable during exposure to ethanol. Clearly, ethanol is not lethal to any of these cells.

2.10.2 Indirect toxicity by ethanol

In order to investigate the possible effects of toxicity due to acetaldehyde, it was first decided to establish whether mutant (or wild-type) cells could produce acetaldehyde from ethanol. Mutant and wild-type cells were treated as described in Materials and Methods, section 1.3.5.1.

Figure 11A shows that no acetaldehyde was produced by RY124 during incubation in ethanol, whereas YPH499 (*ALD6*) was capable of producing significant concentrations of this metabolite. A similar pattern was observed for both strains during

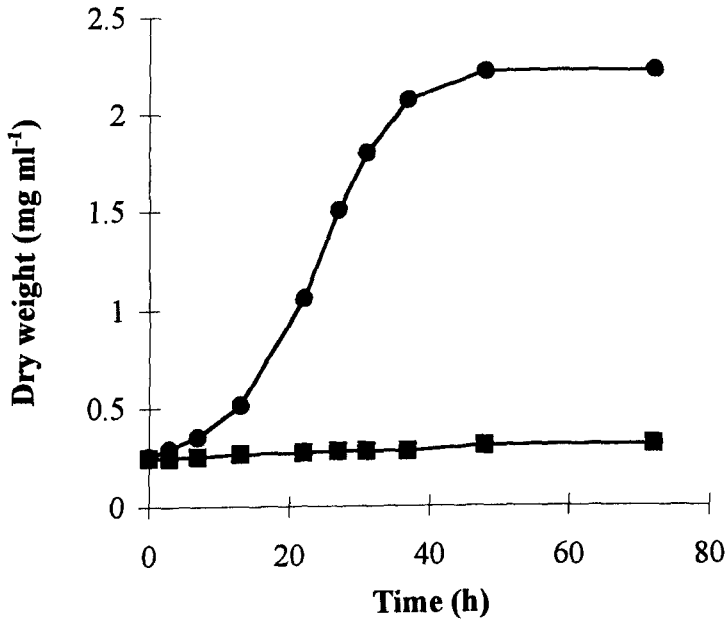

Figure 10A: Increase in cell density for *S. cerevisiae* YPH499 (*ALD6*) –●–, and RY124 (*ald6*) –■– during prolonged exposure to 1% (v/v) ethanol in minmal medium.

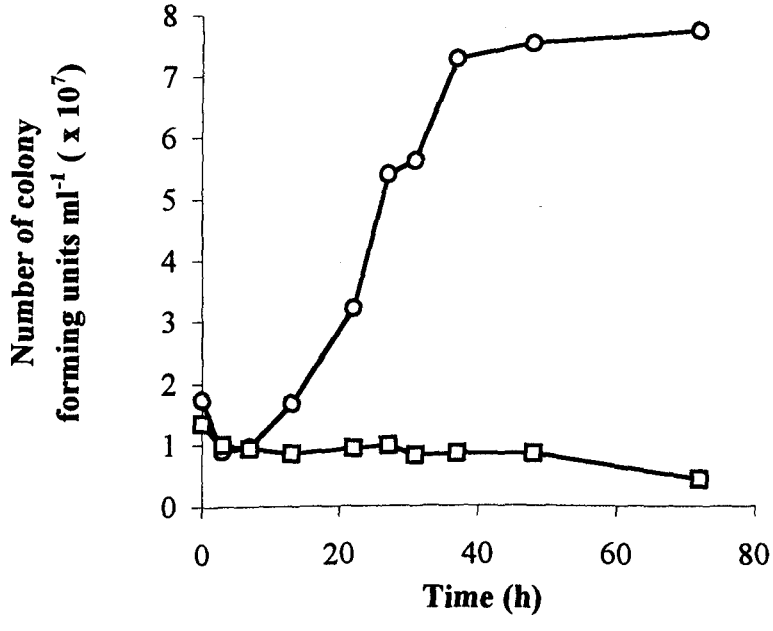

Figure 10B: Viability of *S. cerevisiae* YPH499 (*ALD6*) –O–, and RY124 (*ald6*) –□– during prolonged exposure to 1% (v/v) ethanol in minmal medium.

Table 11: Viability of *ald6* mutants exposed to 1% (v/v) ethanol

Strain tested	Number of colony forming units ml^{-1}			% Change in viability
	0 h	4 h	20 h	
RY125	2.6×10^7	2.84×10^7	2.22×10^7	15% Decrease
RY126	2.69×10^7	2.48×10^7	3.25×10^7	21% Increase
RY127	2.42×10^7	2.74×10^7	3.07×10^7	27% Increase

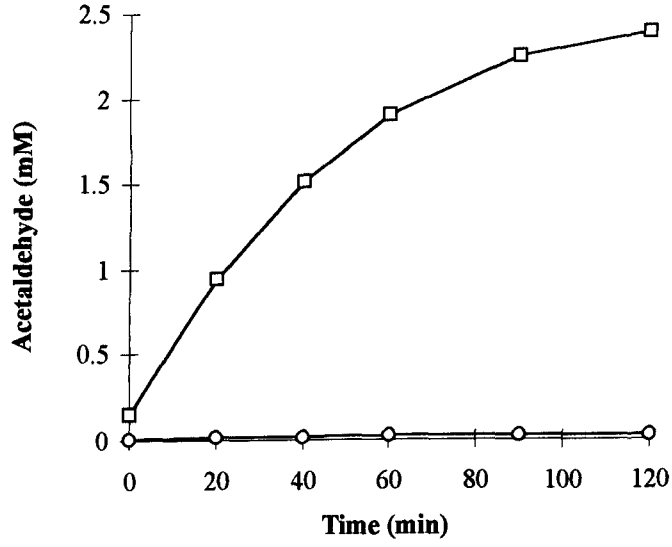

Figure 11A: Acetaldehyde secretion by YPH499 (*ALD6*) –□– ; and RY124 (*ald6*) –O– ; during incubation in minimal medium containing 1% (v/v) ethanol.

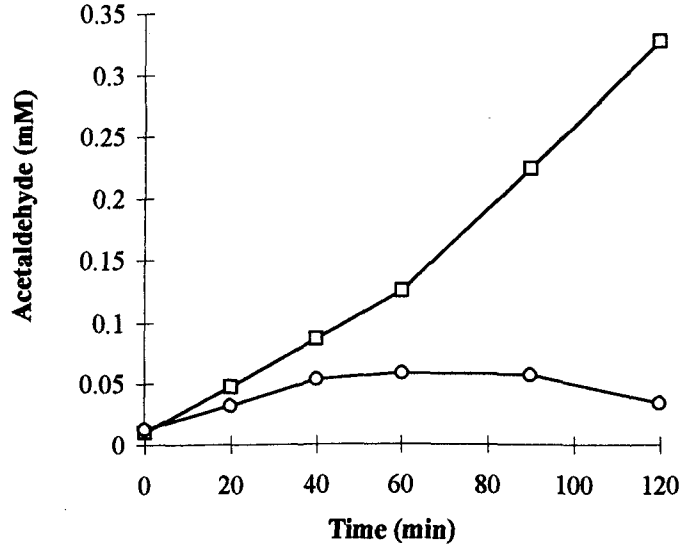

Figure 11B: Acetaldehyde secretion by YPH499 (*ALD6*) –□– ; and RY124 (*ald6*) –O– ; during incubation in minimal medium containing 0.5% (w/v) glucose.

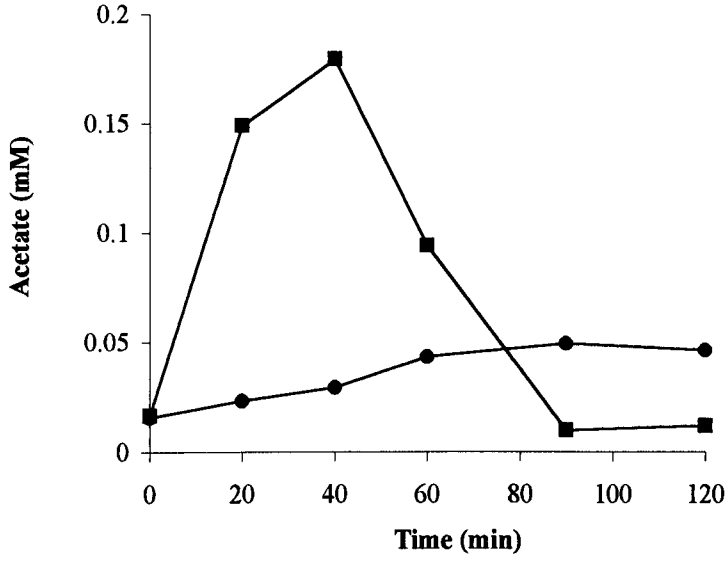

Figure 11C: Acetate secretion by YPH499 (*ALD6*) –■– ; and RY124 (*ald6*) –●– ; during incubation in minimal medium containing 1% (v/v) ethanol.

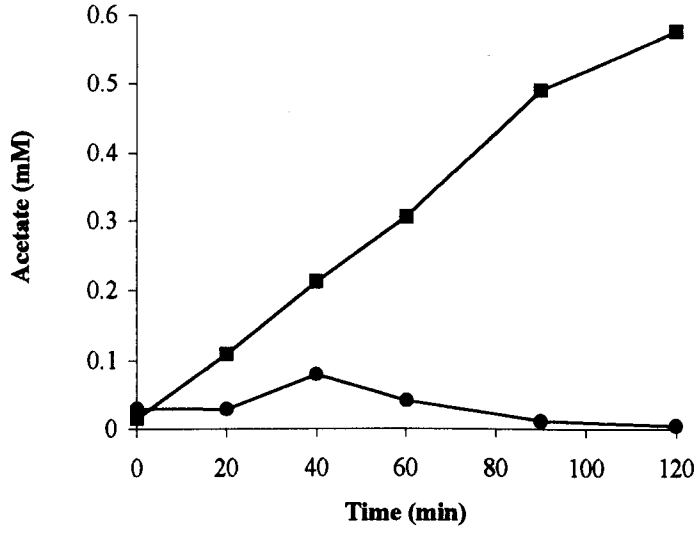

Figure 11D: Acetate secretion by YPH499 (*ALD6*) –■– ; and RY124 (*ald6*) –●– ; during incubation in minimal medium containing 0.5% (w/v) glucose.

incubation on glucose (Figure 11B). The lack of acetaldehyde accumulation could be attributable to the immediate production of acetate. However, the *ald6* mutant also failed to produce this compound during incubation in ethanol (Figure 11C) or glucose (Figure 11D). Other results (not shown) which are consistent with those above, indicate that ethanol did not disappear from mutant growth medium over longer incubation periods (16 h) with this compound.

The parental strain was shown to produce acetate during incubation in glucose or ethanol (Figure 11C and 11D respectively). Interestingly, during incubation in ethanol, wild-type cells appeared to excrete acetate at first and then later assimilate it. This is a phenomenon that occurs with brewing yeast in brewery fermentations (Coote and Kirsop, 1976), the reason for which is not understood. All of the above observations were also made with RY125.

2.10.3 Response of wild-type (*ALD6*) and mutant (*ald6*) cells of *S. cerevisiae* to direct addition of acetaldehyde.

Although RY124 did not produce acetaldehyde, it was decided to test the effect on this and the wild-type strain of addition of acetaldehyde (at various concentrations) to glucose media (i) prior to growth and (ii) during active growth. This might reveal for instance increased sensitivity of the mutant to acetaldehyde.

(i) Addition prior to growth: Wild-type and mutant cells were inoculated into shake flasks containing minimal media with 0.5% (w/v) glucose and either 25 mM, 50 mM or 100 mM acetaldehyde. Growth was then monitored over 48 h. Figure 12A shows that for wild-type cells, as the concentration of added acetaldehyde was increased, the onset of growth was increasingly delayed, after which cultures grew to cell densities as achieved by cultures without acetaldehyde added. The same was true for RY124 (Figure 12B), except that greater inhibition of growth was observed with 100 mM acetaldehyde

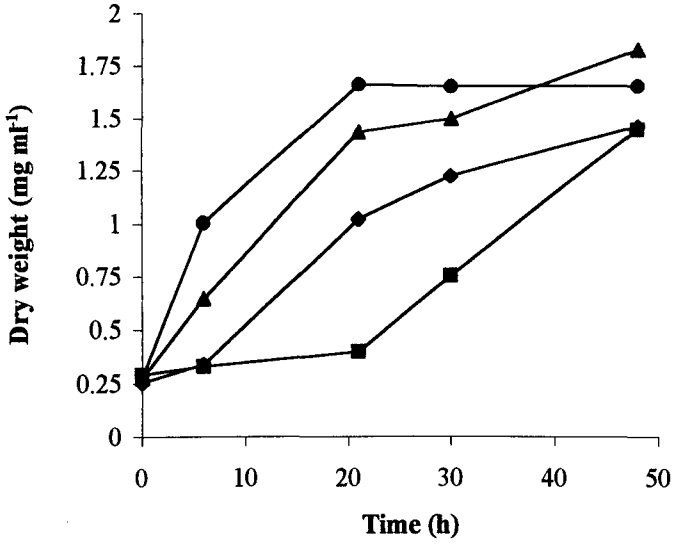

Figure 12A: Growth of YPH499 (*ALD6*) on minimal medium containing 0.5% (w/v) glucose with the following: –●– no additions; –▲–25 mM acetaldehyde; –◆– 50 mM acetaldehyde; –■– 100 mM acetaldehyde.

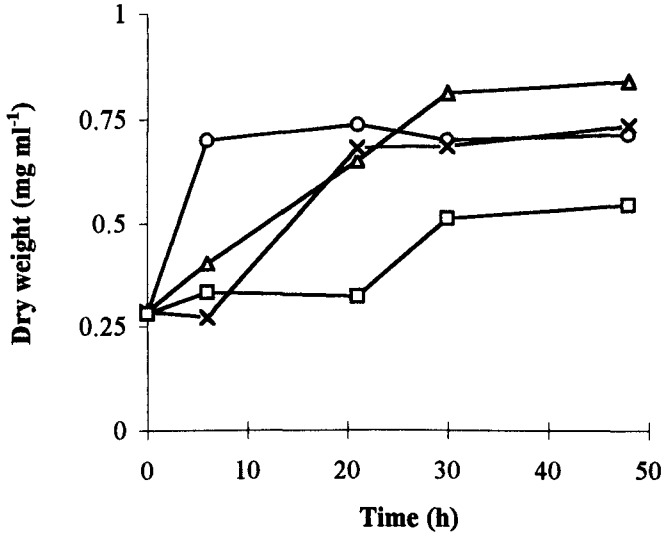

Figure 12B: Growth of RY124 (*ald6*) on minimal medium containing 0.5% (w/v) glucose with the following: –O– no additions; –Δ– 25 mM acetaldehyde; –X– 50 mM acetaldehyde; –□– 100 mM acetaldehyde

than was seen in the wild-type. In general, *ald6* mutant cells were not significantly more or less susceptible to acetaldehyde than parental cells. It was surprising that both strains were able to grow at all in the presence of such high acetaldehyde concentrations. Previous workers have described toxicity at levels as little as 10 mM (Jones, 1989).

A potential problem with such experiments was that acetaldehyde could be lost to evaporation after 48 h at 30°C, due to its volatile nature. However, control flasks revealed that over such a period, no more than 40% total acetaldehyde was lost, with only 5% being typically lost during the first 24 h.

(ii) Addition during growth: The effect of adding acetaldehyde during active growth of the mutant and wild-type strain was tested.

Duplicate cultures were inoculated and when both cultures were seen to be in the phase of active growth (as deduced from preliminary growth curves), one culture was left to grow whilst a small volume of acetaldehyde (1 M) was added to the duplicate culture to arrive at a final concentration of 50 mM. Growth of both cultures was then monitored by optical density measurements.

Figure 13A shows that acetaldehyde caused a reduction in growth rate for the parental strain although cells were able to recover and reach normal cell density levels. The mutant on the other hand did not recover as successfully as the wild-type. Figure 13B reflects this. Addition of acetaldehyde caused a reduction in both growth rate and final cell density.

2.11 Metabolic response of RY124 (*ald6*) to ethanol

Mutant yeast cells that lack the Mg^{2+}-ACDH cannot grow on ethanol and during exposure to this compound cells do not produce significant amounts of acetaldehyde and remain viable. In order to extend the viability investigation and to obtain a greater understanding of the response of such mutants to ethanol, it was decided to study the

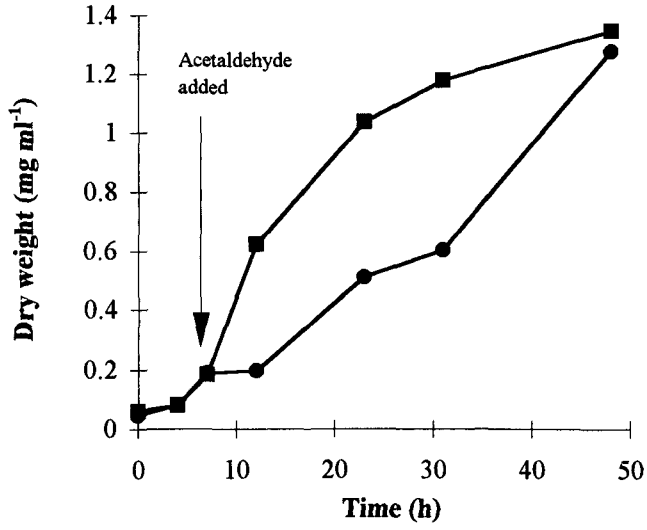

Figure 13A: Effect of adding 50 mM acetaldehyde to a culture of *S. cerevisiae* YPH499 (*ALD6*) growing on 0.5% (w/v) glucose. –■– control culture, –●– culture with acetaldehyde added.

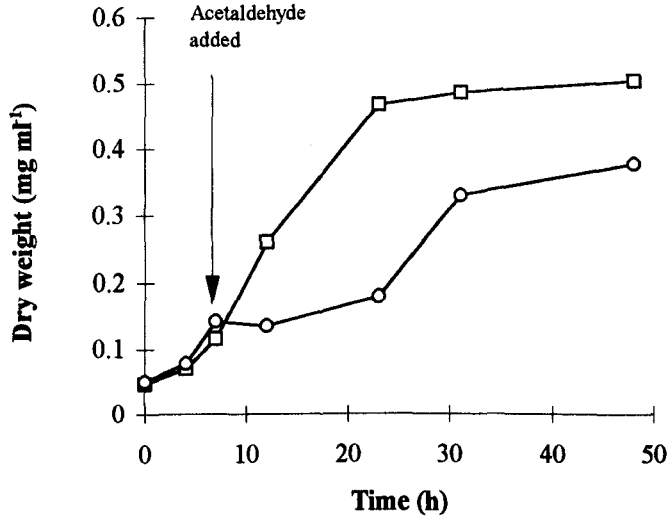

Figure 13B: Effect of adding 50 mM acetaldehyde to a culture of *S. cerevisiae* RY124 (*ald6*) growing on 0.5% (w/v) glucose. –O– control culture, –□– culture with acetaldehyde added.

expression of the two ACDHs and some well-known ethanol-inducible enzymes.

To this end, parental and RY124 cells were incubated in ethanol overnight (16 h) and the activities of the following enzymes determined before and after incubation; K^+-ACDH, Mg^{2+}-ACDH, CAT and isocitrate lyase (ICL). CAT and ICL were measured because they are known to be induced by ethanol. The CAT assay used here, measures total CAT activity so that if only one out of the three isozymes is active, this can be detected. ICL was measured because of its peroxisomal location and the presumed involvement of these organelles in ethanol catabolism.

On exposure of parental cells to ethanol, the specific activities of K^+-ACDH, Mg^{2+}-ACDH, CAT and ICL were all seen to increase (Table 12A) as expected. However, the outcome of incubating mutant cells in ethanol was rather different (Table 12B). As expected, the Mg^{2+}-ACDH was absent before and after incubation, but no induction of the K^+-ACDH was observed. Indeed, the specific activity actually decreased upon exposure, whilst neither CAT nor ICL was induced by ethanol. These findings were surprising although consistent with the fact that RY124 did not grow on ethanol. One would expect that deleting the *ALD6* gene would not result in any other genetic changes that could bring about a change in the regulation of such enzymes.

