ISOLATION OF LOW ETHANOL PRODUCING YEAST STRAINS USING ADAPTIVE EVOLUTION

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Isolation of Low Ethanol Producing Yeast Strains Using Adaptive Evolution

by
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DECLARATION

"I, Dariusz Roman Kutyna, declare that the PhD thesis entitled 'Isolation of Low Ethanol Producing Yeast Strains Using Adaptive Evolution' is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

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ABSTRACT

This thesis describes the application of a non-genetic engineering approach, adaptive evolution, to generate variants of *Saccharomyces cerevisiae* that produce reduced levels of ethanol relative to the strains they were derived from. Sub-lethal concentrations of sulfite, which sequesters acetaldehyde, thereby limiting ethanol production, was used as a selection pressure. However, removal of acetaldehyde not only limits ethanol production, it also compromises redox balance by preventing the fermentative oxidation of NADH. To compensate for this, the cell regenerates NAD^+ by producing increased amounts of glycerol; glycerol production is an NADH-driven reductive process. It was demonstrated in this thesis that culturing yeast in presence of sulfite for a number of generation, results in the generation of genetically stable variants with increased glycerol and reduced ethanol production.

The 'low-ethanol' variants generated from this work were analyzed for ethanol, glycerol and acetic acid production in wide range of experimental settings. Most importantly, under all conditions, the 'low-ethanol' phenotype of the variants was conserved; all variants produced less ethanol compared to their parental strains. The greatest redirection of metabolism away from ethanol production was noted for a variant of an industrial wine strain AWRI1628, which produced 2.9 g/l (0.36 % v/v) less ethanol than its parent, while grown on 100 g/l glucose.

A genetic analysis of two 'low-ethanol' variants was performed. It was determined that in each strain at least two mutations were involved in conferring the 'low-ethanol' phenotype. Attempts were made, using gene arrays, to characterize these genes, but without success.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Historical overview

Fermentation-based processes have been used by humans for different purposes for thousands of years. Archeological findings suggest that alcoholic beverages were fermented from fruits as early as 7000 B.C. Analysis of organic residues from pottery jars found in an early Neolithic village in the Henan province of China provided evidence of such beverages fermented from rice, honey and fruits, including grapes and hawthorn fruit (McGovern et al. 2004). Further archeological evidence of alcoholic beverage production comes from the regions of Iran and Turkey dated from around 6000 - 5000 B.C. (Berkowitz 1996). However, substantial archeological proof, showing wine was made and used in everyday life, comes from excavations around two millennia later in ancient Egypt, where grape cultivation and winemaking were advanced and a very important part of the culture (Poo 1995). In the following centuries the art of winemaking spread to all continents, wherever the climate was suitable for grape cultivation.

Very little was known about the process of fermentation in ancient times; such fermentations were presumably autochtenous, being initiated by microorganisms associated with the fruit, the fermentation vessel and/or the surroundings. A milestone in uncovering the biochemical functions of yeast metabolism in fermentation was achieved by Louis Pasteur. In 1860 Pasteur proved that the synthesis of glycerol during fermentation occurs as a part of yeast metabolic activity, when sugar is broken down to ethanol and carbon dioxide (Barnett 2003). Since then, great progress has been made in understanding yeast biochemistry, biology and fermentation.

Modern applications of yeast are diverse and fall into two main groups. First, yeast are used as a model organism for a wide range of biological research areas, such as studies on cancer (Hartwell 2004, Simon and Bedalov 2004), cell physiology, cell cycle and various cellular and biochemical processes. Secondly, yeast are used for industrial and pharmaceutical purposes, encompassing bio-fuels production (Alper et al. 2006, Bro et al. 2006), cosmetics (Keller et al. 1991, Zulli et al. 1998),

production of heterologous proteins (Cereghino et al. 1999, Azizi and Doyle 2006) and drug development (Mckanzie et al. 1994, Melese and Hieter 2002). It is often the case that yeast technologies developed in one field are borrowed for application in others. Of course, one of the more significant industrial applications of yeast is for the production of alcoholic beverages such as beer and wine.

Until the 1980s wine was mainly produced in a traditional way, relying on microfloral residents found on grapes for the initiation of fermentation. Even today some wineries still use this approach despite the potential risks and unpredictability of this practice; wines produced in this way are considered by some to display distinct sensory properties and a broader palate structure. Wine production on a larger, industrial scale, however, has no place for unpredictability and a greater understanding of yeast biology and fermentation processes has enabled modern wineries to use pure yeast inocula with known properties. This makes it possible to produce predictable and reliable wines within established quality criteria (Pretorius 2000, Pretorius et al. 2003).

Constantly facing new demands by the market, the wine industry is involved in the ongoing development of new yeast strains, with specific and stable desirable characteristics, that can be used in industrial-scale wine production. The need for yeast strains with new and/or improved characteristics is also driven by the emergence of novel viticultural and winemaking practices. The ongoing development of *S. cerevisiae* for wine fermentations has focused mainly on the early initiation of fermentation, improving stress tolerance, and increasing fermentation efficiency (Pretorius 2000, Rainieri and Pretorius 2000). More recently the wine industry has been particularly interested in the development of wine yeast that produce less ethanol, to be used for the production of wines with moderate alcohol levels (de Barros Lopes et al. 2003).

1.2 Why the interest in wine with lower ethanol content?

Grapes grown in warm climates can produce rich, full bodied vintages with ripe fruit flavor profiles. However a warm climate and lengthy maturation can lead to grapes with high sugar levels and this, in turn, leads to wines with high levels of alcohol;

some wines may have ethanol concentrations above 15% v/v (Godden 2000, Day et al. 2002). High alcohol content in wine has several important consequences: excessive alcohol consumption is associated with health issues, selling costs are higher in countries where taxes are levied according to ethanol content and wine flavor is often compromised (de Barros Lopez et al. 2003)

The negative effects of excess alcohol consumption on human health are well known and supported by results from extensive research. In 2004 the World Health Organization (WHO) released the 'Global Status Report on Alcohol', highlighting the range of possible dangers related to excessive alcohol consumption (www.who.org). According to that report: *Alcohol is not an ordinary commodity. While it carries connotations of pleasure and sociability in the minds of many, harmful consequences of its use are diverse and widespread. As documented in this report, globally, alcohol problems exert an enormous toll on the lives and communities of many nations, especially those in the developing world. Research has shown that when extrapolating from historical trends, the role of alcohol as a major factor in the burden of disease will be increasing in the future. Particularly worrying trends are the increases in average volume of drinking predicted for the most populous regions of the world (e.g. in China and India) and the emerging trend of more harmful and risky patterns in drinking especially among young people.*
(http://whqlibdoc.who.int/publications/2004/9241562722_(425KB).pdf)

In some countries taxes for alcoholic beverages are levied according to alcohol content. In such countries 'high-alcohol' wines are relatively expensive and this will impact negatively on consumer demand. In fact this is recognized by the Alcohol and other Drugs Council of Australia (ADCA), which has proposed that a volumetric tax be imposed on alcoholic beverages: *The current alcohol taxation system is confusing, inequitable and provides economic incentives for the production and consumption of the alcohol products that cause the most harm to the community. A volumetric tax on all alcohol products would result in a simplified system whereby all alcohol products, regardless of type, would be taxed according to alcohol content* (http://www.adca.org.au/policy/facts/Alcohol_tax_fact_sheet.pdf). Thus, there are economic pressures to produce wines with reduced ethanol levels that retain high-quality flavor and sensory characteristics.