The survey of inducible enzymes was widened to include the alcohol dehydrogenases. Laboratory strains of *S. cerevisiae* contain three functional isozymes of alcohol dehydrogenase (ADH), namely ADH I (associated with cytosolic production of ethanol), ADH II (associated with cytosolic consumption of ethanol) and ADH III (a mitochondrial enzyme with the same function as ADH II). It is generally assumed that ADH I is constitutive, but that ADH II and III are induced by ethanol (Lutstorf & Megnet, 1968). Thus the activity of the total ADH complement before and after exposure to ethanol was determined by using polyacrylamide gel electrophoresis (PAGE)

Table 12A: Enzyme induction during incubation in ethanol for YPH499 (*ALD6*)

Enzyme	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]	
	Pre-incubation	Post -incubation (16 h)
K^+-ACDH	14	204
Mg^{2+}-ACDH	44	59
CAT	21	810
ICL	0	148

Table 12B: Enzyme induction during incubation in ethanol for RY124 (*ald6*)

Enzyme	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]	
	Pre-incubation	Post -incubation (16 h)
K^+-ACDH	65	43
Mg^{2+}-ACDH	0	0
CAT	8	4
ICL	0	0

and a suitable activity stain. Gels were used instead of conventional enzyme assays because it is difficult to distinguish between the different isozymes even using different assay conditions. Gels were loaded with cell-free extracts prepared from cells prior to and after incubation in 1% (v/v) ethanol. The experiments were carried out for wild-type cells and for the mutant RY124 using the same incubation protocol as in the preceding section.

Similar findings were made with the ADHs as were made with the other ethanol inducible enzymes. In parental cells, the complement of ADH isozymes was seen to 'switch over' upon exposure to ethanol (Figure 14). The major change was assumed to be the induction of the ethanol inducible ADH II and III accompanied by repression of the ADH I. Conversely, the complement of ADH in mutant cells-free extracts did not change during incubation in ethanol. No 'switching over' was observed. This difference between the strains was similar to the lack of inducibility seen for CAT and ICL.

The concept of 'switching over' or 'non switching over' of isozymes was partially reflected in total ADH assays performed prior to loading gels. These were initially carried out so that a known activity (0.1 unit) could be loaded onto the gel. However, by measuring activity at two different concentrations of ethanol (5 mM or 1 M) it was possible to observe how the ratio of these two activities changed on exposure of the yeast to ethanol. The two strains behaved differently (shown in Table 13). For the wild-type, prior to incubation, the ratio of activities using 5 mM ethanol and 1 M ethanol was 1 : 3.2. and after incubation 1 : 1.7. In RY124 (*ald6*) the ratios of values prior to and following incubation were 1 : 3.8 and 1 : 3.2.

The wild-type ratio noticeably decreased, whereas the mutant ratio barely altered. This agreed well with the activity gels. It seems that in wild-type cells, the change seen on the gels was due to the production of the ethanol inducible ADH II and III. With

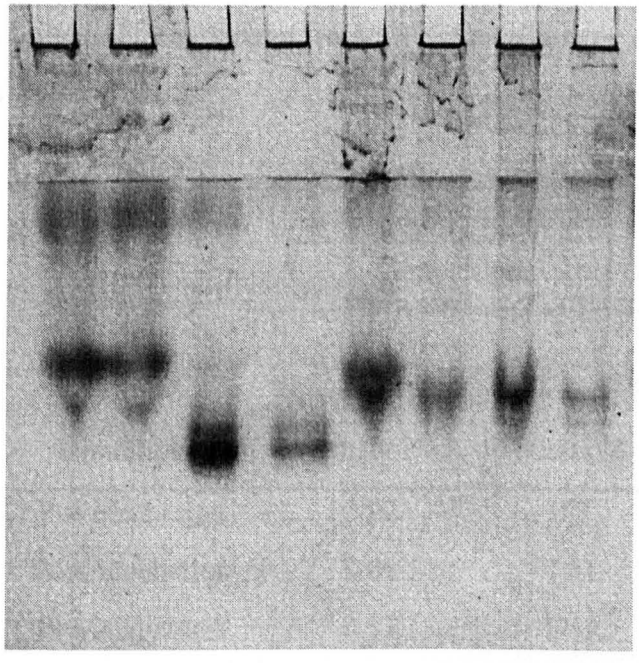

Figure 14: A PAGE gel stained for alcohol dehydrogenase activity in *S. cerevisiae* YPH499 (*ALD6*) and RY124 (*ald6*).

From left to right: Lanes 1 & 2 = YPH499 - pre incubation

Lanes 3 & 4 = YPH499 - post incubation

Lanes 5 & 6 = RY124 - pre incubation

Lanes 7 & 8 = RY124 - post incubation

Note: In each pair of lanes, the right hand lane has been loaded with 50% activity of left hand lane.

Table 13: Total alcohol dehydrogenase activity in wild-type and mutant cells of *S. cerevisiae*

Strain	Culture conditions	Specifc activity [μmoles min^{-1} $(mg\ protein)^{1}$]		
		Using 5 mM ethanol*	Using 1 M ethanol*	Ratio of activities
YPH499 (*ALD6*)	Pre-incubation	0.22	0.7	1 : 3.2
	Post-incubation	2.60	4.51	1 : 1.7
RY124 (*ald6*)	Pre-incubation	0.37	1.42	1 : 3.8
	Post-incubation	0.78	2.52	1 : 3.2

Cells were incubated in 1% (v/v) ethanol for 16 h.

* Using this concentration of ethanol as substrate in the fluorometric assay.

mutant cells little or no change was seen.

Since exposure to ethanol did not induce any response in RY124, it was decided to investigate the possibility that this was specific to ethanol by exposing this mutant to other two carbon (C2) compounds.

2.12 Further attempts to induce CAT and ICL in *S. cerevisiae* RY124

Cells of RY124 and YPH499 were grown on 5% (w/v) glucose, harvested aseptically and then transferred to minimal medium containing either acetaldehyde (10 mM) or 1% (w/v) acetate for overnight incubation (16 h). Cell-free extracts were prepared from cells prior to and following incubation and the specific activities of the following enzymes were measured: CAT, ICL, Mg^{2+}-ACDH and K^{+}-ACDH.

Tables 14A and 14B show the pattern of enzyme expression for wild-type and mutant cells (respectively) incubated in acetaldehyde. The results clearly show that like ethanol (Tables 12A and 12B), acetaldehyde failed to induce CAT, ICL and the K^{+}-ACDH in cells of RY124 yet successfully induced these enzymes in cells of YPH499. Nevertheless, in the wild-type, differences were observed between the effects of ethanol and those of acetaldehyde. These were that the latter caused a decrease in Mg^{2+}-ACDH activity and that the extent of K^{+}-ACDH expression was less. The patterns observed after incubation in acetate were similar (Tables 15A and 15B).

Since it was obvious that a C2 compound was not able to stimulate expression of these enzymes in RY124, oleic acid was used. Oleic acid is a fatty acid and so can be degraded to form acetyl-CoA. Since β-oxidation of fatty acids in yeast occurs in peroxisomes (McCammon *et al*, 1990), CAT may be used in transporting acetyl-CoA to the cytosol and / or mitochondria. ICL is an ideal marker enzyme for peroxisomal activity. To test this theory, RY124 and YPH499 were incubated overnight in minimal media containing 0.1% (w/v) oleic acid.

Table 14A: Enzyme induction during incubation in 10 mM acetaldehyde for YPH499 (*ALD6*)

Enzyme	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]	
	Pre-incubation	Post -incubation (16 h)
K^+-ACDH	11	74
Mg^{2+}-ACDH	58	26
CAT	0	301
ICL	0	53

Table 14B: Enzyme induction during incubation in 10 mM acetaldehyde for RY124 (*ald6*)

Enzyme	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]	
	Pre-incubation	Post -incubation (16 h)
K^+-ACDH	61	68
Mg^{2+}-ACDH	0	0
CAT	0	0
ICL	0	0

Table 15A: Enzyme induction during incubation in 1% (w/v) acetate for YPH499 (*ALD6*)

Enzyme	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]	
	Pre-incubation	Post -incubation (16 h)
K^+-ACDH	7	69
Mg^{2+}-ACDH	49	29
CAT	0	277
ICL	0	70

Table 15B: Enzyme induction during incubation in 1% (w/v) acetate for RY124 (*ald6*)

Enzyme	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]	
	Pre-incubation	Post -incubation (16 h)
K^+-ACDH	39	48
Mg^{2+}-ACDH	0	0
CAT	0	0
ICL	0	0

Table 16A illustrates that in wild-type cells, oleic acid caused slight induction of CAT and ICL but did not induce the ACDHs (completely repressing the Mg^{2+}-activated enzyme). Presumably, if oleic acid provides acetyl-CoA (and NADPH), the Mg^{2+}-ACDH would not be required. Perhaps a higher concentration of oleic acid was required to cause greater expression of ICL and CAT. The wild-type did grow though, increasing in dry weight by 30% over the 16 h.

As with the C2 compounds, oleic acid failed to induce CAT, ICL and the K^+-ACDH in RY124 (Table 16B). Indeed, as with YPH499, the K^+-ACDH was not induced in the mutant but in this case completely repressed. However, no growth on oleic acid was observed for RY124 which may explain the lack of K^+-ACDH activity. Since growth on some compounds resulted in no activity being detected for certain enzymes, mixed extracts were used to test for the presence of inhibitors. No such inhibition was found.

Since none of the compounds tested induced CAT, ICL or the ACDHs, it was conceivable that in disrupting the *ALD6* gene, a secondary mutation had occurred leading to regulatory changes concerned with C2 metabolism. Rather than attempting to elucidate further this rather complex situation, it was decided to turn attention to the other Mg^{2+}-ACDH mutants that had been generated from the original disruption. These were RY125, RY126 and RY127, expected to be identical to RY124 and each other.

2.13 Response to ethanol in other *ald6* mutants of *S. cerevisiae*

Strains RY125 to RY127 were tested for their ability to respond to incubation in ethanol by way of inducing CAT, ICL and the K^+-ACDH. This was performed under identical conditions to those used for RY124 (Results, section 2.11).

Table 17A shows the pattern of enzyme expression in strain RY125 as a result of incubation in ethanol. Like the wild-type, this strain was able to induce CAT, ICL and

Table 16A: Enzyme induction during incubation in 0.1% (w/v) oleic acid for YPH499 (*ALD6*)

Enzyme	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]	
	Pre-incubation	Post -incubation (16 h)
K^+-ACDH	7	11
Mg^{2+}-ACDH	49	0
CAT	0	106
ICL	0	15

Table 16B: Enzyme induction during incubation in 0.1% (w/v) oleic acid for RY124 (*ald6*)

Enzyme	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]	
	Pre-incubation	Post -incubation (16 h)
K^+-ACDH	39	0
Mg^{2+}-ACDH	0	0
CAT	0	0
ICL	0	0

Table 17A: Enzyme induction during incubation in 1% (v/v) ethanol for RY125 (*ald6*)

Enzyme	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]	
	Pre-incubation	Post -incubation (16 h)
K^+-ACDH	56	118
Mg^{2+}-ACDH	0	0
CAT	6	2111
ICL	0	234

Table 17B: Enzyme induction during incubation in 1% (v/v) ethanol for RY126 (*ald6*)

Enzyme	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]	
	Pre-incubation	Post -incubation (16 h)
K^+-ACDH	77	9
Mg^{2+}-ACDH	0	0
CAT	32	1756
ICL	1	351

Table 17C: Enzyme induction during incubation in 1% (v/v) ethanol for RY127 (*ald6*)

Enzyme	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]	
	Pre-incubation	Post -incubation (16 h)
K^+-ACDH	72	8
Mg^{2+}-ACDH	0	0
CAT	25	2129
ICL	2	371

the K^+-ACDH. This confirmed that the original disruption procedure had generated at least two different mutant strains. The remaining two strains were also investigated. The results shown in Table 17B show that for RY126, CAT and ICL were expressed but the K^+-ACDH was not. Similar results were obtained for RY127 (Table 17C). In terms of ability to induce these three enzymes, there were three different varieties of mutant strain generated by the original disruption procedure.

This investigation was also extended to include the alcohol dehydrogenases. Figure 15 shows a PAGE gel stained for ADH activity from cells of RY125 before and after incubation in ethanol. As with wild-type cells (also Figure 15), the complement of ADHs in RY125 was seen to 'switch over'. This was in contrast to RY124. ADH gels were also obtained for RY126 and RY127 and found that the ADHs in these two strains could 'switch over' as a result of incubation in ethanol (data not shown).

Regarding the response to ethanol, the mutants were clearly not identical. However, the essential similarities were that none of them were able to use ethanol as a carbon source, all of them were viable on exposure to ethanol and none of them produced acetaldehyde during this exposure. It is therefore possible to conclude from studying any *ald6* mutant that either the Mg^{2+}-ACDH or both ACDHs are required for normal growth on ethanol.

2.14 An alternative route for cytosolic acetyl-CoA production in the Mg^{2+}-ACDH mutants RY124 and RY125

For growth on glucose an alternative pathway for generation of cytosolic acetyl-CoA must exist in all the *ald6* mutants. For RY124 and indeed all of the mutants, it was concluded that the lack of repression of the K^+-ACDH during growth on 5% (w/v) glucose pointed to a role for this enzyme.

Thus, as was previously discussed, the ultimate aim of the work at this juncture

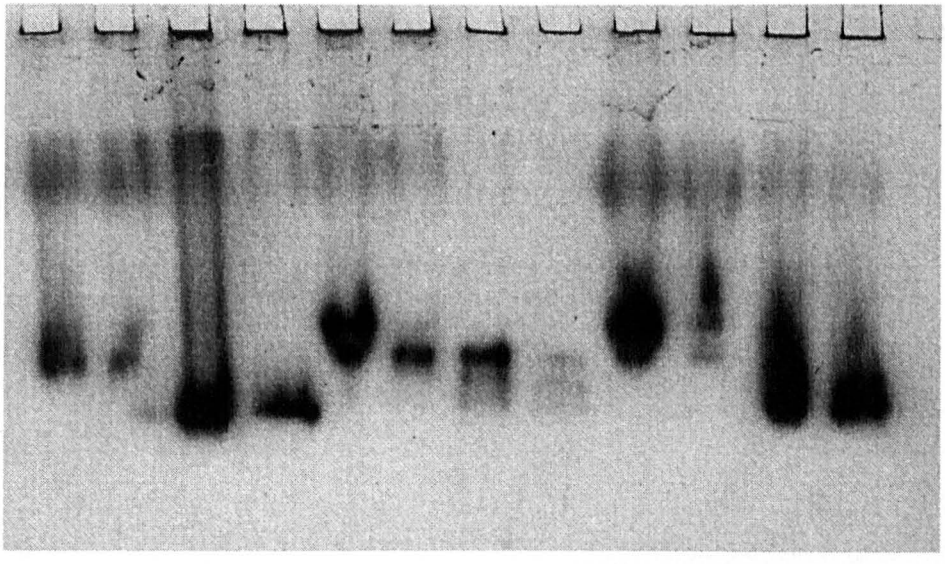

Figure 15: A PAGE gel stained for alcohol dehydrogenase activity in *S. cerevisiae* YPH499 (*ALD6*) RY124 (*ald6*) and RY125 (*ald6*).

From left to right: Lanes 1 & 2 = YPH499 - pre incubation
Lanes 3 & 4 = YPH499 - post incubation
Lanes 5 & 6 = RY124 - pre incubation
Lanes 7 & 8 = RY124 - post incubation
Lanes 9 & 10 = RY125 - pre incubation
Lanes 11 & 12 = RY125 - post incubation

Note: In each pair of lanes, the right hand lane has been loaded with 50% activity of left hand lane.

had to be to create a mutant lacking both ACDHs. This would then prove or disprove the following hypothesis: that in the absence of the Mg^{2+}-ACDH, cytosolic acetyl-CoA production and hence survival is enabled by compensating with the K^{+}-ACDH. To obtain a double mutant, it was first necessary to disrupt only the K^{+}-ACDH in the same wild type strain. This work is described in Results, section 4.

3. The existence of an ALDH family in *Saccharomyces cerevisiae*

In order to disrupt the *ALD6* gene it was first necessary to identify the gene by using the N-terminal sequence of the protein encoded by *ALD6* (Results, section 2). During this identification procedure, it became obvious that the translated products of 9 other open reading frames (ORFs) contained within the GenBank- and *Saccharomyces* Genome- Databases, shared significant homology with the product of *ALD6* i.e. Ald6p (the Mg^{2+}-ACDH). The origin of these ORFs was widespread and included the *Saccharomyces* Genome sequencing project, functional complementation studies and identification during physiological studies of osmotic stress. 8 of the ORFs were identified by conducting a FASTA search of translation of standard ORFs (ORF-Trans at SGD). The remaining ORF was identified in a TFASTA search of Genbank.

Together, these homologous gene products form the aldehyde dehydrogenase (ALDH) family in *Saccharomyces cerevisiae*. The term *aldehyde*, instead of *acetaldehyde* is used because some of these proteins could be specific for long chain aldehydes, whereas others may be specific for acetaldehyde. This ALDH family is listed in Table 18. The encoded products of each gene are phylogenetically related and the extent of this relationship is illustrated by using a phylogenetic tree (Figure 16). What follows is a short explanation of the origin of each family member.

The product of YMR110c has 26.3% homology with Ald6p over 338 amino acids. The *PUT2* gene product encodes a δ-1-pyrroline-5-carboxylate dehydrogenase and was first identified by Brandriss (1983). The product of YHR039c is most closely related to that of YMR110c and PUT2, as is the product of YBR006w. The sequence of the gene *ALD1* has been deposited in the gene bank on the basis that it restored growth on ethanol to a strain lacking the K^{+}-ACDH (Saigal *et al*, 1991). However, this gene has not been identified in the sequencing of the yeast genome and its origin and authenticity

Table 18. The aldehyde dehydrogenase (ALDH) family of *Saccharomyces cerevisiae*

ORF and / or gene name[a]	GenBank accession number	Comments on gene product	Reference
YPL061w (*ALD6*)	U39205	Cytosolic, acetaldehyde dehydrogenase, Mg^{2+}-activated	This work
YMR110c	Z49702		
YHR037w (*PUT2*)	M10029[c]	δ-1-pyrroline-5-carboxylate dehydrogenase	Brandriss (1983); Krzywicki and Brandriss (1984)
YHR039c	U00062		
YBR006w	Z35875		Feldman *et al* (1994)
ALD1	M57887	Mitochondrial	Saigal *et al* (1991)
YMR169c (*ALD3*)[b]	Z49705		
YMR170c (*ALD2*)	X85987	Induced by osmotic stress	Miralles and Serrano (1995)
YER073w	U18814	Mitochondrial (predicted)	
YOR374w (*ALD7*)	Z75282	Mitochondrial (predicted)	Chalmers *et al* (1991); Larsson *et al* (1997).

[a] The gene names *ALD1*, *ALD2*, *ALD3*, *ALD6* and *ALD7* are those currently assigned by the Saccharomyces Genome Database.

[b] The product of this gene is 92% identical to that of *ALD2*

[c] The systematic sequencing is reported in GenBank accession number U00062.

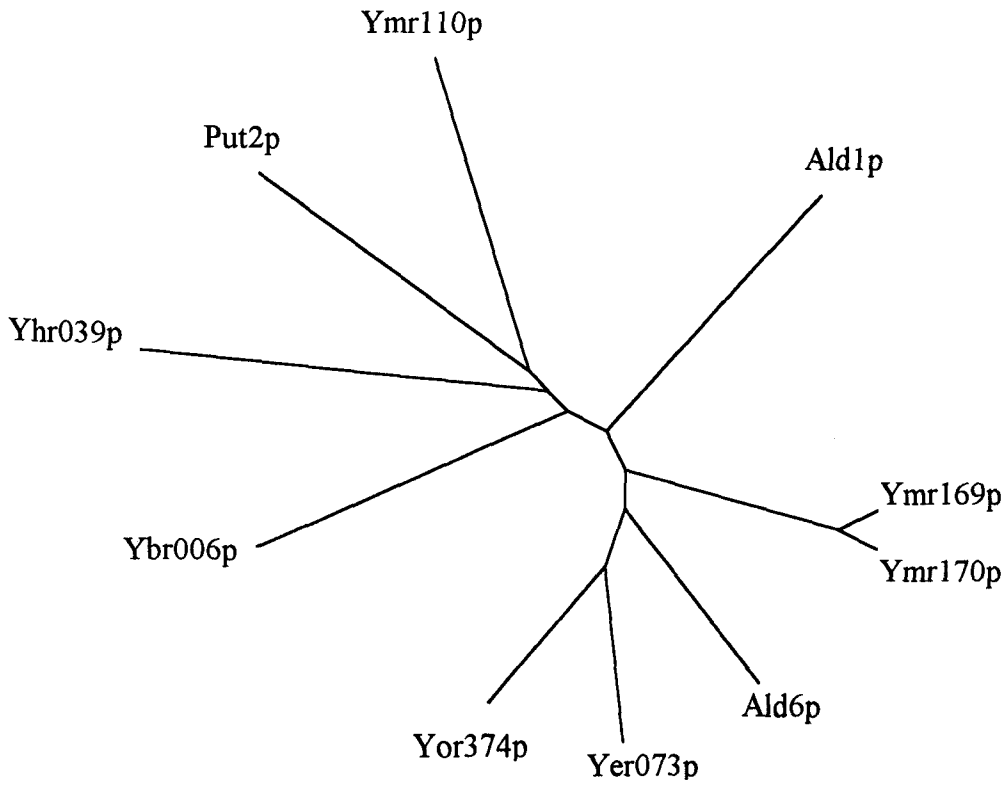

Figure 16: The phylogenetic relationship of the aldehyde dehydrogenase (ALDH) family of *S. cerevisiae* represented as a radial tree. Modified from an original version by P. Meaden (Heriot-Watt University).

are unclear. The products of YMR170c and YMR169c are very similar and possibly arose as a result of gene duplication, a suggestion supported by the fact that the genes are contiguous on chromosome XIII. The products of the two genes YER073w and YOR374w (*ALD7*) are candidates for aldehyde dehydrogenases have been proposed as mitochondrially located from their targetting sequences. Additionally, *ALD7* has been proposed to encode the K^+-ACDH from the work of two separate studies (Larsson *et al*, 1997; Chalmers *et al*, 1991). However, this designation conflicts with the proposed roles of *ALD1* and YER073w and may be a result of using different strains. This subject is dealt with in greater detail in the following section (Results, section 4).