According to some studies, high alcohol content has a negative influence on the sensory attributes of wine (Guth and Sies 2002). The flavor of wine is determined by its chemical composition, and high ethanol levels can increase the so-called "masking effect" on some flavor-related volatile compounds. Ethanol was shown to act as a solvent for many volatile compounds developed during fermentation and maturation of wines. At lowered ethanol concentrations, some volatiles may be released resulting in a richer palate (Williams and Rosser 1981). Ethanol may also give wine a burning sensation and increase the perception of weight or body in wines. At higher levels, ethanol may also increase a perception of bitterness and decrease the astringency of tannins (Lea and Arnold, 1978).

Research into the solubility of volatile aroma/flavor compounds in alcohol has discovered that the "masking effect" is the result of key compounds drastically losing their volatility (and thus their perceptibility to nose and palate) as alcohol rises beyond an optimum level.
(http://www.conetech.com/Downloads/ConeTech_Technical_Brochure.pdf)

1.3 Possible ways to reduce the alcohol content in alcoholic beverages

There are a number of techniques that can be used to reduce the alcohol content in wine. These fit broadly into one of three main groups: viticultural, physical and biological.

1.3.1 Viticultural practices leading to reduced ethanol levels in wine

Whilst not the subject of this thesis, there are several viticultural practices that can contribute to reduced alcohol levels in wine. These practices mainly lead to decreased sugar development in ripening grapes, thus resulting in lowered ethanol content of wine. One approach is earlier grape harvest, i.e. before sugar levels have fully developed. Such practice, however, may lead to the incomplete development of flavor and aroma compounds, which may negatively affect wine quality (Gallander 1983).

Increased grape yields at harvest accompanied by a decrease in sugar content of the berries at maturity may be achieved by increased irrigation of the vines before

veraison, simply leading to dilution of sugar content of the berries (from Jackson and Lombard 1993). However, such practice may also lead to the dilution of flavor and aroma compounds, such that wine made from berries with higher water content may have compromised palate characteristics. A critical factor responsible for sugar content in the berries is the [leaf area] / [fruit weight] ratio. Thus, adjustment of this ratio via partial defoliation of vines may result in lower sugar levels in mature berries (Kaps and Cahoon 1992, Kliewer and Antcliff 1970). This practice is, however, problematic, as defoliation of vines can undesirably alter the content of soluble solids in grapes and negatively influence the vines health.

Shading of vines is also an alternative for reducing sugar development in grapes (Kliewer et al. 1967). According to some studies, however, shading of vines may result in the increased perception of the 'grassiness' in the wines, which is perceived as a negative trait (Smith et al. 1988). Also shading a large portion of vineyards may be problematic from practical application point of view. Another possible solution to obtain grapes with lowered sugar content is planting vines close to each other, since these vines must develop deeper roots systems and 'work harder' to get adequate nutrients (www.jancisrobinson.com). This can also be problematic as vine replacement is usually conducted approximately every 30 years, which does not leave much flexibility for testing which setting would work best under certain conditions.

1.3.2 Physical processes used to reduce alcohol content in beverages

Physical processes used to reduce alcohol content in alcoholic beverages include distillation, freeze concentration and filtration-based techniques. These are described below.

1.3.2.1 Distillation techniques

In general terms, most distillation techniques are based on distillation columns and/or evaporators (Boucher 1988, Thumm 1975). The reduction of alcohol in wine occurs simply by heating and evaporating some of the volatile components of wine, including ethanol. Some of these distillation methods have been improved in recent times to reduce processing time, decrease operating temperature and increase the recovery of volatile aromatic compounds. In general, however, distillation techniques compromise

desirable wine properties, mostly due to the loss of desirable wine volatiles (Pickering 2000).

1.3.2.2 Freeze concentration

Wine is mostly water, which can be frozen and the ice crystals separated from other wine components. The removal of water leaves a residual concentrated liquid containing most of the main wine compounds, including ethanol, which subsequently can be removed using a method such as vacuum distillation. After ethanol separation from the concentrated liquid fraction, the earlier separated water fraction is returned to the concentrate, restoring wine with lowered ethanol content (from Pickering 2000).

1.3.2.3 Reverse osmosis, membrane extraction and dialysis

Reverse osmosis (RO) is a form of membrane filtration, and is one of the most commonly used techniques for the reduction of ethanol content in wines (Bui et al. 1986, Villettaz 1986), (www.memstar.com.au). The filtration process removes alcohol and water, the alcohol is then separated from the water by osmotic perstraction or distillation, and the water portion added back to wine concentrate, resulting in a wine with a lower final alcohol content (from Pickering 2000).

Reverse osmosis is distinguished from the other filtration methods by its use of high pressure, which is required if filtration is to occur across highly selective membranes with very small pore size. "Crossflows", also known outside the wine industry as tangential flow ultrafiltration, are introduced across the surface of the filtration membranes causing their "scrubbing" and reducing the incidence of blockage. It is claimed that when using this method in a wine context, the permeate, consisting mostly of water and alcohol, does not contain any flavor or color compounds that are essential for good quality wine. The method, therefore, should not compromise the characteristics of the final product at the end of the process (Wollan 2003). However, there is no published scientific evidence supporting this claim and it is likely that the composition of processed wine will be changed, over and above reductions in ethanol content.

RO-based alcohol adjustment systems have been developed by a number of companies throughout the world. One such system, (U.S Patent No.08/218920), is shown in Figure 1.1 The permeate, comprising mainly water and alcohol, is distilled and a portion of the alcohol fraction is removed leaving the remaining ethanol and water to be returned to the wine concentrate. The wine itself does not undergo distillation, minimizing the loss of desirable flavor and color compounds. Furthermore, the entire process occurs in a closed system so there is no involvement of external factors, which could compromise the wine's original characteristics (www.vinovation.com), It is unlikely however that the processed wine will posses all of the desirable properties that were present in the original wine.

Another membrane-based method used to reduce the ethanol content of alcoholic beverages is membrane extraction. This technology uses semi-permeable membranes designed to selectively remove ethanol, leaving behind all desired components such as flavor and color compounds. The membrane can be used with ethanol-receiving, non-toxic extraction fluids, such as non-water soluble organic solvents, that absorb the ethanol as it migrates across the membrane. The properties of membranes and extraction fluids may be adjusted to reduce the loss of desirable wine components (Matson 1989). Despite the apparent advantages of this method, there is a risk that the original properties of the wine may be lost and there is no supportive evidence on the sanctity of the method in retaining wine properties.

Dialysis is also a process that uses semi permeable membranes and it has been used for selective ethanol removal from wine. Unlike reverse osmosis, dialysis relies on a concentration gradient across the membrane, driving ethanol diffusion across the membrane, lowering ethanol levels in the wine. Other desirable, low molecular mass compounds may, however, also diffuse across the membrane reducing wine quality.

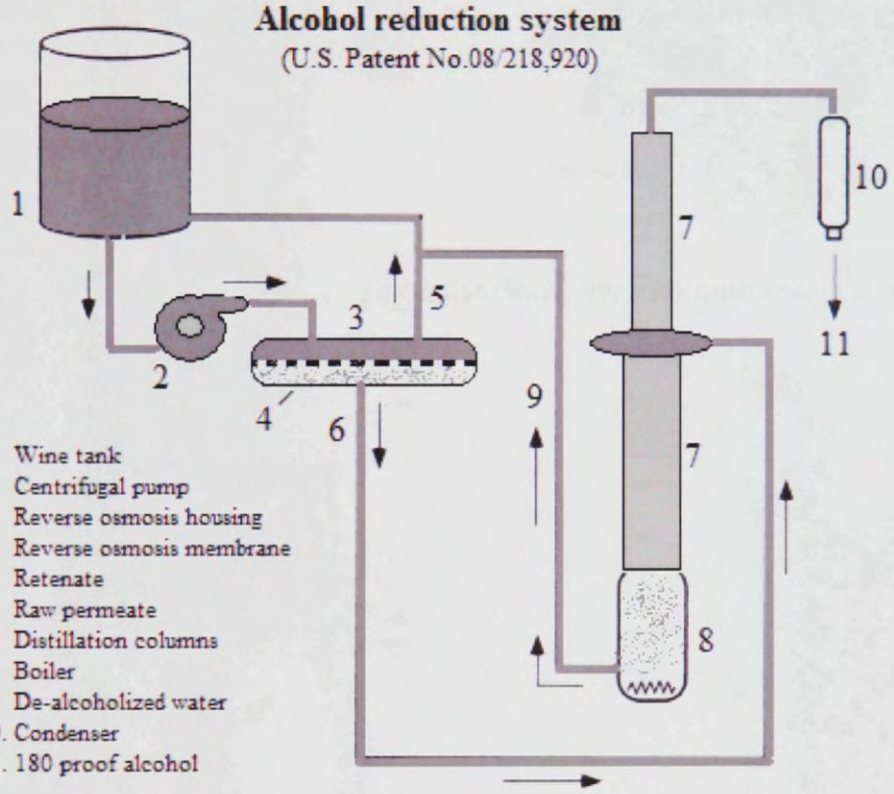
1.3.2.4 "Spinning cone" column technology

"Spinning cone column" (SCC) (Figure 1.2) is based on the same principles as distillation, with the use of gas or steam to remove volatile compounds from a portion of the treated beverage. SCC was first developed in the USA in the 1930s and has

Figure 1.1 Illustration of an alcohol reduction system using reverse osmosis (from www.vinovation.com)

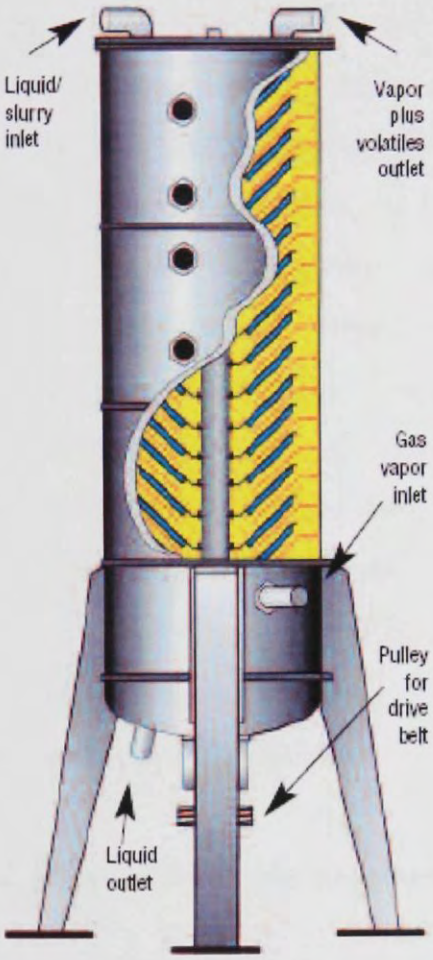
Alcohol reduction system

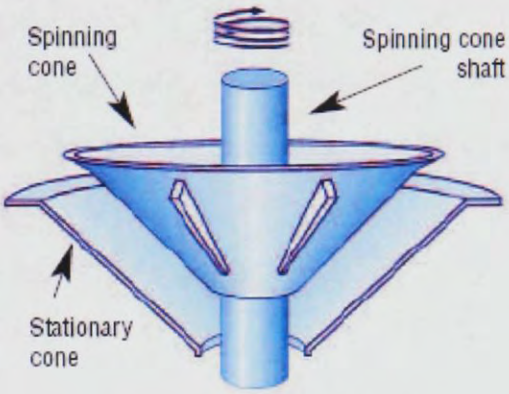
(U.S. Patent No.08/218,920)

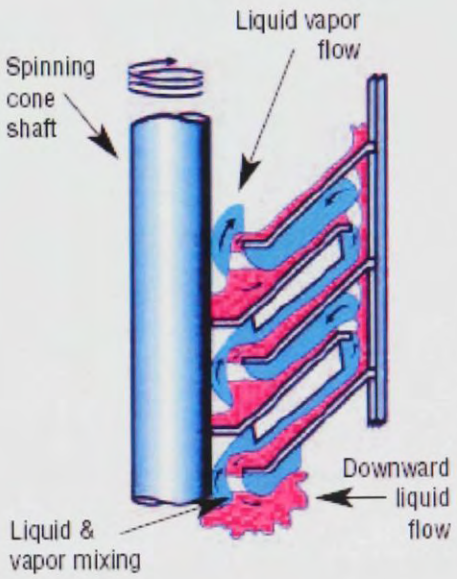


1. Wine tank
2. Centrifugal pump
3. Reverse osmosis housing
4. Reverse osmosis membrane
5. Retenate
6. Raw permeate
7. Distillation columns
8. Boiler
9. De-alcoholized water
10. Condenser
11. 180 proof alcohol

Figure 1.2 Spinning cone column (www.conetech.com)