Lindahl (1992) identified three completely conserved amino acid sequences in an analysis of mammalian ALDHs. These sequences are highly conserved amongst the majority of ALDH family members in *S. cerevisiae* (Figure 17). One suspects that such sequences could represent functional motifs or parts of them. The identification of such motifs supports the grouping of these genes and their encoded products as a family.

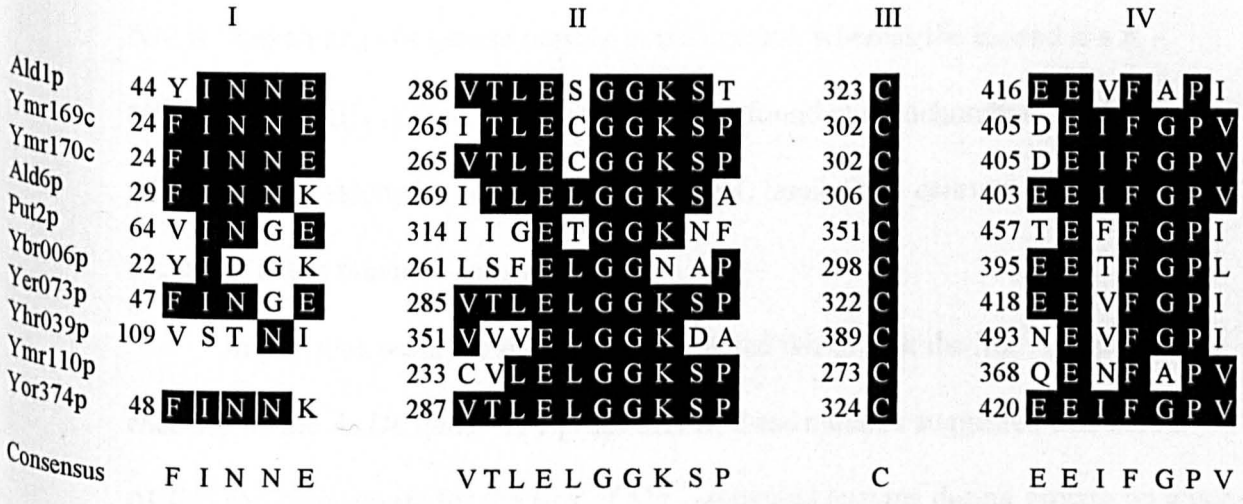

Figure 17: Conserved sequences in aldehyde dehydrogenases (ALDHs) from *S. cerevisiae.* Modified from an original version by P. Meaden (Heriot-Watt University). Aligned sequences are those cited by Lindahl (1992) for mammalian ALDHs. Numbers to the left of each sequence refer to the position of the N-terminal residue in the amino acid sequence of the protein. For each column in the alignments, the consensus amino acid is indicated by a black box.

4. Identification and disruption of the *ALD7* gene of *Saccharomyces cerevisiae*

Two acetaldehyde dehydrogenases (ACDHs) have been characterised in *Saccharomyces cerevisiae* (Llorente and de Castro, 1977). One is the Mg^{2+}-activated, $NADP^+$-linked dehydrogenase present in the cytosol, whereas the second is a K^+-activated, $NAD(P)^+$-linked enzyme (K^+-ACDH) found in mitochondria. Both are members of an aldehyde dehydrogenase (ALDH) family in *S. cerevisiae* that has been discussed in the previous section.

In previous work, strains were constructed which lack the Mg^{2+}-ACDH that is encoded by the *ALD6* gene. The properties of these mutants suggested that the K^+-ACDH can compensate for the lack of Mg^{2+}-activated enzyme during growth on glucose. One way to test this theory would be to construct a mutant lacking both enzymes. First the gene has to be identified, thus allowing the construction of a mutant lacking only the K^+-ACDH.

However, Table 18 (Results, section 3) shows that at least three genes have been proposed to encode this enzyme. *ALD1* was put forward on the basis that it could restore growth on ethanol to a mutant lacking K^+-ACDH activity (Saigal *et al*, 1991) but this sequence cannot be found either in the strain used to construct the *ALD6* disruption (P. Meaden, Heriot-Watt University; personal communication), or in the published yeast genome sequence. Two other candidates also exist based on strong homology of the deduced products to the Mg^{2+}-ACDH; these are YER073w and YOR374w. The latter gene (Genbank accession number Z75282) has been implicated from two separate studies (Chalmers *et al*, 1991; Larsson *et al*, 1997) and has been assigned the name *ALD7* by the *Saccharomyces* Genome Database.

The following experiments describe the purification of K^+-ACDH, its N-terminal sequencing and the identification and disruption of the gene. The work resolves any

ambiguity about the identity of the gene encoding the enzyme.

4.1.1 Purification of the K^+-ACDH

This enzyme was purified from several diverse strains of *S. cerevisiae* and the N-terminal amino acid sequence determined. The progress of a typical purification is shown in Table 19. This shows a purification from YPH499 of 140-fold and an overall yield of 17%. The enzyme was also purified from RY124, a commercial baker's yeast and two commercial brewing strains (BB1 and BB11).

The K^+-ACDH preparations were on average ≥95% pure as determined by densitometry of an SDS-PAGE gel. Figure 18 shows a typical gel for the enzyme purified from the parental strain YPH499. The preparation was completely stable for up to 6 months stored in liquid N_2 but on storage at room temperature the protein was seen to degrade considerably (Figure 19) presumably because of traces of proteolytic activity.

4.1.2 Sequencing of the purified K^+-ACDH

The N-terminal amino acid sequences from several strains of *S. cerevisiae* are shown in Figure 20. They are aligned with the translated products of the three genes proposed to encode the K^+-ACDH (including *ALD7*). Using this information and a TFASTA search of GenBank, the best match for YPH499 (and all of the strains) was found unequivocally to be the product of the gene *ALD7* (YOR374w). This gene is situated on the right arm of chromosome XV of *S. cerevisiae* and the product shows significant homology to other members of the *S. cerevisiae* ALDH family (Figure 16, Results, section 3).

4.1.3 Disruption of *ALD7* encoding the mitochondrial, K^+-ACDH

The *ALD7* gene was disrupted as described in Appendix 1. The disruption was verified as detailed in Appendix 2. The disruption was carried out in the haploid host strain YPH499 and yielded six mutants: RY270 to RY275.

Table 19: Purification of K^+-activated acetaldehyde dehydrogenase from *S. cerevisiae* YPH499

Step	Volume (ml)	Activty (units)	Protein (mg)	Specific activity (units mg^{-1})	Yield (%)
Extract	25	178	1190	0.15	100
Heat	24	140	437	0.32	79
DEAE-Sephacel	9	37	35	1.10	21
Hydroxyapatite	4.5	30	1.5	20	17

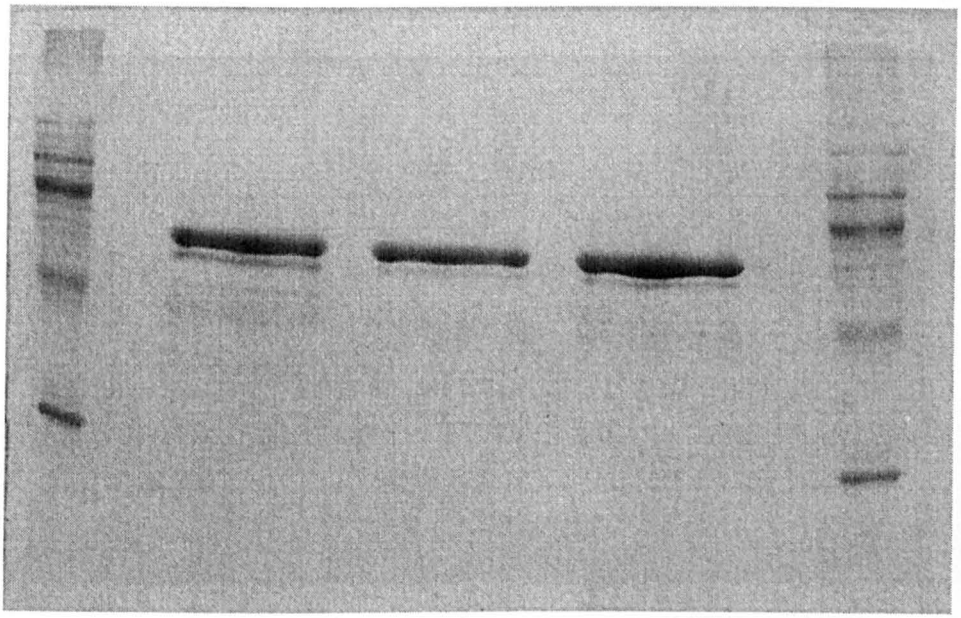

Figure 18: SDS-PAGE gel for K^+-ACDH purified from *S. cerevisiae* YPH499.

Left to right: Lane 1 - Molecular weight standards*

Lane 2 - K^+-ACDH post hydroxyapatite (3rd most active fraction)

Lane 3 - K^+-ACDH post hydroxyapatite (Most active fraction)

Lane 4 - K^+-ACDH post hydroxyapatite (2nd most active fraction)

Lane 5 - Molecular weight standards*

* Molecular weight standards were as follows:

1: Myosin (205-kd)

2: β-Galactosidase (116-kd)

3: Phosphorylase *b* (97.4-kd)

4: Bovine serum albumin (66-kd)

5: Ovalbumin (45-kd)

6: Carbonic anhydrase (29-kd)

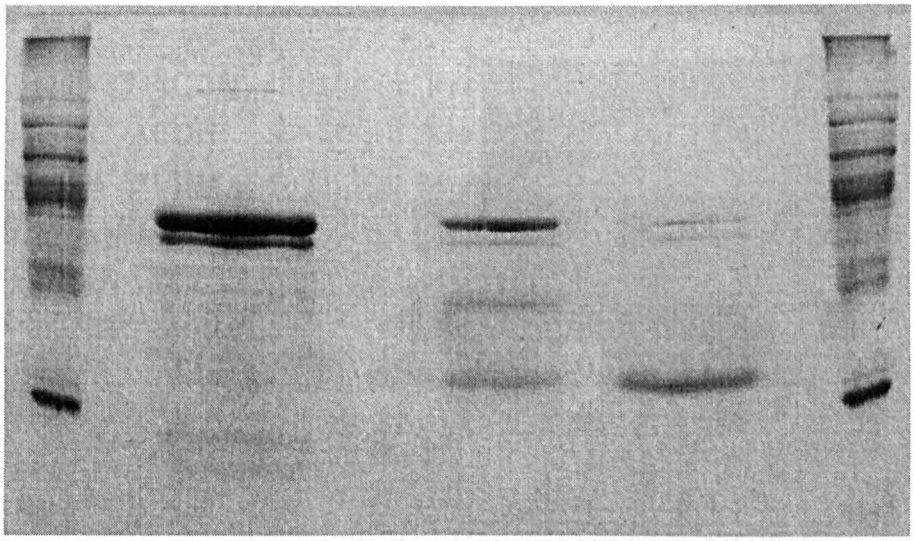

Figure 19: SDS-PAGE Gel for K^+-ACDH purified from *S. cerevisiae* YPH499. Illustration of proteolysis in preparations stored at room temperature.

Left to right: Lane 1 - Molecular weight standards*

Lane 2 - K^+-ACDH (Freshly prepared)

Lane 3 - K^+-ACDH (Stored room temp 5 min)

Lane 4 - K^+-ACDH (Stored room temp 40 h)

Lane 5 - Molecular weight standards*

* Molecular weight standards were as follows:

1: Myosin (205-kd)

2: β-Galactosidase (116-kd)

3: Phosphorylase *b* (97.4-kd)

4: Bovine serum albumin (66-kd)

5: Ovalbumin (45-kd)

6: Carbonic anhydrase (29-kd)

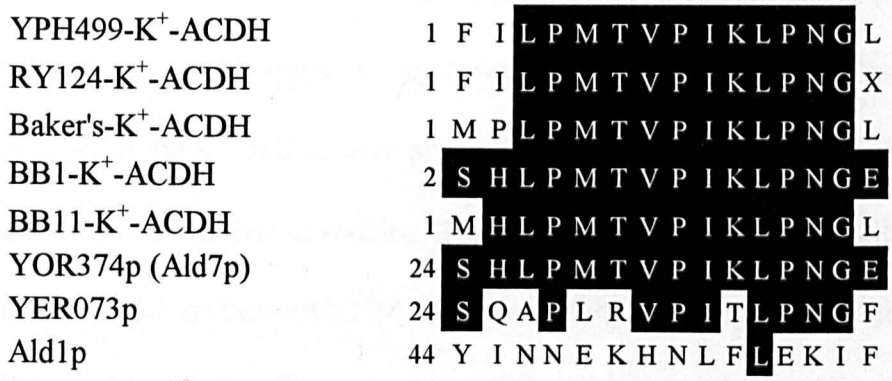

Figure 20: N-terminal amino acid sequences of (i) K^+-activated acetaldehyde dehydrogenase (ACDH) purified from strains of *S. cerevisiae* aligned with (ii) the predicted translation products of three genes proposed to encode the K^+-ACDH of *S. cerevisiae*. Numbers to the left of each sequence refer to the position of the N-terminal residue in the amino acid sequence of the protein. For each column in the alignments, the consensus sequence is indicated by a black box.

4.2 Confirmation for the loss of the K^+-ACDH

The activities of the K^+- and Mg^{2+}-ACDHs were measured in cell-free extracts prepared from both the wild-type and *ald7* mutant strains (Table 20). In mutant cells there was no measurable activity for the K^+-ACDH whereas the cytosolic Mg^{2+}-ACDH was readily detected. Although this result was expected, it was nevertheless possible that inhibitors of the K^+-ACDH were present, or that the Mg^{2+}-ACDH was masking small amounts of K^+-activated activity. The cell-free extracts from the wild-type strain and one of the *ald7* mutants (RY270) were clarified by centrifugation and then applied to an hydroxyapatite column. Two separate activity peaks were detected in an elution profile for the wild-type strain YPH499 (*ALD7*) whereas only one peak was detected for the profile from an *ald7* disruption mutant (Figure 21).

4.3 Localisation of the Mg^{2+}-ACDH in a K^+-ACDH mutant

Work with an *ald6* mutant showed that the K^+-ACDH was located in the mitochondrion as normal (Results, section 2.7.3). The same method was used to check the location of the remaining Mg^{2+}-ACDH in an *ald7* mutant. Table 21 shows that the Mg^{2+}-ACDH was located solely in the cytosol of an *ald7* mutant. The distribution of the marker enzymes pyruvate decarboxylase (PDC) and citrate synthase (CS) suggests that the fractionation experiments were reliable.

4.4 Growth of *ald7* mutants on glucose

An analysis of all six *ald7* mutants (RY270 to RY275) showed no significant difference in maximum specific growth rate, compared to the parental strain, during shake flask growth on glucose minimal media. The maximum specific growth rate (μ_{max}) for the wild-type was 0.33 h^{-1} and 0.30 h^{-1} for 0.5% (w/v) glucose and 5% (w/v) glucose respectively. The μ_{max} values obtained for the mutants fell into the range 0.29 h^{-1} to 0.32 h^{-1} and 0.27 h^{-1} to 0.31 h^{-1} for 0.5% (w/v) glucose and 5% (w/v) glucose

Table 20. **K^+- and Mg^{2+}-ACDH activities in cell-free extracts of *ALD7* and *ald7* strains of *S. cerevisiae***

	Specific Activity [nmoles min^{-1} (mg protein)$^{-1}$]	
Strain	K^+-ACDH	Mg^{2+}-ACDH
YPH499 (*ALD7*)	45	57.5
RY270 *(ald7)*	ND	58
RY271 *(ald7)*	ND	52
RY272 *(ald7)*	ND	60
RY273 *(ald7)*	ND	58
RY274 *(ald7)*	ND	59
RY275 *(ald7)*	ND	55

ND: not detected

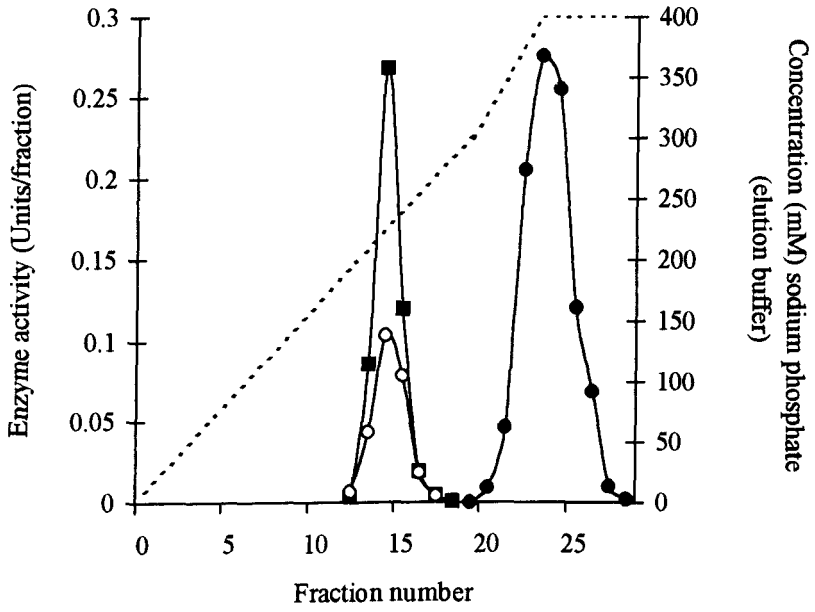

Figure 21. Separation of mitochondrial (K^+-activated) and cytosolic (Mg^{2+}-activated) acetaldehyde dehydrogenases (ACDHs) from *S.cerevisiae* by adsorption to an hydroxyapatite column (at pH 7.5). Key: ● K^+-activated, mitochondrial ACDH from YPH499 (*ALD7*); ■ Mg^{2+}-activated, cytosolic ACDH from YPH499 (*ALD7*); O Mg^{2+}-activated, cytosolic ACDH from RY270 *(ald7)*; dashed line, concentration profile (mM) of sodium phosphate in the elution buffer. Fraction volume was 1.5 ml.

Table 21: Activities of Mg^{2+}- and K^+-ACDH and marker enzymes pyruvate decarboxylase and citrate synthase in RY270 (*ALD7*)

		Activity fraction^{-1} (μmoles min^{-1})			
Fraction	Protein (mg)	PDC	CS	Mg^{2+}-ACDH	K^+-ACDH
'Total'	62.5	36.6	26.9	2.57	0
P1	4.1	0.9	12.2	0.03	0
P2	5.4	0.20	8.3	0.02	0
S	59.4	42	4.4	2.66	0

P1 and P2 are mitochondrial fractions.

S is the soluble or cytosolic fraction.

respectively.

4.5 Growth of parental and mutant cells on a range of carbon sources

Lack of the *ALD7* gene did not affect growth on glucose. However, this gene has long been attributed a role in ethanol growth and mutants lacking the gene would not be expected to grow on this compound. Growth on ethanol and other carbon sources was examined. Table 22 shows that the only real difference between wild-type and mutant cells was in the growth characteritics on ethanol. The mutant cells only grew very slowy on ethanol. This finding warranted further investigation.