Liquid/
slurry
inlet
Vapor
plus
volatiles
outlet
Gas
vapor
inlet
Pulley
for
drive
belt
Liquid
outlet


Spinning
cone
Spinning cone
shaft
Stationary
cone


Liquid vapor
flow
Spinning
cone
shaft
Downward
liquid
flow
Liquid &
vapor mixing

been improved over the years (Sykes et al. 1992, Gray 1993, Pyle 1994, Pickering 2000). The column is usually built of stainless steel and contains two different types of cones; stationary cones attached to the internal surface of the column, and rotatingcones, which are attached to the central spinning shaft of the column. The beverage is pumped through an inlet at the top of the column, and pulled down by gravity and a vacuum pump. The spinning cones create a centrifugal force, which aggregates a thin layer of the wine on each spinning cone, and pushes it up. When the liquid layer reaches the end of the cone surface it overflows and runs down and accumulates on the next rotating spinning cone. This process is repeated, down the cylinder until wine reaches the bottom outlet. Vapor introduced into the column during the process flows upwards and is in constant contact with the thin layer of wine, collecting volatile compounds. Rotating cones are also fitted with fins that create turbulence in the vapor and liquid fluids, greatly increasing the contact area between the processed beverage and the vapor. The process occurs in two steps. First wine is passed through the column to capture its aroma and flavor compounds, which can be stored in concentrated form. The process is then repeated removing ethanol from the wine. In the final stages, aromas and flavors are returned to the treated wine, creating a final product with lower ethanol content and, it is claimed, possessing the original flavor and textural characteristics (www.conetech.com). Although the SCC has been successfully applied at commercial scale, operational and equipment costs of this technique are very high, and it is unlikely that the original characteristics of wine remain intact after treatment.

1.3.2.5 Miscellaneous physical methods for ethanol adjustment in beverages

There are number of alternative approaches that can be used to reduce the alcohol content of alcoholic beverages. These include: dilution of either wine or grape juice with water, blending of wine with fruit juices, and early arrest of fermentation. Most of these approaches are not routinely used in wine industry, however, because they compromise wine quality (Pickering 2000).

1.3.2.6 Disadvantages of physical methods for alcohol removal from wine

Physical methods used to remove alcohol from beverages incur higher production expenses, increasing overall production costs. Many producers are reluctant to introduce these processes because of these additional costs. Furthermore, ethanol extraction methods irreversibly change the original characteristics inherent in the original wine. Some approaches used for reducing ethanol concentration in wine, such as dilution of either wine or grape juice with water, blending of wine with fruit juices, and early arrest of fermentation, are not allowed by law in some countries. Such practices, even if permitted, can negatively impact on wine quality and therefore would not be used in the production of premium wines. (http://www.conetech.com/Downloads/ConeTech_Technical_Brochure.pdf).

1.3.3 Biological approaches used to reduce the alcohol content of beverages

A variety of biological approaches have been developed and successfully used to change product profile during yeast fermentation. These are described below.

1.3.3.1 Enzymatic approaches – glucose oxidase

Glucose oxidase (GOX) is an oxygen-dependent dehydrogenase that catalyses the first step of a two-step process associated with the conversion of glucose to gluconic acid. In the reaction catalyzed by GOX, glucose is first oxidized to gluconolactone, which is subsequently hydrolyzed to gluconic acid. Pretreatment of grape juice with GOX results in decreased glucose levels. Wines produced from GOX-treated juices may have an ethanol concentration reduction of up to 40% (Pickering 1999a). During GOX treatment, however, large amounts of gluconic acid are formed and remain in the wine product, creating an acid imbalance that compromises wine quality (Pickering 1999b).

1.3.3.2 Redirecting cellular carbon metabolism to reduce ethanol levels - an outline.

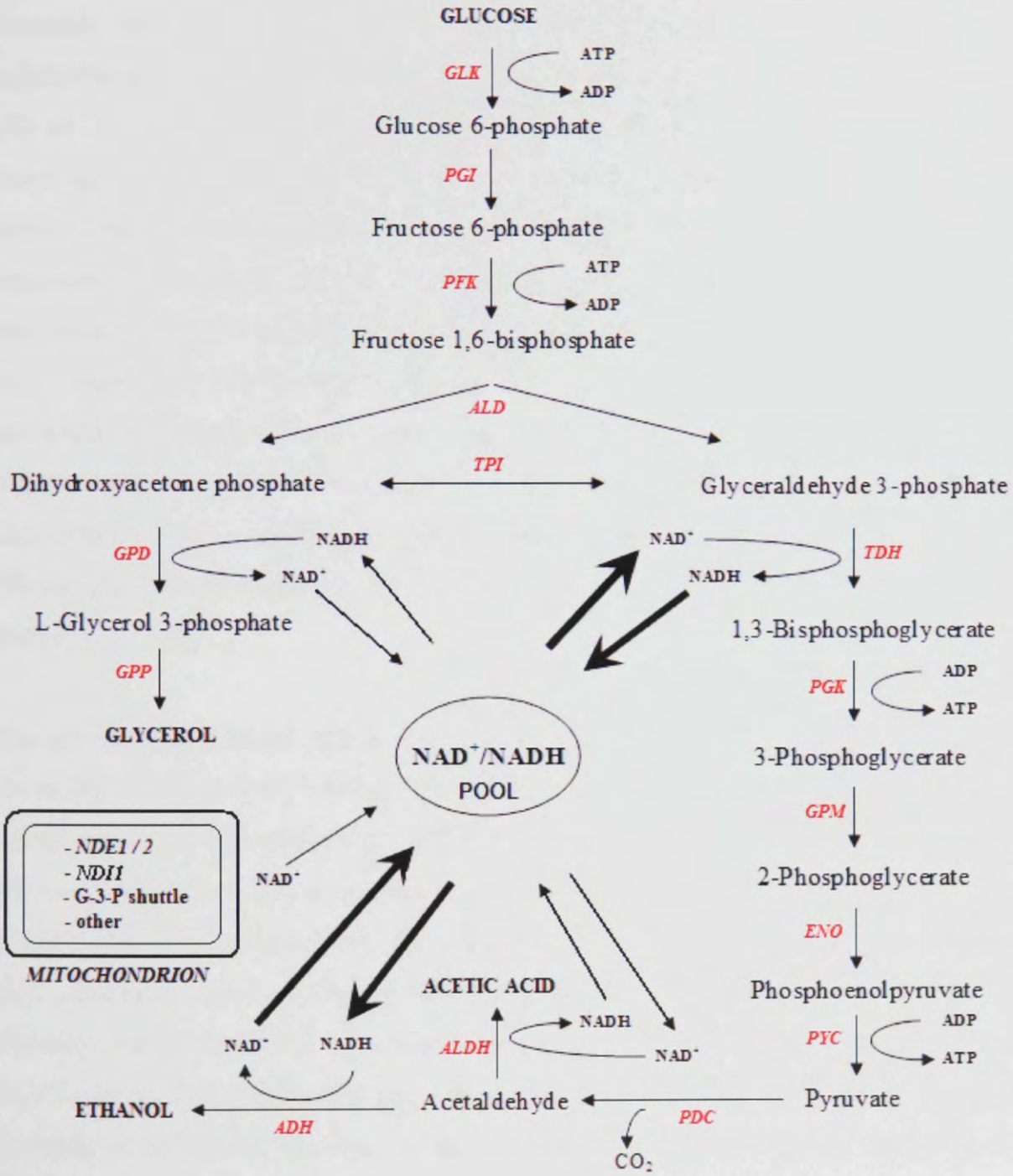
The rationale for redirecting cellular carbon metabolism away from ethanol production is based on complementary cellular strategies to maintain redox balance, particularly with respect to NAD^+/NADH. In yeast cells, NAD^+ and NADH are regulated to maintain a relatively fixed and stable equilibrium (De Koning and

Van Dam, 1992, Richard et al. 1993). In this equilibrium NAD^+, which is continuously reduced during yeast growth, is coupled to the re-oxidation of NADH. In *S. cerevisiae*, the bulk of NAD^+ reduction takes place during glycolysis, when glyceraldehyde-3-phosphate is converted to 1,3-bisphosphoglycerate; the availability of NAD^+ is essential for glycolysis to proceed and cells must re-cycle the reduced form of this co-enzyme, otherwise the glycolytic flux is reduced and this could be lethal for the cell. Although in normally functioning yeast most of the NADH produced during glycolysis is subsequently oxidized during ethanol formation, (see Figure 1.3), yeast can utilize several other ways for oxidizing NADH, including cytosolic production of glycerol catalyzed by glycerol-3-phosphate dehydrogenases *GPD1* and *GPD2*.

Regeneration of NAD^+ may also occur via mitochondrial contributions when yeast are grown under glucose limited conditions and, although mitochondria contribute to cystolic redox balancing when yeast are grown under excess glucose conditions, the extent of this contribution to the redox pool is unclear. Mitochondrial-based contributions to cystolic NADH oxidation can occur according to the following known mechanisms: oxidation via 'internal' mitochondrial NADH dehydrogenase, oxidation via external mitochondrial NADH dehydrogenase and respiration of cytosolic NADH via glycerol-3-P shuttle (Bakker et al. 2001).

'Internal' NADH dehydrogenase, encoded by *NDI1*, is an enzyme that specifically reacts with NADH and is highly glucose repressible. Yeast grown on glucose has much lower transcript levels of Ndi1p compared to growth on non fermentable carbon sources such as acetate or ethanol (Vries and Grivell 1988). Also, highly increased transcription of this gene was observed when growing cells entered diauxic shift (De Risi et al. 1997). Mitochondria isolated from *NDI1*-null mutants failed to oxidize substrates that generate intra-mitochondrial NADH, such as ethanol. *NDI1*-null mutants, however, displayed unaffected growth on ethanol, which was surprising because dissimilation of ethanol would require reoxidation of intramitochondrial NADH (Marres et al. 1991). This observation suggests that other mechanisms are involved in intramitochodnrial NADH oxidation. Glucose repression of this gene suggests it has a minor contribution to cytosolic redox balance during growth on high

Figure 1.3 Central metabolism in *S. cerevisiae* illustrating the main contributions to the NAD^+/NADH redox equilibrium during growth on excess glucose. Black thick lines represent major contributions to the NAD^+/NADH pool, black thin lines represent minor contributions to the NAD^+/NADH pool. The enzymes are marked in red: Glucokinase (GLK), Phosphoglucose isomerase (PGI), Phosphofructokinase (PFK), Aldolase (ALD), Triosephosphate isomerase (TPI), Glycerol-3-phosphate dehydrogenase (GPD), Glycerol-3-phosphatase (GPP), Triosephosphate dehydrogenase (TDH), Phosphoglycerate kinase (PGK), Phosphoglycerate mutase (GPM), Enolase (ENO), Pyruvate decarboxylase (PDC), Aldehyde dehydrogenase (ALDH), Alcohol dehydrogenase (ADH).


GLUCOSE
GLK
ATP
ADP
Glucose 6-phosphate
PGI
Fructose 6-phosphate
PFK
ATP
ADP
Fructose 1,6-bisphosphate
ALD
Dihydroxyacetone phosphate
TPI
Glyceraldehyde 3-phosphate
GPD
NADH
NAD+
L-Glycerol 3-phosphate
GPP
GLYCEROL
NAD+
NADH
TDH
1,3-Bisphosphoglycerate
PGK
ADP
ATP
3-Phosphoglycerate
GPM
2-Phosphoglycerate
ENO
Phosphoenolpyruvate
PYC
ADP
ATP
Pyruvate
NAD+/NADH
POOL
- NDE1 / 2
- NDI1
- G-3-P shuttle
- other
MITOCHONDRION
NAD+
ACETIC ACID
NAD+
NADH
ALDH
NADH
NAD+
ETHANOL
ADH
Acetaldehyde
PDC
CO2

glucose concentrations. Growth of a *NAD1*-null yeast strain in shake-flasks cultures on glucose as a sole carbon source, did not appear to be compromised compared to the wild-type strain, suggesting a minor or no contribution of Ndi1p to the overall redox equilibrium under excess glucose conditions (Marres et al. 1991). Two isoforms of external NADH dehydrogenase has been identified in yeast: *NDE1* and *NDE2* (Luttik et al. 1998). Similarly to *NDI1*, transcription levels of *NDE1* and *NDE2* increased after diauxic shift, when cells started to utilize ethanol (Risi et al. 1997), suggesting that these genes are also glucose repressible and do not have a significant role in yeast exposed to high-glucose growth conditions. *NDE1*- and *NDE2*-null strains grown in shake-flasks on glucose as a sole carbon source grew at a maximum growth rate that was very similar to the wild type strain (Luttik et al. 1998). This suggested that during growth on glucose, when glucose dissimilation occurs predominantly via its fermentative oxidation, external NADH dehydrogenases do not play a significant role in NAD^+ regeneration. Luttik et al. (1998) also showed that mitochondria isolated from an *NDE1*- and *NDE2*-null strain failed to oxidize external NADH, the growth of such mutant was entirely respirative in aerobic glucose-limited chemostats. These observations suggest that cellular systems other than NADH dehydrogenases participate in the oxidation of cytosolic NADH (see, for review, Bakker et al. 2001).