4.6 Growth of an *ald7* mutant on ethanol

Cells of YPH499 and RY270 were inoculated into minimal media containing 1% (v/v) ethanol and growth was monitored. Three different starting cell densities were used and two controls were also included. One control was minimal medium containing 0.5% (w/v) glucose and the second was minimal medium containing no carbon source. The results show that for a strain lacking the K^+-ACDH, the impaired growth on ethanol was independent of starting cell density (Figures 22A and 22B). The parental strain showed a doubling time of 9.7 h whereas RY270 took more than 110 h to increase four-fold in cell number.

4.7 Viability of *ald7* mutants exposed to ethanol

As was discussed for the *ald6* mutants (Results, section 2.10), it was possible that ethanol could have a lethal effect on yeast cells, particularly the *ald7* mutants or that acetaldehyde arising from ethanol could have this effect.

4.7.1 Direct toxicity by ethanol

This was investigated by incubating cells of all the *ald7* mutants for 20 h in minimal medium containing 1% (v/v) ethanol and removing a sample at suitable time intervals to be plated onto YEPD agar. Samples were first diluted appropriately in

Table 22: Growth of wild type (*ALD7*) and mutant (*ald7*) cells on various carbon sources in minimal medium.

Culture conditions	YPH499 (*ALD7*)	RY270 (*ald7*)	RY272 (*ald7*)	RY274 (*ald7*)
Basal medium only	+	+	+	+
0.5% glucose	+++	+++	+++	+++
5.0% glucose	+++	+++	+++	+++
2% sucrose	+++	+++	+++	+++
1% acetate	+	+	+	+
2% glycerol	+	+	+	+
1% ethanol	+++	+	+	+

+ Poor or no growth, corresponding to a cell density of < 0.01 mg (dry weight) ml^{-1}

++ Average growth, corresponding to a cell density of between 0.5 and 0.8 mg (dry weight) ml^{-1}

+++ Good growth, corresponding to a cell density of between 1.2 and 1.5 mg (dry weight) ml^{-1}

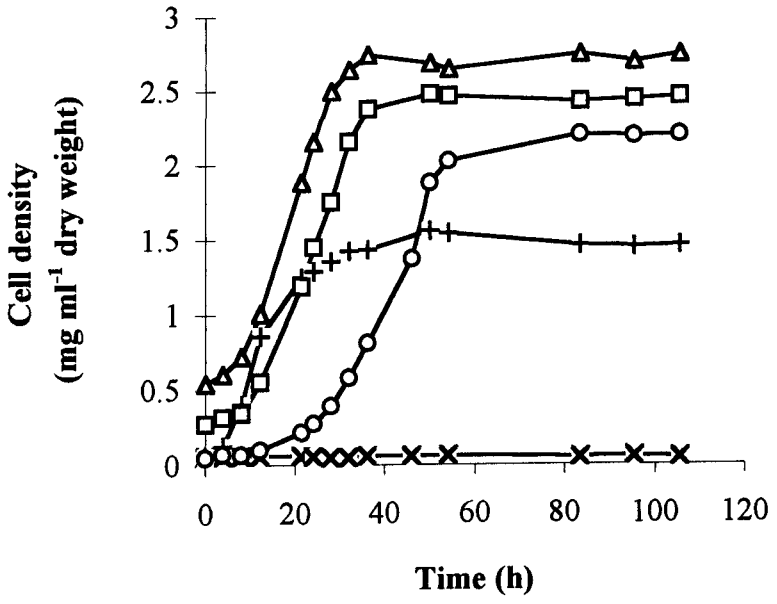

Figure 22A: Growth of YPH499 on minimal medium containing –O–, –□–, –Δ–, 1% (v/v) ethanol (inoculated at progressively higher cell densities); –x– no carbon source and –+–, 0.5% (w/v) glucose.

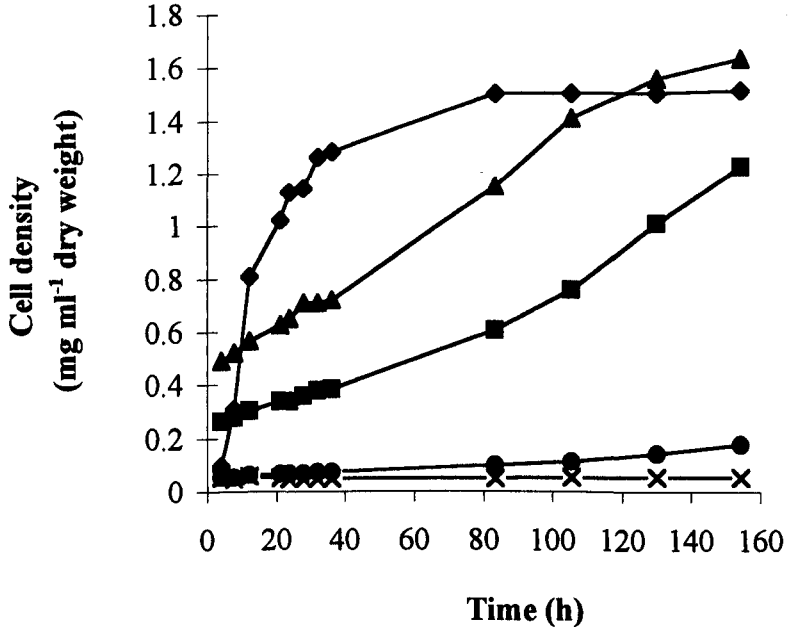

Figure 22B: Growth of RY270 on minimal medium containing –●–, –■–, –▲–, 1% (v/v) ethanol (inoculated at progressively higher cell densities); –x–, no carbon source and –◆–, 0.5% (w/v) glucose.

sterile deionised water. The viability of all six mutant strains was not diminished by this treatment (Table 23).

4.7.2 Indirect toxicity by ethanol

This was tested in mutants lacking the K^+-ACDH and in wild-type cells as a control. As an additional control, cells of both strains were incubated in 0.5% (w/v) glucose. Incubations took place over a 2 h period and samples were removed to determine the concentrations of acetate and acetaldehyde. RY270 was able to produce acetaldehyde to wild-type levels during incubation in ethanol or glucose (Figures 23A and 23B respectively) but it was not able to produce acetate during growth on either ethanol or glucose whereas the wild type could (Figures 23C and 23D respectively).

It is unlikely that acetaldehyde was toxic to RY270 because it accumulated to a similar level as in the wild-type.

4.7.3 Response of K^+-ACDH mutants to added acetaldehyde

This was tested by adding 10 mM acetaldehyde either before growth began or during active growth. For addition before growth, cells of YPH499 and RY270 were inoculated into minimal medium containing 0.5% (w/v) glucose and either 25 mM, 50 mM or 100 mM acetaldehyde added. Growth was monitored over a 48h period. Figure 24 shows that for the K^+-ACDH mutant, as the concentration of acetaldehyde increased, the initial growth lag was prolonged. However, mutant cells were able to recover and proceeded to grow at normal rates (compared to a culture with no acetaldehyde added). Growth was most affected by 100 mM acetaldehyde, but final culture density still reached 78% of that obtained by the control culture. Similar results were observed with wild-type cells (Figure 12A; Results, section 2.10.3).

Figure 25A shows that on addition of acetaldehyde to growing cultures, mutant cells were not significantly more sensitive than the wild type (Figure 25B).

Table 23: Viability of *ald7* mutants exposed to 1% (v/v) ethanol

Strain tested	Number of colony forming units ml^{-1}			% Change in viability
	0 h	4 h	20 h	
RY270	2.18×10^7	2.44×10^7	2.49×10^7	14% increase
RY271	1.85×10^7	2.27×10^7	2.07×10^7	12% increase
RY272	2.23×10^7	2.29×10^7	2.25×10^7	1% increase
RY273	2.10×10^7	2.10×10^7	2.47×10^7	18% increase
RY274	2.63×10^7	2.40×10^7	2.69×10^7	2% increase
RY275	2.14×10^7	2.6×10^7	2.49×10^7	16% increase

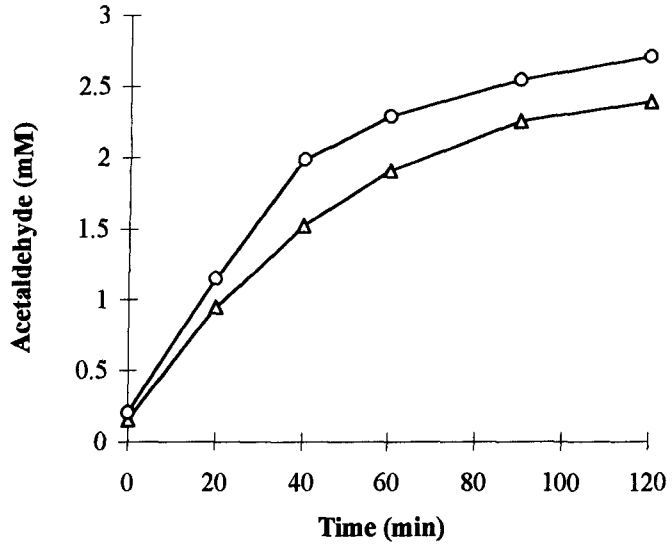

Figure 23A: Acetaldehyde secretion by YPH499 (*ALD7*) –Δ– ; and RY270 (*ald7*) –O– ; during incubation in minimal medium containing 1% (v/v) ethanol.

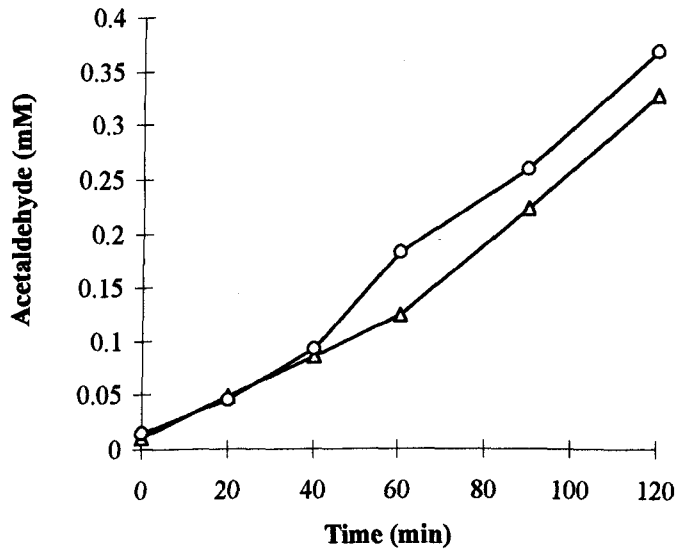

Figure 23B: Acetaldehyde secretion by YPH499 (*ALD7*) –Δ– ; and RY270 (*ald7*) –O– ; during incubation in minimal medium containing 0.5% (w/v) glucose.

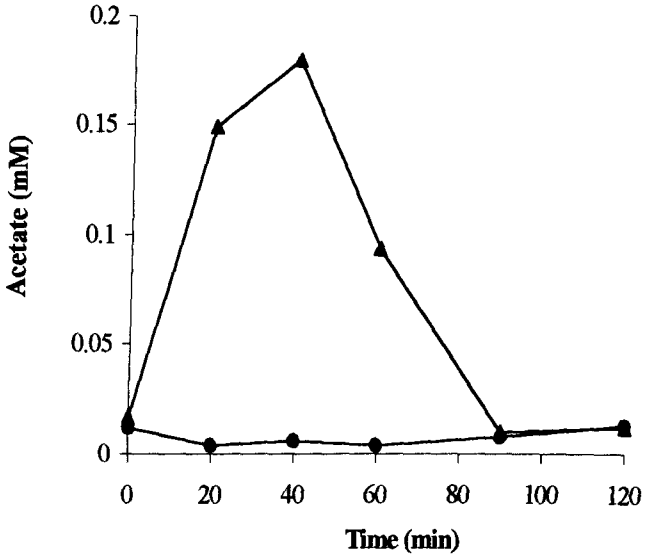

Figure 23C: Acetate secretion by YPH499 (*ALD7*) –▲– ; and RY270 (*ald7*) –●– ; during incubation in minimal medium containing 1% (v/v) ethanol.

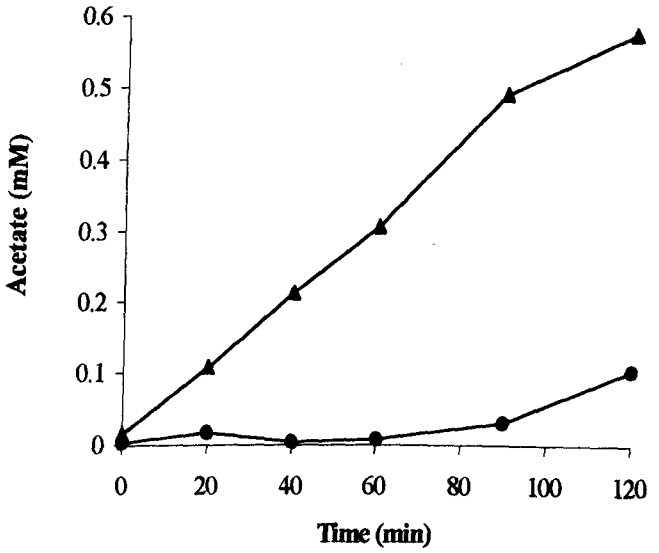

Figure 23D: Acetate secretion by YPH499 (*ALD7*) –▲– ; and RY270 (*ald7*) –●– ; during incubation in minimal medium containing 0.5% (w/v) glucose.

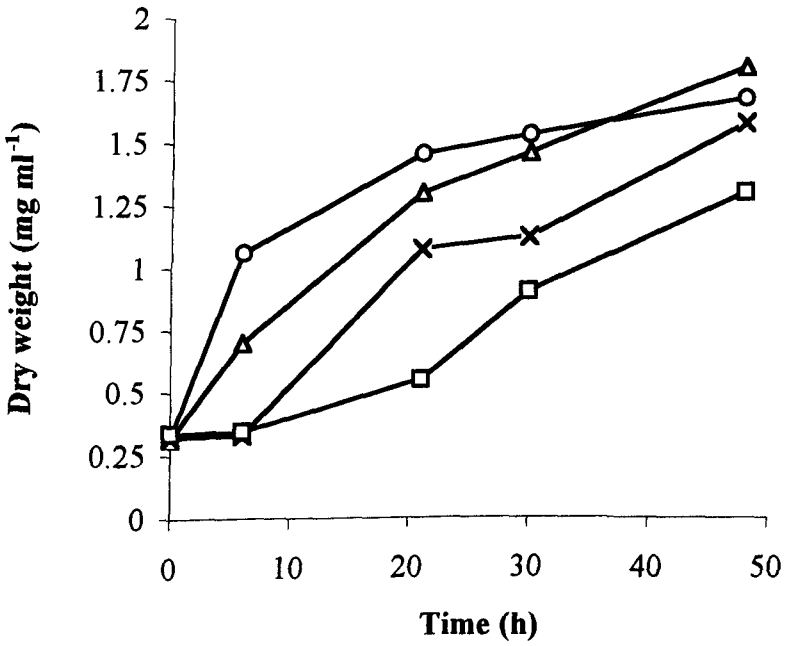

Figure 24: Growth of RY270 (*ald7*) on minimal medium containing 0.5% (w/v) glucose with the following: –O– no additions; –Δ– 25 mM acetaldehyde; –x– 50 mM acetaldehyde; –□– 100 mM acetaldehyde

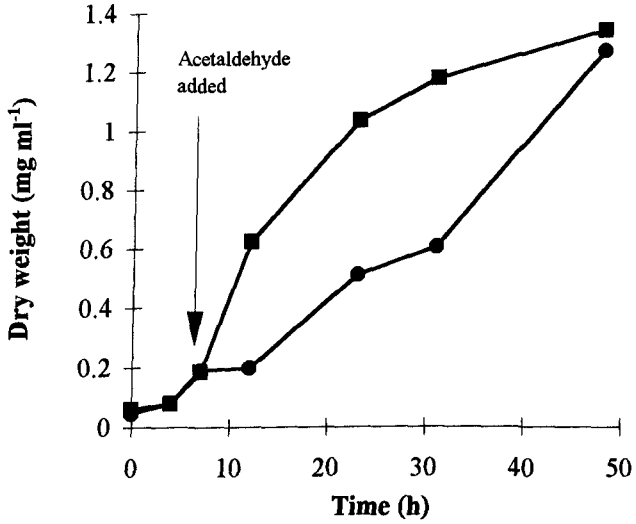

Figure 25A: Effect of adding 50 mM acetaldehyde to a growing culture of *S. cerevisiae* YPH499 (*ALD7*). –■– control culture, –●– culture with acetaldehyde added.

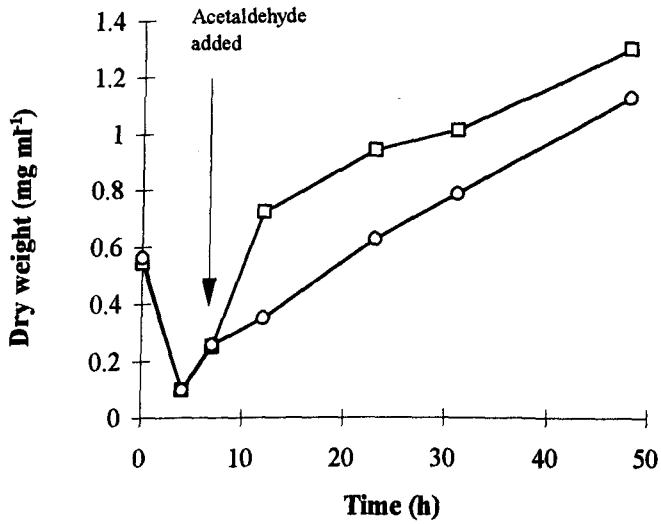

Figure 25B: Effect of adding 50 mM acetaldehyde to a growing culture of *S. cerevisiae* RY270 (*ald7*). –□– control culture, –○–culture with acetaldehyde added.

4.8 Metabolic response of RY270 (*ald7*) to ethanol

A mutant lacking the Mg^{2+}-ACDH did not produce acetaldehyde when growing on ethanol but maintained viability when exposed to this compound (Results, section 2.10.1). However, depending on the particular mutant strain used, cells differed in their ability to regulate the activity of those ethanol inducible enzymes that were tested (Results, section 2.11). This had revealed that one of the four mutants was atypical (Results, section 2.13). The response of these same ethanol-inducible enzymes in the K^+-ACDH mutants was investigated.

Initially, the ethanol inducible enzymes CAT and ICL were measured in addition to the two ACDHs. For all but one of the *ald7* mutants (RY272) , it can be seen (Table 24) that expression of CAT and ICL was very similar to that observed with wild-type cells (Table 12A; Results, section 2.11). As expected, activity of the K^+-ACDH was not detected before or after incubation. For the Mg^{2+}-activated enzyme, expression was seen in all but two of the mutants. One of these exceptions was again RY272, but in the other (RY274) both CAT and ICL were expressed in the normal way.

Secondly, changes in the expression of the ADHs were investigated in the K^+-ACDH mutants. For this, both fluorometric assays and PAGE gels were used. Like the wild-type, RY270 was able to 'switch-over' its complement of ADHs as a result of exposure to ethanol (results not shown). This is reflected in the specific activities listed in Table 25 and was expected because of the ability of this strain to induce CAT and ICL (Table 24). In contrast, 'switching-over' of ADHs was not found in RY272 (data not shown) which was in good agreement with its inability to induce CAT and ICL. Again, this was predictable from the specific activities given in Table 25. Not surprisingly, RY271 and RY273 to RY275 were all capable of inducing the ADH isozymes II and III. This was evident from PAGE gels (not shown) and the specific activities (Table 25).

Table 24: Enzyme induction during ethanol incubation for *S. cerevisiae*

Strain	Enzyme	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]	
		Pre-incubation	Post-incubation
YPH499 (*ALD7*)	CAT	0	1780
	ICL	0	249
	Mg^{2+}-ACDH	66	118
	K^{+}-ACDH	7	300
RY270 *(ald7)*	CAT	15	604
	ICL	0	189
	Mg^{2+}-ACDH	58	82
	K^{+}-ACDH	0	0
RY271 *(ald7)*	CAT	4	1131
	ICL	0	246
	Mg^{2+}-ACDH	50	61
	K^{+}-ACDH	0	0
RY272 *(ald7)*	CAT	0	2
	ICL	0	0
	Mg^{2+}-ACDH	84	36
	K^{+}-ACDH	0	0
RY273 *(ald7)*	CAT	4	1211
	ICL	0	287
	Mg^{2+}-ACDH	56	67
	K^{+}-ACDH	0	0
RY274 *(ald7)*	CAT	5	1221
	ICL	0	233
	Mg^{2+}-ACDH	66	63
	K^{+}-ACDH	0	0
RY275 *(ald7)*	CAT	3	1319
	ICL	0	261
	Mg^{2+}-ACDH	57	71
	K^{+}-ACDH	0	0

Table 25: Total alcohol dehydrogenase activity in wild-type and *ald7* mutant cells of *S. cerevisiae*

Strain	Culture conditions	Specifc activity [μmoles min^{-1} (mg protein)^{1}] Using 5 mM ethanol*	Using 1 M ethanol*	Ratio of activities
YPH499 (*ALD7*)	Pre-incubation	0.22	0.70	3.2
	Post-incubation	2.61	4.50	1.7
RY270 (*ald7*)	Pre-incubation	0.16	0.65	4.1
	Post-incubation	2.19	4.02	1.8
RY271 (*ald7*)	Pre-incubation	0.56	1.96	3.5
	Post-incubation	3.44	7.20	2.1
RY272 (*ald7*)	Pre-incubation	0.94	3.07	3.3
	Post-incubation	1.47	5.42	3.7
RY273 (*ald7*)	Pre-incubation	0.51	1.75	3.4
	Post-incubation	2.58	4.92	1.9
RY274 (*ald7*)	Pre-incubation	0.60	2.23	3.7
	Post-incubation	0.42	0.98	2.3
RY275 (*ald7*)	Pre-incubation	0.52	1.85	3.6
	Post-incubation	4.03	6.67	1.7

* Using this concentration of ethanol as substrate in the fluorometric assay.

It was evident that in this range of mutants, only one out of six was atypical with respect to ethanol catabolism (RY272). Despite this parallel with the *ald6* mutants, it remains surprising because the six strains were derived from three independent transformants of the diploid strain. Thus, RY272 and RY273 share the same parent and should in theory be identical.

4.9 Conclusions regarding the role of *ALD7*

The results presented in this section have confirmed the identity of the gene encoding the K^+-ACDH in a range of *S. cerevisiae* strains as *ALD7*. This finding is consistent with previous work (Chalmers *et al*, 1991; Larsson *et al*, 1997) but not with that of Saigal *et al* (1991) who proposed *ALD1*. The origin and role of *ALD1* remain obscure.

This study has examined the role of the K^+-ACDH by analysing the phenotype of mutants deleted for *ALD7*. The *ald7* mutants showed no measurable activity for the enzyme. Deletion of the gene had no detectable effect on the yeast during growth on glucose, in contrast to the behaviour observed in *ald6* mutants (Results, section 2).

However it is clear that the K^+-ACDH does play a role during growth on ethanol since deletion of *ALD7* altered the growth of mutants on this substrate. This role has until now been speculative (Jacobson and Bernofsky, 1974), especially since the Mg^{2+}-ACDH may also function in ethanol catabolism from the work on *ald6* mutants (Results, section 2). It appears that both ACDHs are required for growth on ethanol, with neither able to compensate for the loss of the other. This contrasts with the findings from work on alcohol dehydrogenases (Lutstorf and Megnet, 1968). A trivial explanation for lack of growth on ethanol has been ruled out, namely, that of accumulation of acetaldehyde to toxic levels, since viability was maintained in *ald7* mutants exposed to ethanol. This was also true for *ald6* mutants (Results, section 2.10). Thus from work on Mg^{2+}-ACDH

mutants and K^+-ACDH mutants, it is apparent that each of the ACDHs has a distinct physiological role, in that neither can completely replace the other.

4.10 Osmotolerance: another proposed role for the K^+-ACDH of *S. cerevisiae*

Some workers have attributed a key role to the K^+-ACDH in the response of yeast to osmotic stress, particularly when induced by high extracellular concentrations of NaCl (Blomberg & Adler, 1989; Larsson *et al*, 1997). Production of internal solutes such as glycerol can help to overcome such stress and in producing glycerol, the activity of glycerol 3-phosphate dehydrogenase (G3PDH) is increased and the concentration of cytosolic NADH diminishes. It has been suggested that because the reaction catalysed by the K^+-ACDH produces NADH, this enzyme is induced by osmotic stress. Blomberg & Adler (1989) showed that on exposure of *S. cerevisiae* to 0.7 M sodium chloride, the specific activity of the K^+-ACDH increased two-fold and that of the G3PDH increased six-fold. These changes correlated with secretion of acetate (the product of the reaction catalysed by the K^+-ACDH) and glycerol (the product of the pathway in which G3PDH is involved). The involvement of the K^+-activated enzyme is surprising because of its mitochondrial location. A cytosolic enzyme might seem more likely to function in this role. The theory of Blomberg and Adler (1989) was tested here by using a mutant which was devoid of the K^+-ACDH enzyme.

4.10.1 Attempts to osmotically stress wild-type and *ald7* mutant cells

It was important to first establish an effective concentration of sodium chloride to use i.e. one that would cause approximately 50% reduction in growth. It was also decided to test the efficacy of sorbitol as an agent of osmotic stress. This compound has an advantage, because secondary effects on metabolism that may be caused by high concentrations of NaCl would not occur. The results in Table 26A show that using

1.2 M NaCl caused approximately 60% to 70% reduction in growth rate. To obtain a similar level of growth inhibition with sorbitol a concentration of 2 M was used (Table 26B).

NaCl was used in an attempt to osmotically stress the wild-type and *ald7* mutant (RY270). Cells were processed as described in Materials and Methods (section 1.3.5.3). Extracts were prepared from cells that were not incubated, cells that were incubated in glucose only and cells that were incubated in glucose with sodium chloride (1.2 M). The activities of the two ACDHs and G3PDH were then measured in each of these extracts (Table 27). In addition, the acetate secreted by cells incubated in either glucose or glucose with sodium chloride was measured (Figure 26). It can be seen from the enzyme activities (Table 27A) that in the wild-type (YPH499), the K^+-ACDH (and Mg^{2+}-ACDH) and G3PDH were not significantly induced when exposed to NaCl. Similar results were seen with mutant cells (Table 27B). Blomberg & Adler (1989) observed a six-fold increase in G3PDH activity and a doubling of K^+-ACDH activity between cells that had not been incubated and cells that had been exposed to NaCl. Not surprisingly then, in this study, cells incubated in glucose and NaCl did not secrete more acetate compared to cells incubated in just glucose (Figure 26).

Likely reasons for the failure of this experiment were considered. One was that the concentration of NaCl was too high and therefore inhibitory; the second was that the wild-type in this study was not an osmotolerant strain. Blomberg (1997) reported that the osmotolerant phenotype is strain-specific in *S. cerevisiae.*

To test the first explanation, the NaCl concentration was lowered to 0.7 M (that used by Blomberg & Adler, 1989). Neither the pattern of acetate secretion nor the induction of G3PDH and K^+-ACDH were as reported by Blomberg & Adler (1989) (results not shown). The strain used here did not possess an osmotolerant phenotype or

Table 26A: Growth inhibition of *ALD7* and *ald7* strains of *S. cerevisiae* by sodium chloride

Culture conditions (All minimal medium)	Maximum specific growth rate (h^{-1})	
	YPH499 (*ALD7*)	RY270 (*ald7*)
0.5% (w/v) glucose	0.32	0.29
0.5% glucose + 0.4 M NaCl	0.27	0.27
0.5% glucose + 0.8 M NaCl	0.17	0.18
0.5% glucose + 1.2 M NaCl	0.11	0.11

Table 26B: Growth inhibition of *ALD7* and *ald7* strains of *S. cerevisiae* by sorbitol

Culture conditions (All minimal medium)	Maximum specific growth rate (h^{-1})	
	YPH499 (*ALD7*)	RY270 (*ald7*)
0.5% (w/v) glucose	0.30	0.30
0.5% glucose + 0.4 M sorbitol	0.30	0.30
0.5% glucose + 0.8 M sorbitol	0.25	0.27
0.5% glucose + 1.2 M sorbitol	0.22	0.22
0.5% glucose + 2.0 M sorbitol	0.12	0.12

Table 27A: Enzyme induction during incubation of wild-type cells in 1.2 M NaCl

	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]		
Enzyme	Pre-incubation	Post-incubation (– NaCl)	Post-incubation (+ NaCl)
G3PDH	136	56	138
K^+-ACDH	97	72	107
Mg^{2+}-ACDH	51	44	52

Table 27B: Enzyme induction during incubation of RY270 cells in 1.2 M NaCl

	Specifc activity [nmoles min^{-1} (mg protein)$^{-1}$]		
Enzyme	Pre-incubation	Post-incubation (– NaCl)	Post-incubation (+ NaCl)
G3PDH	117	79	110
K^+-ACDH	0	0	0
Mg^{2+}-ACDH	60	49	50

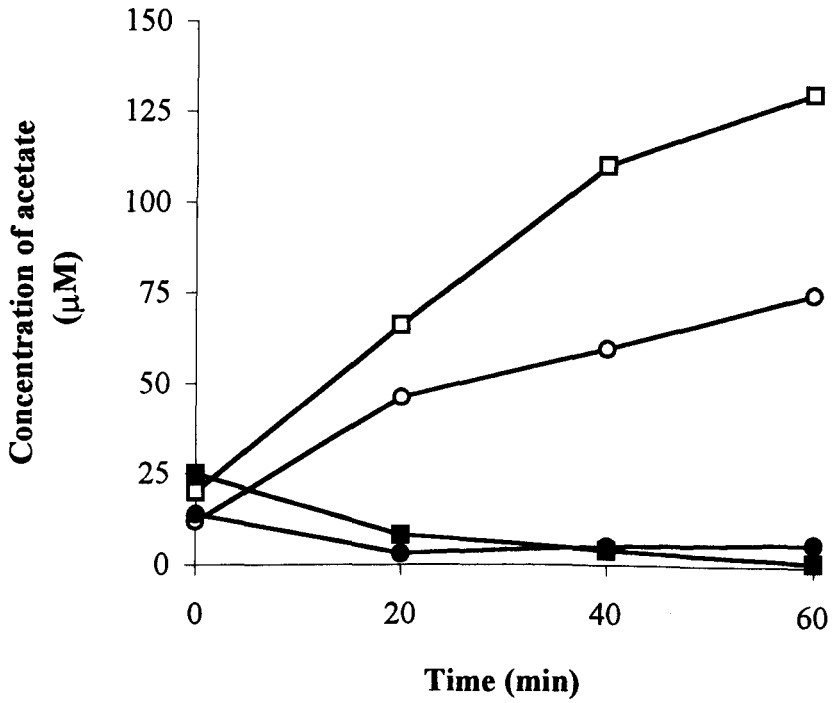

Figure 26: Acetate secretion by wild-type cells during –□– incubation in 0.5% (w/v) glucose and –O– incubation in 0.5% (w/v) glucose + 1.2 M NaCl. Acetate secretion by *ald7* mutant cells during –■– incubation in 0.5% (w/v) glucose and –●– incubation in 0.5% (w/v) glucose + 1.2 M NaCl

did not respond quantitatively in the same way.

4.10.2 Attempts to osmotically stress a known osmotolerant strain of *S. cerevisiae*

The experiment was conducted using *Saccharomyces cerevisiae* strain Y41. This was the strain used by Blomberg and Adler (1989). The experiment gave the expected results with this strain. Both the pattern of enzyme induction (Table 28) and acetate secretion (Figure 27) were similar to those published by Blomberg & Adler (1989) but the degree of enzyme expression and acetate secretion was less in this study.

Additionally the K^+-ACDH activity was not seen to increase in this laboratory. Blomberg & Adler (1989) reported that the specific activity increased from 13 to 26 nmoles min^{-1} (mg protein)$^{-1}$. However, their assay was a spectrophotometric one and the ACDH activities are difficult to measure with this method. The assay used here was a fluorometric one which is far more sensitive and accurate.

Overall, the findings with strain Y41 led to the conclusion that the strain used in this study (YPH499) offered no response to NaCl compared to Y41. Hence it was not possible to make any general conclusions regarding the role of the K^+-ACDH by using an *ald7* mutant.

Table 28: Enzyme induction during incubation of *S. cerevisiae* Y41 in 0.7 M NaCl

	Specifc activity [nmoles min^{-1} $(mg\ protein)^{-1}$]		
Enzyme	Pre-incubation	Post-incubation (– NaCl)	Post-incubation (+ NaCl)
G3PDH	84	79	319
K^+-ACDH	40	36	42
Mg^{2+}-ACDH	166	130	149

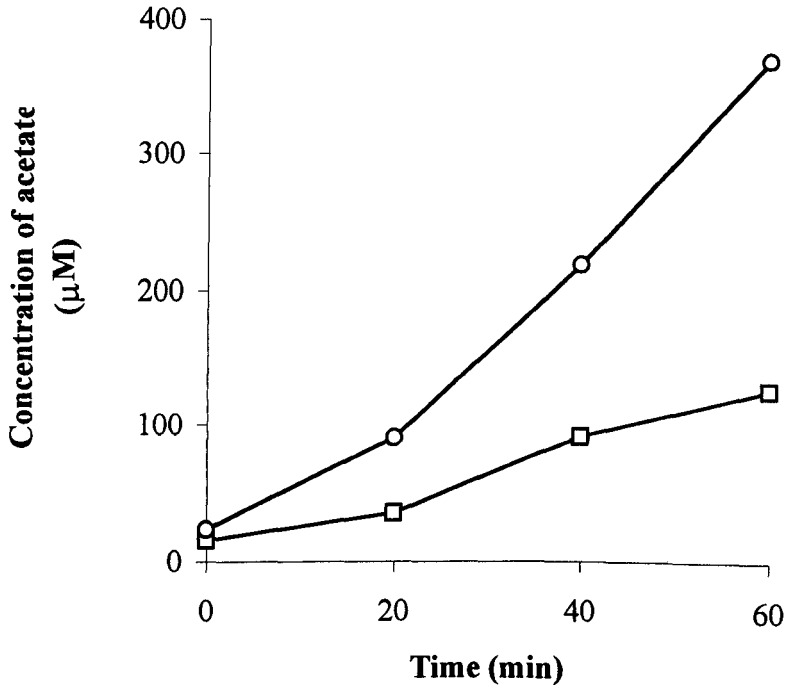

Figure 27: Acetate secretion by *S. cerevisiae* Y41 during –□– incubation in 0.5% (w/v) glucose and –O– incubation in 0.5% (w/v) glucose + 0.7 M NaCl

5. Investigation of the PDH bypass in *S. cerevisiae* under brewing conditions

Several groups have studied the contribution that the PDH bypass makes to the production of cytosolic acetyl-CoA (Holzer, 1961; Pronk *et al*, 1994; van den Berg & Steensma, 1995; Flikweert *et al*, 1996). Many of these studies have used laboratory strains of yeast grown on glucose under defined conditions.

To date little or no work has focused on the enzymes of the PDH bypass under brewing conditions. Many compounds that are central to beer flavour and aroma are derived from the product of this pathway (acetyl-CoA). The environmental conditions presented to yeast in the brewing process are varied and complex and in return, require a range of physiological responses by the micro-organism. It was thought that an investigation into the enzymology of the PDH bypass would assist in understanding the role that it plays during brewery fermentation.

5.1 A study of the PDH bypass in yeast during a pilot scale lager fermentation

A commercial pilot scale lager fermentation (800 l) was set up using an authentic lager wort (prepared at a starting gravity of 1060°) and a brewing strain of yeast (BB11). The vessel was pitched with yeast obtained from a full-scale brewery. From the point of pitching until the yeast began to flocculate, samples were withdrawn from the vessel and processed as described in Material and Methods (section 1.3.4).

Figures 28A to 28F show the specific activity profiles throughout the sampling period for pyruvate decarboxylase, acetyl-CoA synthetase, alcohol dehydrogenase, the Mg^{2+}-ACDH, carnitine acetyl-transferase and the K^{+}-ACDH respectively. The activities given at time zero are the activities determined in the pitching yeast. The results show unequivocally that under brewing conditions, yeast possess the necessary enzymes to produce cytosolic acetyl-CoA. PDC and ACS were present throughout the phase of active growth (Figures 28A and 28B respectively), illustrating that the brewing yeast has

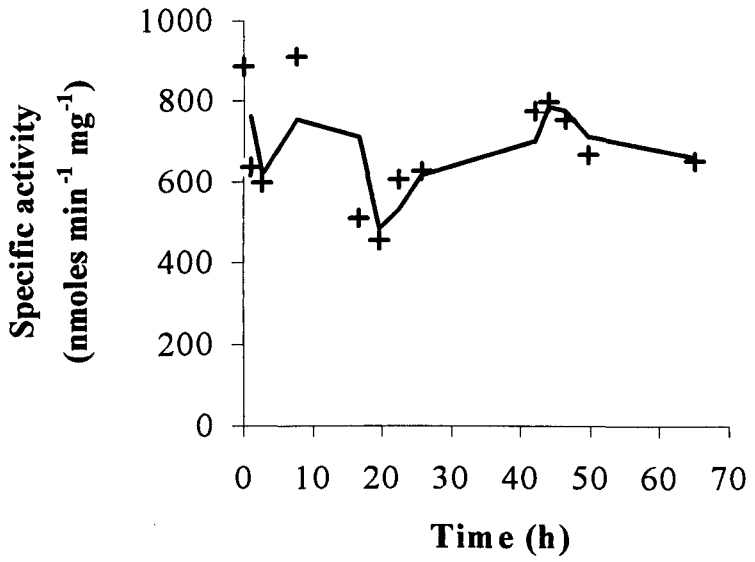

Figure 28A: Specifc activity profile for pyruvate decarboxylase during a pilot scale lager fermentation

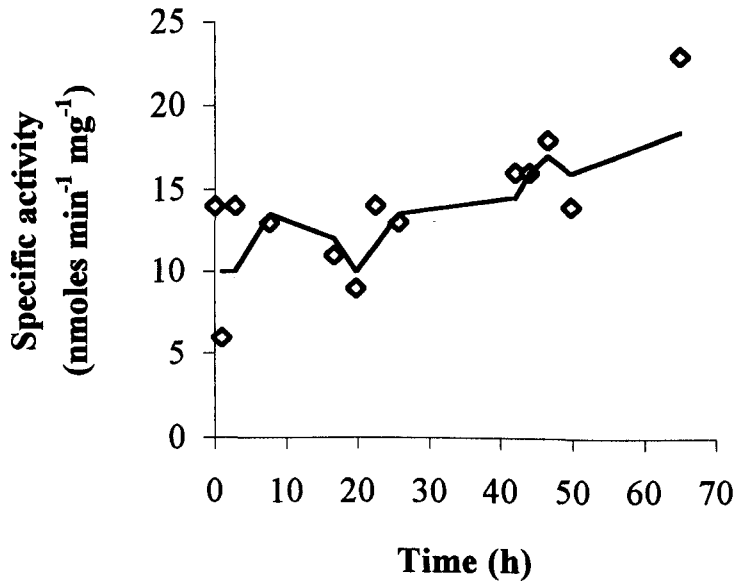

Figure 28B: Specifc activity profile for acetyl-CoA synthetase during a pilot scale lager fermentation

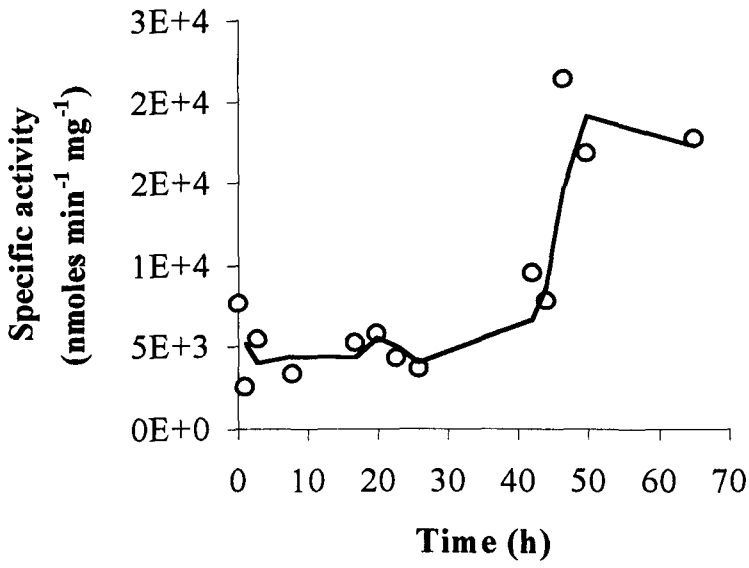

Figure 28C: Specifc activity profile for alcohol dehydrogenase I during a pilot scale lager fermentation