The glycerol-3-phosphate shuttle is an indirect mechanism that can regenerate NAD^+ via oxidation of cytosolic NADH. This system contains two major components: NAD^+ linked glycerol-3-phosphate dehydrogenases, *GPD1* and *GPD2*, and mitochondrial glycerol-3-phosphate dehydrogenase *GUP2*. However, this shuttle is not likely to have a significant contribution in the NAD^+/NADH redox balance of yeast cells grown in high external glucose as Gup2p was previously shown to be glucose repressible (Nevoigt and Stahl 1997). Also, the same levels of glycerol were produced by the *GUP2*-null mutant and the wild type strain during growth in batch cultures on glucose (Larsson et al. 1998). Growth in glucose-limited chemostat did not significantly influence the phenotype of the *GUP2*-null mutant.

The mechanisms of NADH oxidation described above, although proved to have a role in aerobic conditions under glucose limitation, are unlikely to have a significant contribution to intracellular NAD^+/NADH redox balance in yeast grown on high

glucose concentrations. However, as mentioned earlier, the extent of mitochondrial contributions to the NAD^+/NADH redox balance of yeast grown on excess glucose is unclear.

The redox balance of the yeast cell is firmly linked to the production of metabolic by-products, such as glycerol, ethanol and acetic acid, and changes in the flux of their metabolic pathways are well known to have an impact on intracellular equilibrium, especially the NAD^+/NADH redox balance. This need by yeast to maintain a redox balance has been investigated and used in recent years, to design controlled and predictable metabolic rerouting systems for yeast, that allow the redirection of carbon flux towards desired end points, e.g., glycerol overproduction. A variety of GM-based approaches have been successfully introduced to reroute metabolic carbon flux towards increased glycerol synthesis at the expense of ethanol production (Michnick et al. 1996, Remize et al. 1999, de Barros Lopes et al 2000).

1.3.3.3 GMO-based approaches

Targeted changes can be made to the genome that lead to a redirection of metabolic flux away from ethanol synthesis and toward other end-points. Such approaches have, for example, allowed researchers to successfully manipulate the glycerol/ethanol ratio during yeast-driven alcoholic fermentation, enabling the development of yeast that produce increased levels of glycerol which, due to an increase in NAD^+ production, subsequently results in a reduction in ethanol production.

1.3.3.3.1 Over-expression of glycerol 3-phosphate dehydrogenase enzymes (GPD1 or GPD2).

One approach used to enhance glycerol production is the over-expression of *GPD1* and/or *GPD2* genes, which encode isozymes of glycerol 3-phosphate dehydrogenase (Gpd) (Nevoigt and Stahl 1996, Michnick et al. 1997, Remize et al. 1999 and 2001, de Barros Lopes et al. 2000, Eglinton et al. 2002, Cambon et al. 2006) (Figure 1.4). Gpd converts dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate (G-3-P), which is subsequently dephosphorylated to glycerol by glycerol 3-phosphatase. Over-expression of *GPD1* or *GPD2* has been shown to redirect yeast metabolism such that

Figure 1.4 Diagram of central metabolism in *S. cerevisiae* with *GPD* overexpression illustrating the main contributions to the NAD^+/NADH redox equilibrium during growth on excess glucose. Broken lines represent reduced carbon flow via glycolysis and fermentation, and reduction in contributions to NAD^+/NADH pool. Red lines indicate increased carbon flow towards glycerol and acetic acid production, and increased contributions to NAD^+/NADH pool. The enzymes are marked in red and they are: Glucokinase (GLK), Phosphoglucose isomerase (PGI), Phosphofructokinase (PFK), Aldolase (ALD), Triosephosphate isomerase (TPI), Glycerol-3-phosphate dehydrogenase (GPD), Glycerol-3-phosphatase (GPP), Triosephosphate dehydrogenase (TDH), Phosphoglycerate kinase (PGK), Phosphoglycerate mutase (GPM), Enolase (ENO), Pyruvate decarboxylase (PDC), Aldehyde dehydrogenase (AlDH), Alcohol dehydrogenase (ADH).

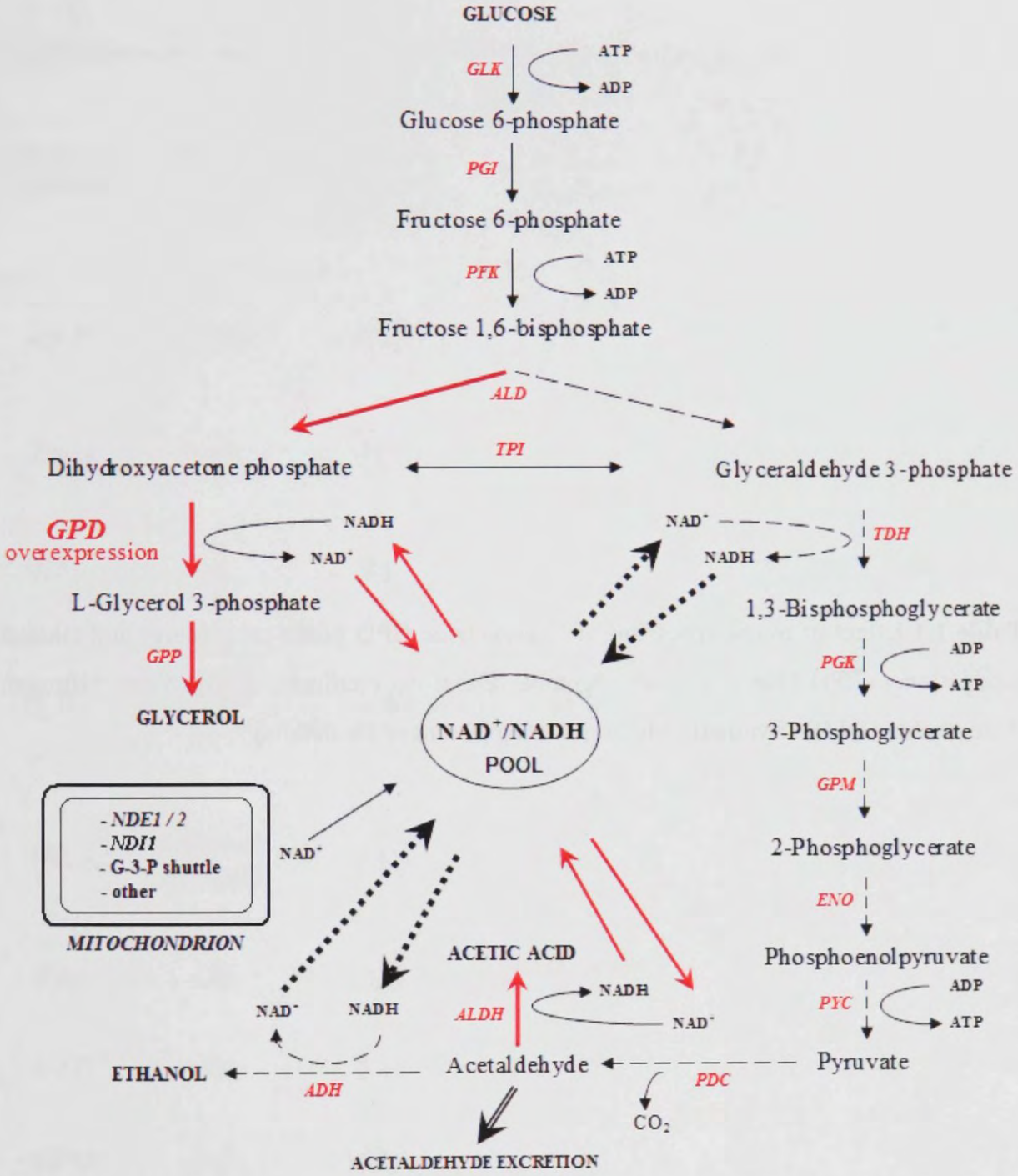

GLUCOSE
GLK
ATP
ADP
Glucose 6-phosphate
PGI
Fructose 6-phosphate
PFK
ATP
ADP
Fructose 1,6-bisphosphate
ALD
TPI
Dihydroxyacetone phosphate
Glyceraldehyde 3-phosphate
GPD
overexpression
NADH
NAD+
L-Glycerol 3-phosphate
GPP
GLYCEROL
NAD+
NADH
TDH
1,3-Bisphosphoglycerate
PGK
ADP
ATP
3-Phosphoglycerate
GPM
2-Phosphoglycerate
ENO
Phosphoenolpyruvate
PYC
ADP
ATP
Pyruvate
NAD+/NADH
POOL
- NDE1 / 2
- NDI1
- G-3-P shuttle
- other
MITOCHONDRION
NAD+
NAD+
NADH
ACETIC ACID
ALDH
NADH
NAD+
ETHANOL
ADH
Acetaldehyde
PDC
CO2
ACETALDEHYDE EXCRETION

Table 1.1 Effect of over-expression of *S. cerevisiae* GPD genes on glycerol and ethanol production. YEPD-(Yeast Extract, Peptone, Dextrose medium), YNB-(Yeast Nitrogen Base medium), MS-(Synthetic Medium – see references for details)

Gene over-expressed	Medium used	Increase in glycerol yield after *GPD* over-expression (%)	Decrease in ethanol yield after *GPD* over-expression (%)	Increase in acetic acid yield after *GPD* over-expression (%)	Source of data
GPD1	YEPD	***548***	***35***	***194***	Nevoight and Stahl 1996
GPD1	YNB	***225***	***22***	***207***	Michnick et al. 1997
GPD1	MS	***123***	***3***	***181***	Remize et al. 1999
GPD2	Grape juice	***109***	***4.5***	***76***	de Barros Lopes et al. 2000
GPD2	Synthetic Leu-free	***163***	***24***	***115***	Eglinton et al. 2002
GPD1	MS	***275***	***17.5***	***363***	Cambon et al. 2006
GPD1	MS	***214***	***11.5***	***158***	Cambon et al. 2006
GPD1	MS	***129***	***10.5***	***259***	Cambon et al. 2006

it synthesizes up to 548% more glycerol depending on the yeast strain, medium and fermentation conditions (Table 1.1). Increased glycerol levels were observed to cause decreases in ethanol production, with ethanol yields decreasing by as much as 35% (Table 1.1). Although increased glycerol production reduces ethanol yield due to an oversupply of NAD^+, it is often the case that this decrease is not sufficient to restore the steady state NAD^+/NADH ratio. In such cases, the surplus NAD^+ is reduced by aldehyde dehydrogenase resulting in higher acetate production rates, which has an undesirable impact on wine quality (see Figure 1.4)

Along with significant increases in glycerol and acetic acid biosynthesis, and corresponding decreases in ethanol yield observed with GPD mutant strains, many other alterations in the flux of central carbon metabolism were reported in *GPD1/2* overexpressing strains. For example, such modified strains were reported to produce elevated levels of succinate, acetaldehyde, acetoin and 2,3-butanediol (Michnick et al. 1997, Remize et al. 1999, Eglinton et al. 2002, Cambon et al. 2006). Further genetic modifications of the yeast need to occur to avoid producing excessive amounts of these metabolites, thus preserving wine quality. For example, acetic acid is produced mainly via oxidation of acetaldehyde, which involves acetaldehyde dehydrogenase (*ALD*), of which five isozymes have been reported in *S. cerevisiae* (Saint-Prix et al. 2004). During fermentation, acetic acid is synthesized mainly by oxidation of acetaldehyde via cytosolic, NADPH dependent, Mg^{2+}-activated Ald6p, encoded by the *ALD6* gene (Meaden et al. 1997, Remize et al. 2000, Saint-Prix et al. 2004). Deletion of the *ALD6* gene in a *GPD2* over-expressing laboratory strain of *S. cerevisiae* S288C, resulted in a significant decrease in acetic acid formation compared to the parent strain, while glycerol production increased. Surprisingly, *GPD2* overexpression in an *ALD6* deletion background resulted in a phenotype that produced slightly increased ethanol yields relative to either of these modifications introduced separately, but still less than the parent strain (Eglinton at al. 2002). Similarly, in a study using three wine yeast strains that were modified to overexpress *GPD1*, deletion of *ADL6* gene caused a reduction of acetic acid production with glycerol was overproduction. A significant reduction in acetate formation was also noted for wild type strains carrying deletions of *ADL6* (Cambon at al. 2006).