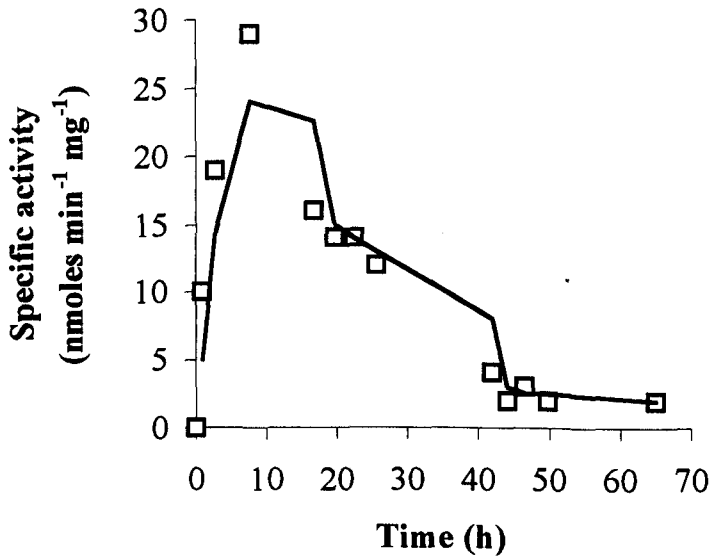

Figure 28D: Specifc activity profile for Mg^{2+}-ACDH during a pilot scale lager fermentation

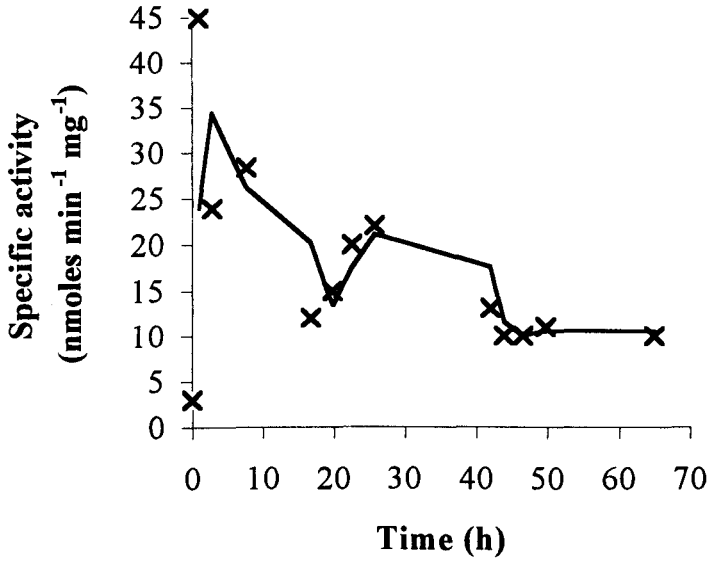

Figure 28E: Specifc activity profile for carnitine acetyl-transferase during a pilot scale lager fermentation

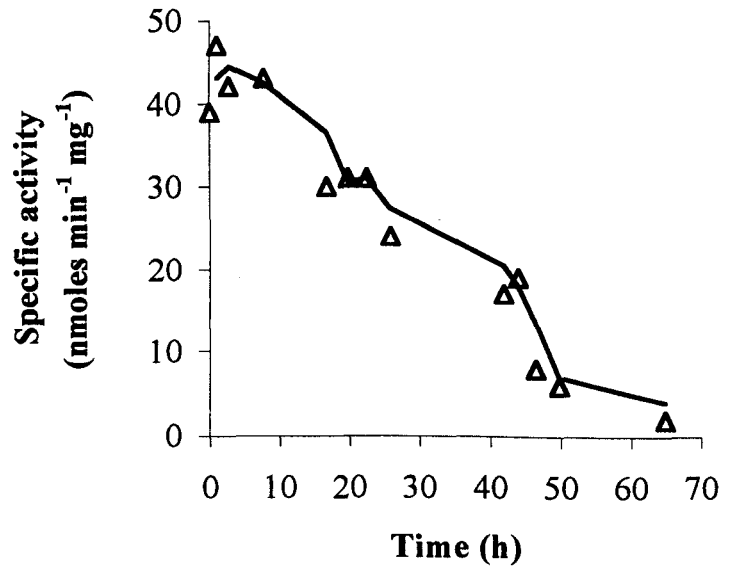

Figure 28F: Specifc activity profile for K^+-ACDH during a pilot scale lager fermentation

the potential to make cytosolic acetyl-CoA by this pathway during the aerobic (approximately the first 10 h (data not shown)) and anaerobic phase of fermentation. PDC must also be active to enable production of ethanol. Consistent with this was the presence of ADH (Figure 28C). This enzyme became more active as the fermentation proceeded, which is in good agreement with a typical profile for ethanol production (Boulton, 1991).

The Mg^{2+}-ACDH showed variable activity throughout the sampling period (Figure 28D). An early peak at 10 h was followed by a rapid decrease, with very low specific activity after 45 h. This was surprising, since several workers have identified the enzyme as a constitutive one (Mauricio *et al*, 1997; Mauricio & Ortega, 1993; Llorente and de Castro, 1977). The finding raised doubts as to whether or not the yeast could continue to produce acetyl-CoA in the absence of the enzyme. At the lowest point of Mg^{2+}-ACDH activity, the vessel was anaerobic (data not shown). It is assumed that acetyl-CoA could not be produced by the action of pyruvate dehydrogenase with export to the cytosol via CAT. This is consistent with the small amount of CAT activity observed (Figure 28E). The presence of any CAT activity was surprising, because the typical concentration of maltose that is present in wort should repress this enzyme as glucose would (Claus *et al*, 1983). There seemed to be no compensating change in K^+-ACDH activity in response to the change in Mg^{2+}-ACDH (Figure 28F). The presence of K^+-ACDH some 30 h after total oxygen depletion was unexpected because of the reported repression of this enzyme under hypoxia (Llorente and de Castro, 1977). In addition, this enzyme is known to be repressed by glucose and so the presence of maltose (even more repressing than glucose) would be expected to exhibit the same effect. However, it seemed possible that the K^+-ACDH was active in order to counteract the osmotic stress induced by the high sugar content (typically 150 g l^{-1}) of wort (see

Results, section 4.10).

The above results are consistent with the potential to produce cytosolic acetyl-CoA early on in the fermentation or possibly during the aerobic phase only. Thereafter, it can be envisaged that the absence of the Mg^{2+}-ACDH and the lack of compensation by either the K^{+}-activated enzyme or CAT could prevent further production of acetyl-CoA. Perhaps the acetyl-CoA is produced largely during the early part of fermentation and then used to derive the unsaturated fatty acids and sterols that are essential for anaerobic growth (Andreasen and Stier; 1953, 1954). The intracellular concentration of acetyl-CoA reportedly increases later on in a fermentation (Quain *et al*, 1981) which conflicts with the results obtained here, since there would be no obvious route for production.

The patterns of ACDH activity (both Mg^{2+}- and K^{+}-activated) during the fermentation left two questions unanswered; (i) does the Mg^{2+}-ACDH activity remain at or near zero for the remainder of the fermentation? and (ii) when does the K^{+}-ACDH activity increase to the level that is seen in the pitching yeast? It was also important to examine the reproducibility of these profiles and so it was decided to follow a full lager pilot scale fermentation and sample as before. On this occasion, only the activity of ADH and the ACDHs was determined. Additionally, supernatants (beer) from cell samples were retained and analysed for the presence of organic acids, alcohols, carbonyls and flavour compounds. This it was thought, might establish a correlation for ACDH / ADH activity and production of key metabolites, such as acetyl-CoA and those essential to beer flavour and aroma.

5.2 Attempts to correlate *in vitro* ADH and ACDH activity to metabolite formation during a pilot scale lager fermentation

Figure 29A shows that the activity profile for the Mg^{2+}-ACDH was indeed reproducible and that the activity did eventually fall to zero after 90 h. The K^{+}-ACDH

activity (Figure 29B) was similar to that for the first experiment (Figure 28F) but did not fall to such low levels. After reaching its lowest point, it was then seen to rise quite rapidly and fluctuate thereafter. This demonstrated that the K^+-ACDH regains its starting level of activity by the end of a fermentation, rather than during storage of the yeast (a period of typically 2 days). The profile for ADH activity (Figure 29C) also seemed to reproduce the first experiment (Figure 28), with an activity peak occurring at approximately 50 h although this seems out of place with the profiles for ethanol (Figure 29D) and esters (Figures 29E and 29F) production. It can be seen that whilst ADH activity had peaked and was falling, synthesis of these compounds had only just begun to increase. The profiles for the two ACDHs were consistent with the patterns of acetaldehyde (Figure 29G) and acetate (Figure 29H) production. The concentration of acetaldehyde peaked very early on (2 h) and then decreased, presumably as it was utilised by one or both of the ACDHs. However, as the ACDH activities decreased, acetaldehyde peaked again (50 h) and then steadily declined, which may have been due to diacetyl formation (Figure 29I) and/or ethanol formation (Figure 29D). Diacetyl is formed (via α-acetolactate) from acetaldehyde and pyruvate. Thus the production of diacetyl may correlate with the plateau of pyruvate production that is seen after approximately 50 h (Figure 29J).

Overall, it can be seen that the production of diacetyl and its derivative flavour-active compounds may well occur throughout the fermentation. However, of primary interest here was the formation of cytosolic acetyl-CoA and it appears from the ACDH profiles, that the capacity to produce this metabolite via the Mg^{2+}-ACDH diminishes after 50 h (Figure 29A). This enzyme also produces NADPH which is required for biosynthesis (such as fatty acid production). However, the K^+-ACDH remains active when the Mg^{2+}-ACDH activity has diminished and this enzyme (the K^+-ACDH) can use

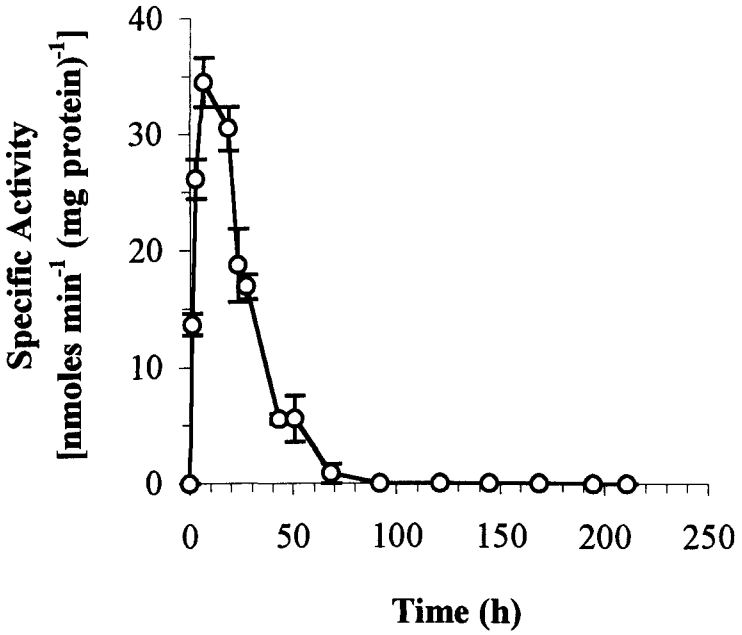

Figure 29A: Mg^{2+}-ACDH activity during a pilot-scale lager fermentaion

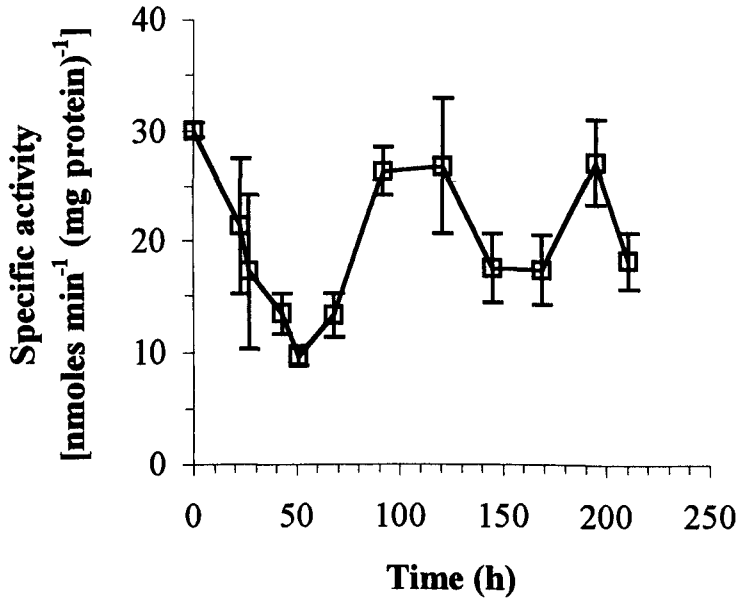

Figure 29B: K^{+}-ACDH activity during a pilot-scale lager fermentation

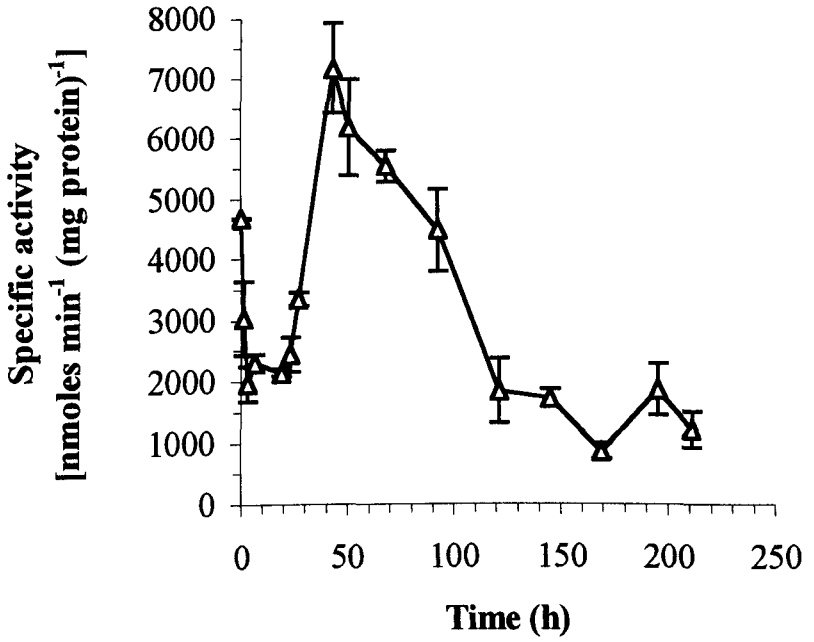

Figure 29C: ADH activity during a pilot-scale lager fermentation

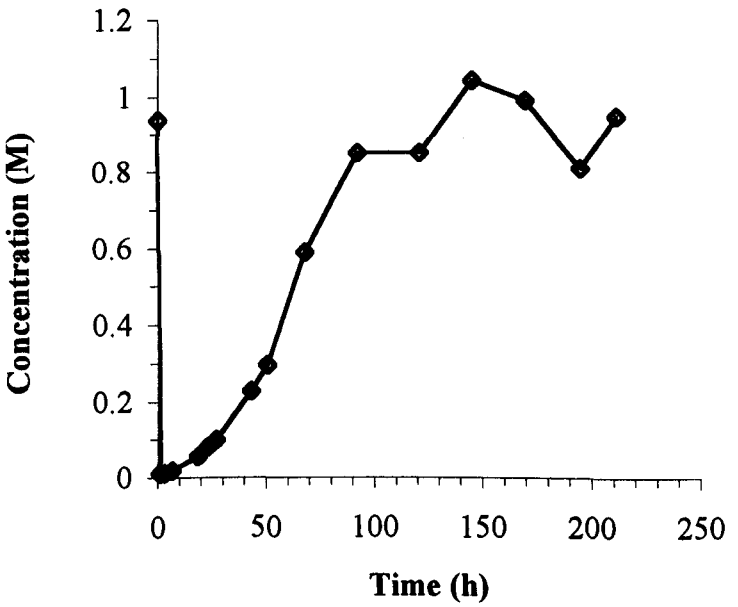

Figure 29D: Ethanol production during a pilot-scale lager fermentation

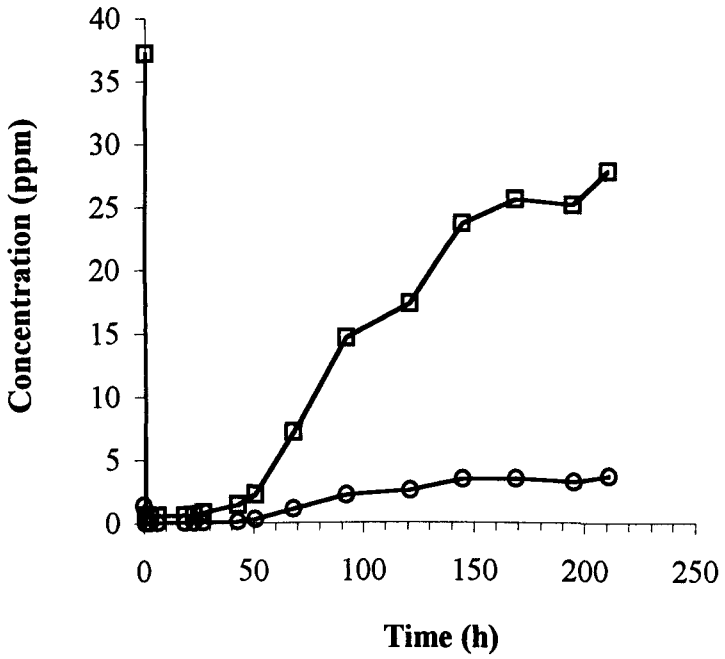

Figure 29E: Ester production during a pilot-scale lager fermentation. –□– Ethyl acetate, –O– iso amyl acetate

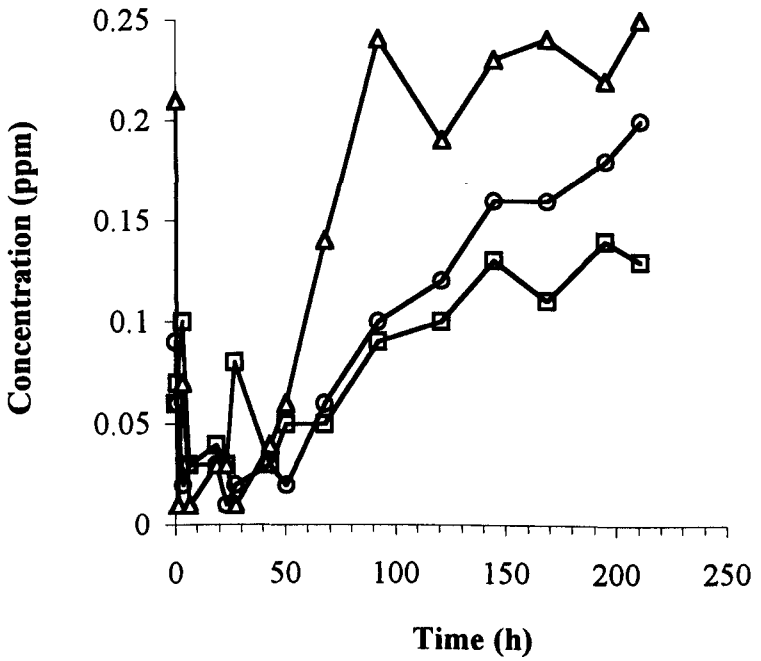

Figure 29F: Ester production during a pilot-scale lager fermentation. –□– Iso butyl acetate, –O– ethyl butyrate, –Δ– ethyl hexanoate

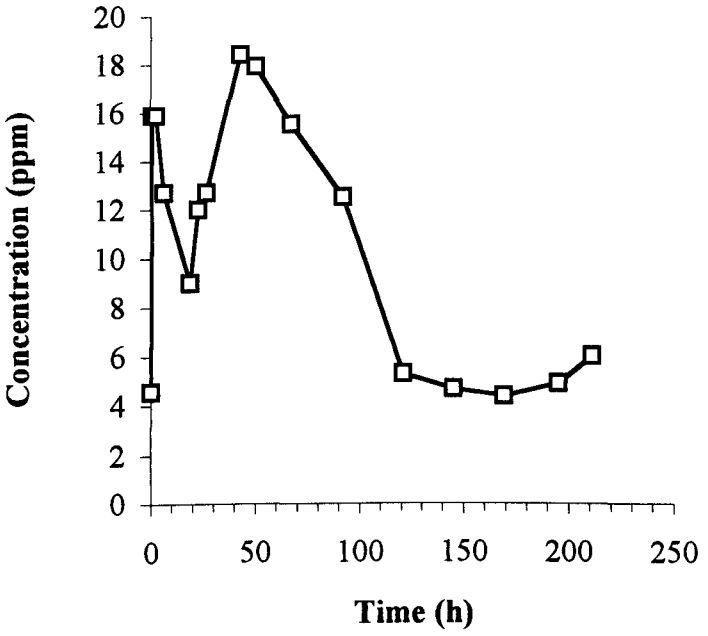

Figure 29G: Acetaldehyde production during a pilot-scale lager fermentation

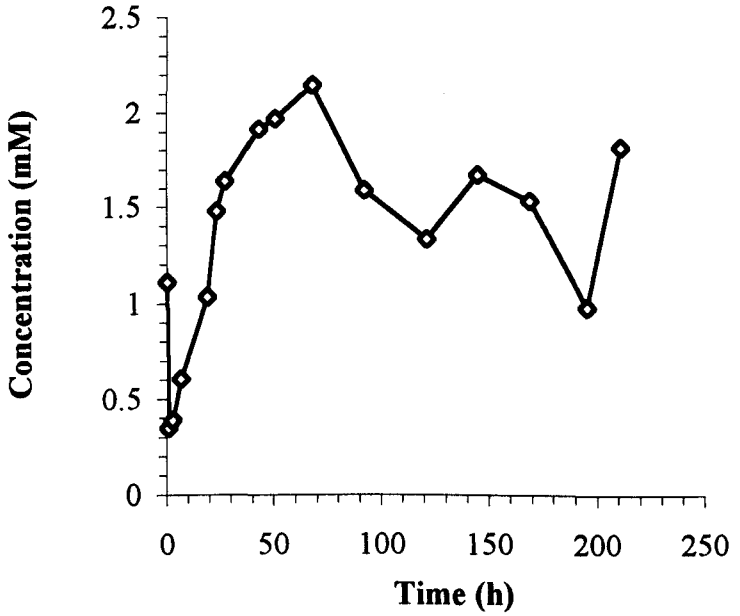

Figure 29H: Acetate production during a pilot-scale lager fermentation

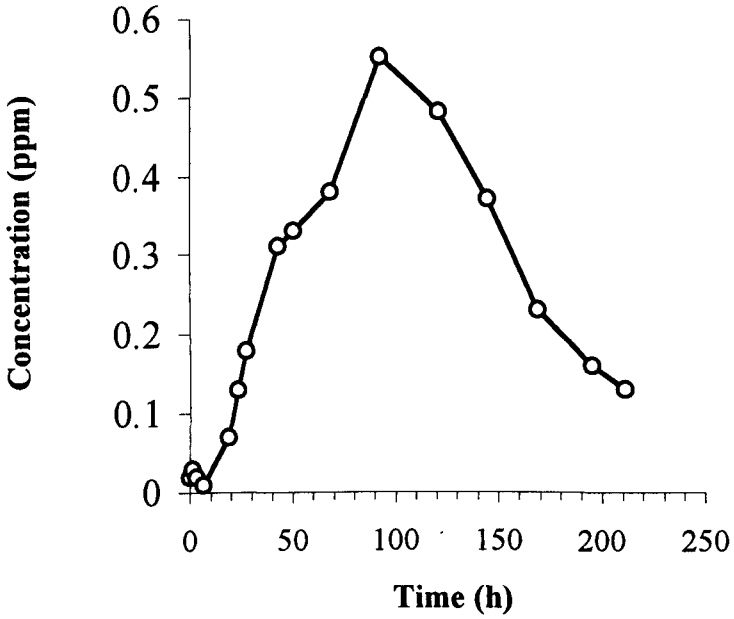

Figure 29I: Diacetyl production during a pilot-scale lager fermentation

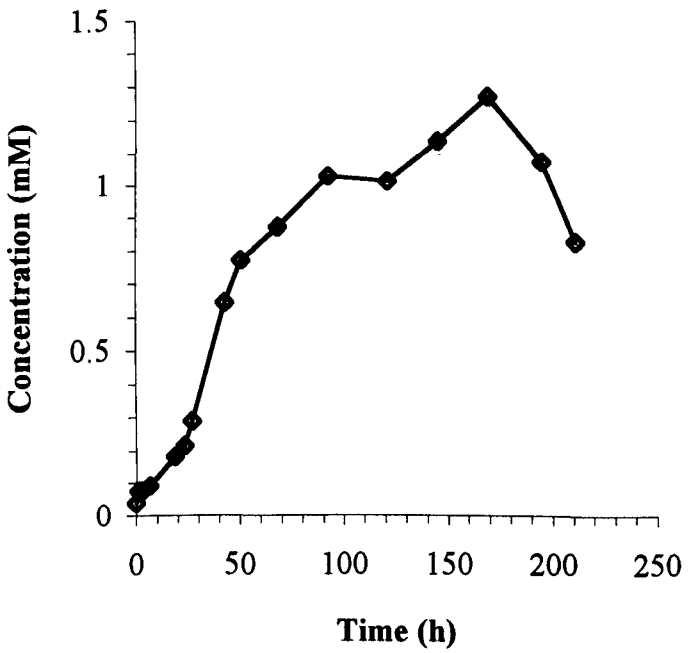

Figure 29J: Pyruvate production during a pilot-scale lager fermentation

$NADP^+$. I have found by using pure enzyme, that the K^+-ACDH functions at 20% efficiency when provided with $NADP^+$ rather than NAD^+ (results not shown). This would of course generate a mitochondrial pool of NADPH, not a cytosolic one.

Production of acetyl-CoA is essential to sterol and unsaturated fatty acid synthesis, which only occurs whilst oxygen is present. It appears likely that most acetyl-CoA production is concentrated into the early part of fermentation (which conflicts with the work of Quain *et al*, 1981) but it may continue after 50 h and be produced via the K^+-ACDH (which would agree with Quain *et al*, 1981). It is not clear though, why the Mg^{2+}-ACDH activity disappears. An obvious suggestion would be the absence of oxygen, but this enzyme is a fermentative one. The idea has not, however, been tested. Nonetheless, the activity of the ACDHs and their regulation is central to acetyl-CoA production in brewing yeast.

5.3 Further work

It was originally planned to set up a small scale mock fermentation using authentic wort but using a mutant deleted for either the K^+ or Mg^{2+}-ACDH and compare enzyme and metabolite profiles to the wild-type strain. However, this was not possible because of the inability of the wild-type to grow on maltose (YPH499 is known to not possess the *MAL* genes). To enable this work to take place, the mutations would have to be created in a strain possessing a maltose$^+$ (*MAL*$^+$) background.

However, at present, work is underway in this laboratory to investigate the factor(s) influencing the pattern of ACDH activity.

DISCUSSION

1. Studies of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae*

At the outset of this work, it was not clear whether during growth of *S. cerevisiae* on glucose, cytosolic acetyl-CoA is produced directly in the cytosol or by export from the mitochondrion. Holzer (1961) and Pronk *et al* (1994) had suggested that only a small proportion of cytosolic acetyl-CoA was formed directly by the PDH bypass. They supposed that the majority was formed in the mitochondrion by the PDH complex and subsequently exported to the cytosol through the action of carnitine acetyltransferase.

The work presented here has shown that under certain growth conditions, both laboratory and brewing strains of *S. cerevisiae* must produce acetyl-CoA directly by the PDH bypass. In aerobic, batch growth on 5% (w/v) glucose in defined medium there was no detectable carnitine acetyltransferase activity and export of acetyl-CoA from the mitochondria using this enzyme would not be possible. It was not clear from the same piece of work if this was true for aerobic, batch growth on galactose or 0.5% (w/v) glucose. This ambiguity arises because of the small amounts of CAT activity that were detected under these conditions.

Flikweert *et al* (1996) showed unequivocally that only the PDH bypass could produce sufficient amounts of cytosolic acetyl-CoA to satisfy cellular requirements. They achieved this by constructing a mutant devoid of all pyruvate decarboxylase activity (the first enzyme of the PDH bypass). This mutant could not grow on glucose as a sole carbon source, presumably because it could not produce enough acetyl-CoA via an alternative pathway. This work confirmed the findings described in the first part of this thesis and also the results of van den Berg and Steensma (1995). Their work focused on

acetyl-CoA synthetase (ACS), an enzyme that is also part of the PDH bypass. The work on this enzyme by van den Berg and Steensma showed that mutants lacking the cytosolic ACS could not grow on 2% (w/v) glucose. No results were shown for growth on other concentrations of this substrate.

2. Identification and disruption of the *ALD6* gene of *S. cerevisiae*

Despite much work on PDC and ACS, little genetic work had been carried out on the Mg^{2+}-ACDH, the third component of the PDH bypass. Thus, the second part of this thesis was concerned with identifying and disrupting the gene encoding this enzyme. Identification was made possible by matching the N-terminal protein sequence with the predicted product of a gene (YPL061w), identified as part of the systematic sequencing of the yeast genome. The N-terminal sequence was obtained from homogenous enzyme, purified as described by Dickinson (1996).

In a collaborative piece of work with Dr. P. G. Meaden (Heriot-Watt University, Edinburgh) the *ALD6* gene was named and disrupted in the diploid yeast strain YPH501. Mutants lacking the gene were found to possess no measurable activity for the Mg^{2+}-ACDH, but did display K^{+}-ACDH activity. Throughout this study no Mg^{2+}-ACDH activity was detected in any of the constructed mutants (RY124 to RY127).

The mutants did not use ethanol as a carbon source and this was independent of starting cell density or growth period. Consistent with this, Mg^{2+}-ACDH mutants respired poorly with ethanol compared to the wild-type. Direct toxicity by ethanol was disproved as an explanation for lack of growth, as was toxicity by acetaldehyde. Indeed, both parental and mutant cultures were able to tolerate concentrations of exogenous acetaldehyde up to and including 100 mM. This was much higher than previously described (Jones, 1989). During incubation in 1% ethanol (w/v), cultures of the mutants secreted insignificant amounts of acetaldehyde compared to wild-type cultures.

The metabolic response of the mutants to ethanol was varied and highlighted the importance of working with more than one strain. Carnitine acetyltransferase, isocitrate lyase and K^+-ACDH are all known to be induced by ethanol. However, in one out of four mutants (RY124), these enzymes were not expressed following 16 h incubation with ethanol. Similarly, PAGE gels stained for alcohol dehydrogenase activity in RY124 showed that the complement of ADHs did not 'switch over' in the same way as the parental cultures and other mutant strains. RY124 was also unable to express ICL, CAT and K^+-ACDH during incubation in oleic acid, acetaldehyde and acetate.

The maximum specific growth rates of the mutants on glucose were typically reduced by between 50 and 70%. This was consistent with the proposed involvement of this enzyme in the PDH bypass (van den Berg and Steensma, 1995; Flikweert *et al*, 1996). It was not possible to improve the growth characteristics by supplementation of glucose or sucrose cultures with either acetate or oleic acid.

Since it was impossible to make cytosolic acetyl-CoA by the PDH bypass in these mutants, other routes for production were tested. The most obvious route (that involving PDH and CAT) was not found to be operative. However, a study of the K^+-ACDH in wild-type and mutant strains under defined growth conditions led to the suggestion that a novel pathway exists. This requires diffusion of acetaldehyde into the mitochondrion, where acetate is produced by the action of the K^+-ACDH and subsequently passively diffuses to the cytosol (Lowenstein, 1967) for conversion to acetyl-CoA by the action of ACS. It would have been informative to construct a mutant lacking both the Mg^{2+}-ACDH and the K^+-ACDH. This work is still in progress.

3. The existence of an ALDH family in *S. cerevisiae*

In order to disrupt the gene for the K^+-ACDH it was first necessary to establish its identify from the information in the available databases. In attempting to make this

identification, it became obvious that an aldehyde dehydrogenase (ALDH) family existed in *S. cerevisiae*, of which *ALD6* was just one member. Saigal *et al* (1991) had reported the mutation and identification of the gene encoding the K^+-ACDH and had named the gene *ALD1*. However, this gene showed no significant homology to any other member of the ALDH family. The most likely members of the family to encode a mitochondrial ACDH were YER073w and YOR374w. YOR374w has been proposed to encode the K^+-ACDH from a study by Larsson *et al* (1997). The product of YOR374w was consistent with a sequence obtained from a commercially available K^+-ACDH by Chalmers *et al* (1991). However, neither study had disrupted the gene to study the physiological effects caused by the loss of the K^+-ACDH.

4. Identification and disruption of the *ALD7* gene of *S. cerevisiae*

The gene encoding the K^+-ACDH was identified unequivocally as YOR374w (*ALD7*). The work of this thesis therefore questions the origin, existence and identity of *ALD1* (Saigal *et al*, 1991). Identification of *ALD7* was made possible by purifying and N-terminal sequencing the K^+-ACDH from laboratory, baker's and brewing strains of yeast. The purification involved the development of a rapid and novel protocol.

Mutants were constructed which lacked the K^+-ACDH, but retained the Mg^{2+}-ACDH. The growth rate of mutants on glucose was very similar to the wild-type, but growth on ethanol was severely impaired. This confirmed the proposed role of the enzyme in ethanol catabolism (Jacobson and Bernofsky, 1974). From viability measurements, it was apparent that ethanol was not toxic to the mutants (RY270 to RY275). Similarly, acetaldehyde was not toxic, when added to lag-phase or actively growing cultures. Unlike the Mg^{2+}-ACDH mutants though, these mutants were able to produce acetaldehyde at wild-type levels when incubated in ethanol.

As with the Mg^{2+}-ACDH mutants, it seemed that not all of the *ALD7* mutants

were identical. This was revealed during the investigation of expression of ethanol-inducible enzymes. Strain RY272 was unable to induce CAT and ICL and did not 'switch-over' its complement of ADHs. All other mutants behaved as the wild-type did. The role of the K^+-ACDH in osmotolerance was also investigated but the wild-type was found to be unsuitable for work of this nature. This was due to the fact that it did not respond to osmotic stress in the same way as *S. cerevisiae* strain Y41 did in a previous study (Blomberg & Adler, 1989). Therefore, no conclusions could be made regarding the response of the K^+-ACDH mutants.

A study published after the work for this thesis was completed has reported the disruption of *ALD6* and *ALD7* in *S. cerevisiae* (Wang *et al*, 1998). This group were originally responsible for identifying *ALD1* as the gene encoding the K^+-ACDH (Saigal *et al*, 1991). Wang *et al* (1998) also disrupted a gene called YER073w, which out of the ALDH family members is the most homologous to *ALD7*.

The paper shows that disruption of YER073w, *ALD6* or *ALD7* has no effect on growth of the mutants on ethanol or glucose. I have no data for disruption of YER073w but the findings of my study with *ald6* and *ald7* mutants are inconsistent with those of Wang *et al*.

Construction of a double mutant (lacking *ALD6* and *ALD7)* was also reported by Wang et al. and the mutant did not grow on glucose but barely grew on ethanol. From my results, it is surprising that the mutant grows at all on ethanol but the lack of growth on glucose by the double mutant is consistent with the hypothesis set out in this thesis (that the K^+-ACDH can compensate for the loss of the Mg^{2+}-ACDH). However, construction of a triple mutant (lacking YER073w, *ALD6* and *ALD7)* revealed that a strain lacking both ACDHs and YER073w can not grow on ethanol or glucose.

It must be noted that the method used by Wang et al (1998) to analyse growth

(diameter of yeast colonies over time) is an inaccurate one and technically limited for use with *S. cerevisiae*.

5. Investigation of the PDH bypass in *S. cerevisiae* under brewing conditions

The present work provides the first examination of the roles of the PDH bypass and the ACDHs in brewing yeast. Most work on ACDHs in commercial yeast strains has focused on wine fermentation (Mauricio *et al*, 1997; Mauricio and Ortega, 1993).

The three enzymes of the PDH bypass were found to be active during a commercial lager fermentation. This showed that yeast can produce cytosolic acetyl-CoA, which is then used for the synthesis of unsaturated fatty-acids, sterols and esters. Such compounds are essential to yeast survival and ultimately beer quality. The Mg^{2+}-ACDH peaked sharply during pitching and after 30 h was no longer measurable. This was unexpected from previous reports which have described this enzyme as a constitutive one (Llorente and de Castro, 1977). The early peak in Mg^{2+}-ACDH activity might well be linked to cytosolic acetyl-CoA production that takes place whilst the fermentation vessel is aerobic. The presence of oxygen would enable production of unsaturated fatty-acids and sterols. It is not known though, why the activity of this enzyme is reduced to such low levels thereafter.

The K^{+}-ACDH activity remained relatively constant throughout the fermentation. This was unexpected, as it has been reported to be repressed by hypoxia (Llorente and de Castro, 1977; Jacobson and Bernofsky, 1974). However, it may remain active to compensate for the lack of Mg^{2+}-ACDH activity. This would be consistent with the theoretical route of cytosolic acetyl-CoA production proposed for the Mg^{2+}-ACDH mutants. Consistent with this is the reported peak in acetyl-CoA production later on in a fermentation (Quain *et al*, 1981)

PUBLICATIONS

Meaden, P.G., Dickinson, F. M., Mifsud, A., Tessier, W. D., Westwater, J., Bussey, H. and Midgley, M. (1997). The *ALD6* gene of *Saccharomyces cerevisiae* encodes a cytosolic, Mg^{2+}-activated acetaldehyde dehydrogenase. *Yeast.* 13, 1319-1327.

Tessier, W. D., Meaden, P. G., Dickinson, F. M. and Midgley, M. (1998). Identification and disruption of the gene encoding the K^{+}-activated acetaldehyde dehydrogenase of *Saccharomyces cerevisiae*. *FEMS Microbiology Letters.* In Press.

REFERENCES

Ackrell, B.A.C., Kearney, E.B. and Singer, T.P. (1978). *Methods Enz.* 47, 466-483.

Andreasen, A.A. and Stier, T.J.B. (1953). *J. Cell. Comp. Physiol.* 41, 23-29

Andreasen, A.A. and Stier, T.J.B. (1954). *J. Cell. Comp. Physiol.* 43, 271-276

Armitt, S., McCullough, W. and Roberts, C.F. (1976). *J. Gen. Micro.* 92, 263-282.

Atomi, H., Ueda, M., Suzuki, J., Kamada, Y. and Tanaka, A. (1993). *FEMS Microbiol. Lett.* 112, 31-34

Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F. and Cullin, C. (1993). *Nucl. Acids. Res.* 21, 3329-3330

Berg, P (1956). *J. Biol. Chem.* 222, 991-1013

van den Berg, M.A. and Steensma, H.Y. (1995). *Eur. J. Biochem.* 231, 704-713.

Black, S. (1955). *Methods Enz.* 1, 508-511.

Blomberg, A. and Adler, L (1989). *J. Bact.* 171, 1087-1092.

Boiteux, A. and Hess, B. (1970). *FEBS Lett.* 9, 293-296.

Bostian, K. A. and Betts, G. F. (1978). *Biochem. J.* 173, 773-786.

Boulton, C.A. (1991). *Biotech. Gen. Eng Rev.* 9, 127-181.

Boulton, C.A. and Ratledge, C. (1981a). *J. Gen. Micro.* 127, 169-176.

Boulton, C.A. and Ratledge, C. (1981b). *J. Gen. Micro.* 127, 423-426.

Bradford, M. M. (1976). *Anal. Biochem.* 72, 248-254.

Brandriss, M.C. (1983). *Mol. Cell. Biol.* 3, 1846-1856.

Brown, A. D. (1978). *In A. H. Rose and J. G. Morris (ed.), Advances in Microbial Physiology, vol. 17. Academic Press, Inc., New York.*

Bruinenberg, P. M., van Dijken, J. P., Kuenen, J. G. and Scheffers, W. A. (1985). *J. Gen. Micro.* 131, 1035-1042.

Bussey, H., Kaback, D.B., Zhong, W., *et al.* (1997). *Proc. Natl. Acad. Sci. USA* 92, 3809-3813.

Bussey, H., Storms, R.K., Ahmed, A., *et al* (1997). *Nature* 387, 103-105.

Chalmers, R.M., Keen, J.N. and Fewson, C..A. (1991). *Biochem. J.* 273, 99-107.

Chapman, C. and Bartley, W. (1968). *Biochem. J.* 107, 455-465.

Clark, J. F. and Jakoby, W. B. (1970). *J. Biol. Chem.* 245, 6065-6072.

Claus, R., Kappeli, O. and Fiechter, A. (1983). *FEMS Microbiol. Lett.* 18, 185-188.

Coot, N. and Kirsop, B.H. (1976). *J. Inst. Brew.* 82, 149-153.

Dalziel, K. (1962). *Biochem. J.* 84, 244-255.

De Virgillio, C., Burckert, N., Barth, G., Neuhaus, J.M., Boller, T. and Wiemken, A. (1992). *Yeast* 8, 1043-1051.

Dickinson, F.M. (1996). *Biochem. J.* 315, 393-399.

Dickinson, F.M. and Dalziel, K. (1967). *Biochem J.* 104, 165-172.

Dubois, M., Giles, K., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956). *Anal. Chem.* 28, 350-356.

Elgersma, Y., van Roermund, C. W. T., Wanders, R. J. A. and Tabak, H. F. (1995). *EMBO J.* 14, 3472-3479.

Evans, C. T., Scragg, A. H. and Ratledge, C. (1983). *Eur. J. Biochem.* 130, 195-199.

Feldman, H., Aigle, M., Ajinovic, G., *et al* (1994). *EMBO J.* 13, 5795-5809.

Flikweert, M.T., van der Zanden, L., Janssen, W.M.T.M., Steensma, H.Y., van Dijken, J.P. and Pronk, J.T. (1996). *Yeast* 12, 247-257.

Gancedo, J.M. (1992). *Eur. J. Biochem.* 206, 297-313.

Gietz, R.D., Schiestl, R.H., Willems, A.R. and Woods, R.A. (1995). *Yeast* 12, 247-257.

Hampsey, M. D. and Kohlaw, G. B. (1981). *J. Biol. Chem.* 256, 3791-3795.

Hohmann, S. (1993). *Mol. Gen. Genet.* 241, 657-666.

Holzer, G. (1961). *Cold Spring Hbr. Symp. Quant. Biol.* 26, 277-288.

Holzer, H. and Goedde, W.H. (1957). *Biochem Z.* 329, 175-191

Jacobson, M.K. and Bernofsky, C. (1974). *Biochem. Biophys. Acta* 350, 277-291.

Jones, R.P. (1989). *Enz. Micr. Tech.* 11, 130-141.

de Jong-Gubbels, P., Vanrolleghem, P., Heijnen, J. J., van Dijken, J. P. and Pronk, J. T. (1995). *Yeast* 11, 407-418.

Kawamoto, S., Ueda, M., Nozaki, C., Yamamura, M., Tanaka, A. and Fukui, S. (1978). *FEBS Lett.* 96, 37-40.

Kispal, G., Cseko, J., Aikonyi, I. and Sandor, A. (1991). *Biochim. Biophys. Acta.* 1085, 217-222.

Kispal, G., Sumegi, B., Dietmeier, K., Bock, I., Gajdos, G., Tomcsanyi, T. and Sandor, A. (1993). *J. Biol. Chem.* 268, 1824-1829.

Klein, H. P. and Jahnke, L. (1968). *J. Bact.* 96, 1632-1639.

Klein, H. P. and Jahnke, L. (1971). *J. Bact.* 106, 596-602.

Klein, H.P. and Jahnke, L. (1979). *J. Bact.* 137, 179-184.

Kohlaw, G.B. and Tan-Wilson, A. (1977). *J. Bact.* 129, 1159-1161.

Krzywicki, K.A. and Brandriss, M. (1984). *Mol. Cell. Biol.* 4, 2837-2842.

Kurita, O. and Ito, H. (1994). *Biosci. Biotech. Biochem.* 58, 609-615.

Larsson, N., Norbeck, J., Karlsson, H., Karlsson, K.A. and Blomberg, A. (1997). *Electrophoresis* 18, 418-423.

Lambowitz, A.M., Smith, E.W. and Slayman, C.W. (1972) *J. Biol. Chem.* 247, 4859-4865.

Lindahl, R. (1992). *Crit. Rev. Biochem. Mol. Biol.* 27, 283-335.

Llorente, N. and de Castro, I.N. (1977). *Rev. Esp. Fisiol.* 33, 135-142.

Lowenstein, J. M. (1967). *Biochem. Soc. Symp.* 27, 61-98

Lundquist (1974): In Bergmeyer, H.U., p1555-1559 Methods of enzymatic analysis - Verlag Chemistry, Weinheim, Germany

Lutstorf, U. and Megnet, R. (1968). *Arch. Biochem. Biophys.* 126, 933-944.

Mauricio, J.C. and Ortega, J.M. (1993). *Microbios* 75, 95-106.

Mauricio, J.C., Moreno, J.J. and Ortega, J.M. (1997). *J. Agric. Food Chem.* 45, 1967-1971.

McCammon, M.T., Veenhuis, M., Trapp, S. B. and Goodman, J. M. (1990). *J. Bact.* 172, 5816-5827.

Millan, C. and Ortega, J. M. (1988). *Amer. J. Enol. Vitica.* 39, 107-112.

Miralles, V. J. and Serrano, R. (1995). *Mol. Microbiol.* 653-662.

Nakai, K. and Kanehisa, M. (1992). *Genomics.* 14, 897-911.

Neuberg, C. and Karczag, L. (1911). *Biochem Z.* 36, 68-75

Nordstrom, K. (1965). *Proc. 10th Cong. Eur. Brew. Conv.* 291-295.

Page, R. D. M. (1996). *CABIOS.* 12, 357-358.

Pearson, W. R. and Lipman, D. J. (1988). *Proc. Natl. Acad. Sci. USA.* 85, 2444-2448.

Perlman, P. S. and Mahler, H. R. (1974). *Arch. Biochem. Biophys.* 162, 248-271.

Petrik, M., Kappeli. O. and Fiechter, A. (1983). *J. Gen. Microbiol.* 129, 43-49.

Postma, E., Verduyn, C., Scheffers, W. A. and van Dijken, J. P. (1989). *Appl. Environ. Microbiol.* 53, 468-477.

Pronk, J.T., Wenzel, T.J., Luttik, M.A.H., Klassen, C.C.M., Sceffers, W.A., Steensma, W.A., Steensma, H.Y. and van Dijken, J.P. (1994). *Microbiology* 140, 601-610.

Pronk, J. T. Stennsma, H. Y. and van Dijken, J. P. (1996). *Yeast 12*, 1607-1633.

Quain, D.E., Thurston, P.A. and Tubb, R.S. (1981). *J. Inst. Brew.* 87, 108-113.

Ramsay, R. R. and Tubbs, P. K. (1976). *Eur. J. Biochem.* 69, 299-303.

Ratledge, C. and Gilbert, S. C. (1985). *FEMS Microbiol. Lett.* 27, 273-279.

Ratledge, C. and Evans, C.T. (1989) in Rose, A.H. and Harrison, J.S. (Eds), *The Yeasts, Vol 3: Metabolism and Physiology of Yeasts*, 2nd edn. Academic Press, N.Y. 367-455.

Ramsey, R. R. and Tubbs, P. K. (1975). *FEBS Lett.* 54, 21-25.

Rockmill, B., Lambie, E.J. and Roeder, G.S. (1991). *Methods Enzymol.* 194, 146-149.

van Roermund, C. W. T., Elgersma, Y, Singh, N., Wanders, R. J. A. and Tabak, H, F. (1995). *The EMBO J.* 14 (14), 3480-3486.

Rose, A.H. and Harrison, J.S. (Eds), *The Yeasts, Vol 5: Yeast Technology*, 2nd edn. Academic Press, N.Y. 7-67.

Saigal, D., Cunningham, S. J., Farres, J. and Weiner, H. (1991). *J. Bacteriol.* 173, 3199-3208.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). *Proc. Natl. Acad. Sci. USA* 74, 5463-5467.

Satyanarayana, T., Mandel, A. D. and Klein, H. P. (1974). *Biochim. Biophys. Acta.* 341, 396-401.

Schmalix, W. and Bandlow, W. (1993). *J. Biol. Chem.* 268, 27428-27439.

Seegmiller, J.E. (1953). *J. Biol. Chem.* 201: 629-637.

Seegmiller, J.E. (1955). *Meth. Enz.* 1, 511-514.

Sherman, F. and Hickes, J. (1991). *Methods Enzymol.* 194, 21-37.

Sikorski, R.S. and Hieter, P.(1989). *Genetics* 122, 19-28.

Srere, P.A. (1963). *Methods in Enzymology.* 5, 641-644.

Srere, P. A. and Lipmann, F. (1953). *J. Amer. Chem. Soc.* 75, 4874-4879.

Steinman, C. R. and Jakoby, W. B. (1967). *J. Biol. Chem.* 242, 5019-5023.

Storms, R.K., Wang, Y., Fortin, *et al.* (1997). *Genome* 40, 151-164

Sumper, M. (1974). *Eur. J. Biochem.* 49, 469-474.

Thompson, J. D., Higgins, D. G. and Gibson, T.J. (1994). *Nucl. Acids Res.* 22, 4673-4680.

van Urk, H., Voll, W. S., Scheffers, W. A. and van Dijken, J. P. (1990). *Appl. Environ. Microbiol.* 56, 282-286.

Visser, W., van Spronsen, E. A., Nanninga, N., Pronk, J. T., Kuenen, J. G. and van Dijken, J. P. (1995). *Antonie van Leeuwenhoek* 67, 243-253.

Wales, D. S., Cartledge, T. G. and Lloyd, D. (1980). *J. Gen. Micro.* 116, 93-98.

Wang, X., Mann, C. J., Bai, Y., Ni, L. and Weiner, H. (1998). *J. Bact.* 180, 822-830.

Wenzel, T. J., Luttik, M. A. H., van den Berg, J. A. and Steensma, H. Y. (1993). *Eur. J. Biochem.* 218, 405-411.

Wenzel, T. J., van den Berg, M. A., Visser, W., van den Berg, J. A. and Steensma, H. Y. (1992). *Eur. J. Biochem.* 209, 697-705.

Weusthuis, R. A., Visser, W., Pronk, J. T., Scheffers, W. A. and van Dijken, J. P. (1994). *Microbiology* 140, 703-715.

Wightman, P., Quain, D.E. and Meaden, P.G. (1996). *Lett. Appl. Microbiol.* 22, 90-94.

Woeltje, K. F., Esser, V., Weis, B. C., Sen, A., Cox, W. F., McPhaul, M. J., Slaughter, C. A., Foster, D. W. and McGarry, J. D. (1990). *J. Biol. Chem.* 265, 10720-10725.

Wright, A. P. H., Png, H. L. and Hartley, B. S. (1989). *Curr. Genet.* 15, 171-176.

Young, E. T. and Pilgrim, D. (1985). *Mol. Cell. Biol.* 5, 3024-3034.

APPENDICES

The following appendices contain materials, methods and some results relating to the genetic work carried out for this thesis. This genetic work was performed by Dr P.G. Meaden (Heriot-Watt University, Riccarton, Edinburgh) to whom I am extremely grateful.

APPENDIX 1 - Materials and Methods for disrupting the genes *ALD6* and *ALD7*

1. Disruption of *ALD6*

(i) Yeast strains

The diploid yeast strain YPH501 (*MATa/MATα ura3-52 / ura3-52 lys2-801 / lys2-801 ade2-101 / ade2-101 trp-Δ63 / trp-Δ63 his3-Δ200 / his3-Δ200 leu2-Δ1 / leu2-Δ1*; Sikorski and Hieter, 1989) was the host for the deletion of YPL061w/*ALD6*. YPH499 is an isogenic haploid (*MATa*) and was used as a reference strain for the determination of mitochondrial and cytosolic ACDH activities. RY124 to RY127 (*MATa ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1 ald6::HIS3*) are haploid strains from which the coding region of YPL061w/*ALD6* has been completely deleted. RY124 to RY127 were obtained by sporulation of YPH501 following deletion of one copy of YPL061w/*ALD6* from this strain.

(ii) DNA sequencing

This is described in detail elsewhere (Bussey *et al*, 1995; Storms *et al*, 1997) and summarised here. The source of *S. cerevisiae* strain AB972 DNA used for sequencing this region was a prime-clone cosmid 8460 obtained from L. Riles (Washington

University, St Louis, MO). A library was constructed from this cosmid as follows. Cosmid DNA was purified on ethidium bromide-CsCl density gradients, and then sheared by sonication to an average size of about 2 kbp. The ends of the linearised DNAs were blunted by treatment with T4 and Klenow DNA polymerases. Fragments in the same range 1.5 to 3 kbp were purified from agarose following size fractionation by gel electrophoresis. The purified fragments ligated with *Sma*I-digested Bluescript SKII + DNA were transformed in *Escherichia coli* strain XL1-Blue and about 500 independent transformants, identified as antibiotic-resistant white colonies on minimal medium plates containing ampicillin and X-gal, were stored at -80°C in 15% (w/v) glycerol. This small library was then used to determine the sequence of the cosmid inserts. In an initial shotgun phase, approximately 350 independent clones were sequenced. For this, Bluescript clones containing double stranded inserts were purified using Promega Wizard Miniprep columns (Promega Corporation, Madison, WI) and the method recommended by the supplier. The DNA sequence of both ends of the insert DNA in each clone was determined using the Sanger dideoxy sequencing method (Sanger *et al*, 1977) adapted for automated sequencing. For this, two sequencing reactions were carried out on each Bluescript clone. One was primed with the universal sequencing primer and the other by the reverse sequencing primer. Sequencing reactions were performed using the Applied Biosystems Inc. Prism Ready Reaction Dye Terminator Kit (Perkin Elmer Applied Biosystems Division, Foster City, CA) and the protocol provided by the supplier. The sequencing reaction products were generated in 0.6 ml microcentrifuge tubes using a DNA thermal cycler (Perkin Elmer 9600) and *Taq* DNA polymerase. Unincorporated dye terminators were removed by one or more extractions with an equal volume of phenol. The reaction products were resolved on an ABI 373A automated DNA sequencing machine. The automatically-read sequence results were transferred to a Sun

work-station and assembled into contigs using the Staden DNA assembly program (R.Staden, Medical Research Council, Cambridge, UK). To fill gaps and resolved ambiguities that remained after the shotgun phase, Bluescript clones harbouring the appropriate sequences were used and sequenced as described above.

(iii) Gene Deletion

Two oligonucleotide primers (Gibco-BRL, Bethesda, MA) were constructed for PCR amplification of the *HIS3* gene, based on the method of Baudin *et al* (1993) and using pUC-HIS3 as the template. The forward primer (ALD6-F1) was 5'-AAACATCAAG AAACATCTTTAACATACACAACACATCTCTTGGCCTCCTCTAG-3', in which the underlined portion corresponds to nucleotides -51 to -15 of the YPL061w/*ALD6* sequence (GenBank accession number U39205). The reverse primer (ALD6-R1) was 5'-TTTGTGTATATGACGGAAAGAAATGCAGGTTGGTACATTATCGTTCAG AATGACACG-3' with the underlined portion in this primer being complimentary to nucleotides 1540 to 1501 in the YPL061w/*ALD6* sequence. A digoxigenin (DIG)-labelled probe for the detection of *HIS3* was prepared by PCR by including DIG-11-dUTP (50 mM; Boehringer Mannheim, Germany) in the reaction. Yeast transformation was performed as described by Gietz *et al* (1995). Standard procedures for yeast sporulation (Sherman and Hickes, 1991) and enrichment (Rockmill *et al*, 1991) were used to isolate haploid strains.

(iv) Southern blotting and hybridisation

Total DNA was prepared from yeast using a Kristal DNA extraction kit (Cambridge Molecular Technologies, Cambridge, UK). Southern blotting of yeast DNA was performed using a vacuum blotting system (VacuGene, from Pharmacia Biotech, Uppsala, Sweden) in accordance with the manufacturer's instructions. A DIG-labelled probe for YPL061w/*ALD6* was prepared from YPH501 genomic DNA by PCR using the

primers 5'-ACGACACTGAATGGGCTACC-3' (corresponding to the nucleotides 230 to 249 of the YPL061w/*ALD6* sequence (GenBank accession number U39205) and 5'-CTTCAACATCTTGGCCACCT-3' (corresponding to nucleotides 1356 to 1337 of the YPL061w/*ALD6* sequence and with DIG-11-dUTP (50 mM) included in the reaction. The following cycle was used for the PCR: 2 min at 94°C, 2 min at 55°C and 3 min at 72°C for a total of 35 cycles. Hybridisation of DIG-labelled PCR product to DNA blots was carried out as described previously (Wightman et al, 1996).

2. Disruption of *ALD7*

The methods used were as described for the disruption of *ALD6* except for the following:

(i) Yeast strains

The diploid yeast strain YPH501 (*MAT***a**/*MAT*α *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1*) was the host for deletion of *ALD7*. YPH499 is an isogenic haploid (*MAT***a**) and was used as a reference strain for the determination of Mg^{2+}- and K^{+}-ACDH activities. RY270 to RY275 (*ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 ald7::HIS3*) were obtained by sporulation of YPH501 following deletion of one copy of *ALD7* from this strain.

(ii) Gene deletion

The method of Baudin *et al* (1993) was used for the deletion of *ALD7*, using a PCR product amplified from the *HIS3* gene and flanked by sequences 40 nucleotides in length to direct recombination with *ALD7 in vivo*. The forward primer (ALD7-F1) was:

5'-GGATTAGAAGTATCTGGAAAACCAACCAAGAAAACTACAACTCTTGGC

CTCCTCTAG-3', in which the underlined portion corresponds to nucleotides -59 to -20 of the *ALD7* sequence (relative to the first nucleotide of the translational start codon). The reverse primer (ALD7-R1) was:

5'-TGTAAGCATCGATTGGACACCAGGCTTATTGATGACCTTATCGTTCAG AATGACACG-3', with the underlined portion in this primer being complementary to nucleotides +1597 to +1558 in the *ALD7* sequence.

(iii) Southern blotting and hybridisation

Procedures for the preparation of total DNA, and digoxigenin-labelled DNA probes, and for performing Southern blotting and hybridisation, were as described previously (Wightman *et al*, 1996). A probe for *HIS3* was produced from the HIS3 gene using the primers ALD7-F1 and ALD7-R1 in the PCR. For preparation of the *ALD7* probe, the forward primer was 5'-CCTCCATTGGGAGACTTCAA-3' (corresponding to nucleotides +41 to +60 of the *ALD7* sequence) and the reverse primer 5'-GGATAATGTTTTGCACGGCT-3' (complementary to nucleotides +940 to +921).

APPENDIX 2 - Verification for the deletion of the genes *ALD6* and *ALD7*

1. Verification for the deletion of *ALD6*

The 1.6 kbp PCR product amplified from *HIS3* with the primers ALD6-F1 and ALD6-R1 was used to transform YPH501 to His^+. Of six transformants selected for further study, all contained a 6.4 kbp *Eco*RI fragment when probed with the DIG-labelled PCR product (result not shown). This is the size predicted by the nucleotide sequence of chromosome XVI following deletion of *ALD6* and insertion of *HIS3*. The six transformants were then sporulated but the yield of asci was extremely low (less than 5%) in every case. This was not unexpected as YPH501 sporulates poorly. A spore enrichment technique was therefore used to facilitate the isolation of haploid strains. Following enrichment, the sporulation mixture from each transformant was plated out onto YEPD agar and incubated. By replica plating onto GYNB agar lacking histidine it was found that the proportion of His^+ colonies derived from each transformant varied from approximately 60% to 80%. His^+ colonies (a total of 45) were subcultured onto fresh GYNB agar lacking histidine from the transformant giving the highest proportion of haploids (that is, the lowest proportion of His^+ colonies) and of these, 32 displayed mating ability when tested. Six of these putative haploids were chosen for the preparation of total DNA which, following digestion with *Eco*RI, was analysed by Southern blotting and hybridisation using the DIG-labelled 1.6 kbp PCR product. All six strains generated a band at 6.4 kbp. Probing of *Eco*RI-digested DNA from each of the same six strains with the DIG-labelled *ALD6* probe failed to produce a hybridisation signal whereas YPH499 gave the expected band at 6.9 kbp (result not shown). None of the six strains was able to sporulate. It was concluded from these analyses that all six strains were indeed haploids with the intended deletion in *ALD6*.

2. Verification for the deletion of *ALD7*

The diploid yeast strain YPH501 was transformed to His^+ with the 1.1 kbp PCR product obtained by amplification of *HIS3* with the primers ALD7-F1 and ALD7-R1. Total DNA isolated from three independent transformants was digested with *Xba*I and subjected to Southern blotting and hybridisation analysis using the *HIS3* probe. A 5.4 kbp fragment was detected in all three transformants (results not shown), consistent with the deletion of *ALD7* as predicted from the yeast genome sequence. His^+ haploids were obtained from each of the three diploid transformants, and two from each diploid were selected for analysis by Southern blotting and hybridisation. Total DNA from all six haploid strains contained the expected 5.4 kbp *Xba*I fragment detected with the *HIS3* probe, and failed to show hybridisation with the *ALD7* probe (results not shown).