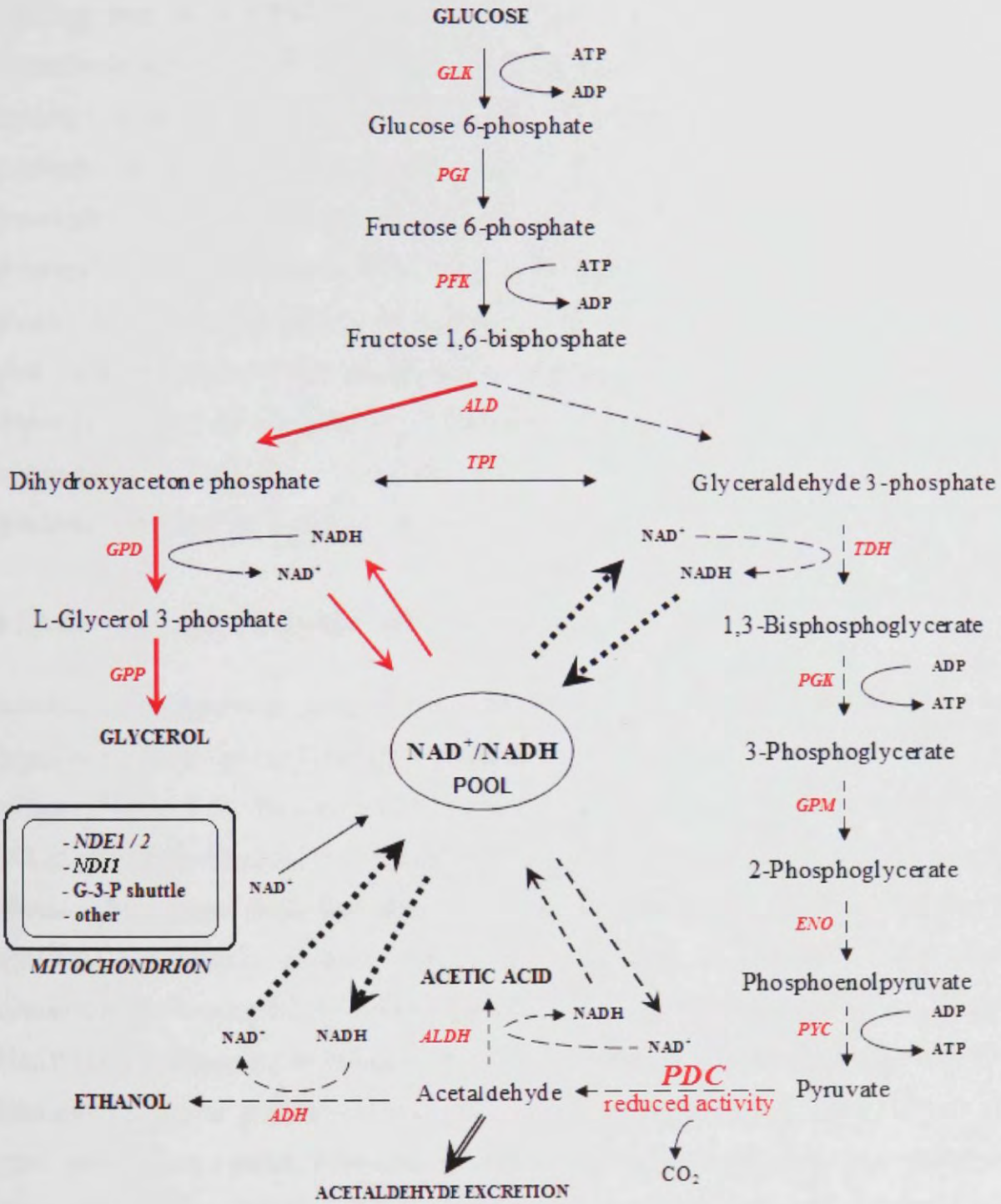
1.3.3.3.2 Pyruvate decarboxylase (*PDC*) mutants.

Pyruvate decarboxylase, (Pdcp), is a homotetrameric enzyme that catalyses the decarboxylation of pyruvate to acetaldehyde and CO_2 (Figure 1.3). There are three pyruvate decarboxylase genes in *S. cerevisiae*: *PDC1*, *PDC5* and *PDC6*, which are regulated by the transcription factor *PDC2*. Of the pyruvate decarboxylases, only *PDC1* and *PDC5* are known to be active in yeast during fermentation (Hohmann 1991, Flikweert et al. 1996 and 1999, Ishida et al. 2006). Deletion of all three *PDC* renders yeast unable to grow in batch cultures in mineral defined medium complemented with glucose as a sole carbon source, and such mutants synthesize extensive amounts of pyruvate. *PDC*-null yeast mutants could only utilize glucose when a supplementary amount of acetate or ethanol (up to 5% of the carbon supplied) was added to the medium.Growth in complex and defined media on ethanol was not impaired in a *PDC*-null strain, and such strain produced the same biomass as the wild-type (Flikweert et al. 1996). Replacement of ethanol by glucose feed in chemostat cultures of *PDC*-null mutants caused growth inhibition and culture wash out. In *PDC*-null mutants, excess NADH production via glycolysis causes NADH accumulation (since it is no longer oxidized by the fermentation pathway) which inhibits glycolytic flux.

Yeast with reduced or abolished pyruvate decarboxylase activity have lower carbon flux in the fermentative pathway; the reduction in fermentation activity occurring due to less pyruvate being converted to acetaldehyde. This may cause several changes in cell function. First, the glycolytic rate upstream of pyruvate is slowed down, due to the accumulation of glycolytic intermediates and their effect on glycolytic enzyme activity and this, in turn, may result in lowered ATP yields. Second, with a reduction in fermentation activity, the NADH oxidation rate will be affected and the cell may compensate for this by increasing glycerol production (see, for review, Pronk et al. 1996), (Figure 1.5).

A significant reduction in ethanol production and an increase in glycerol production were observed when transcription factor *PDC2,* which is required for high level transcription of pyruvate decarboxylase genes, was deleted, most likely due to a decrease in acetaldehyde production and a surplus of intracellular NADH. Unlike the

Figure 1.5 Diagram of central metabolism in *S. cerevisiae* with reduced *PDC* activity illustrating the main contributions to the NAD^+/NADH redox equilibrium during growth on excess glucose. Broken lines represent reduced carbon flow via glycolysis and fermentation, and reduction in contributions to NAD^+/NADH pool. Red lines indicate increased carbon flow towards glycerol and acetic acid production, and increased contributions to NAD^+/NADH pool. The enzymes are marked in red and they are: Glucokinase (GLK), Phosphoglucose isomerase (PGI), Phosphofructokinase (PFK), Aldolase (ALD), Triosephosphate isomerase (TPI), Glycerol-3-phosphate dehydrogenase (GPD), Glycerol-3-phosphatase (GPP), Triosephosphate dehydrogenase (TDH), Phosphoglycerate kinase (PGK), Phosphoglycerate mutase (GPM), Enolase (ENO), Pyruvate decarboxylase (PDC), Aldehyde dehydrogenase (AlDH), Alcohol dehydrogenase (ADH).


GLUCOSE
GLK
ATP
ADP
Glucose 6-phosphate
PGI
Fructose 6-phosphate
PFK
ATP
ADP
Fructose 1,6-bisphosphate
ALD
Dihydroxyacetone phosphate
TPI
Glyceraldehyde 3-phosphate
GPD
NADH
NAD+
L-Glycerol 3-phosphate
GPP
GLYCEROL
NAD+
NADH
TDH
1,3-Bisphosphoglycerate
PGK
ADP
ATP
3-Phosphoglycerate
GPM
2-Phosphoglycerate
ENO
Phosphoenolpyruvate
PYC
ADP
ATP
Pyruvate
NAD+/NADH
POOL
- NDE1 / 2
- NDI1
- G-3-P shuttle
- other
MITOCHONDRION
NAD+
ACETIC ACID
ALDH
NADH
NAD+
NAD+
NADH
ETHANOL
ADH
Acetaldehyde
PDC
reduced activity
CO2
ACETALDEHYDE EXCRETION

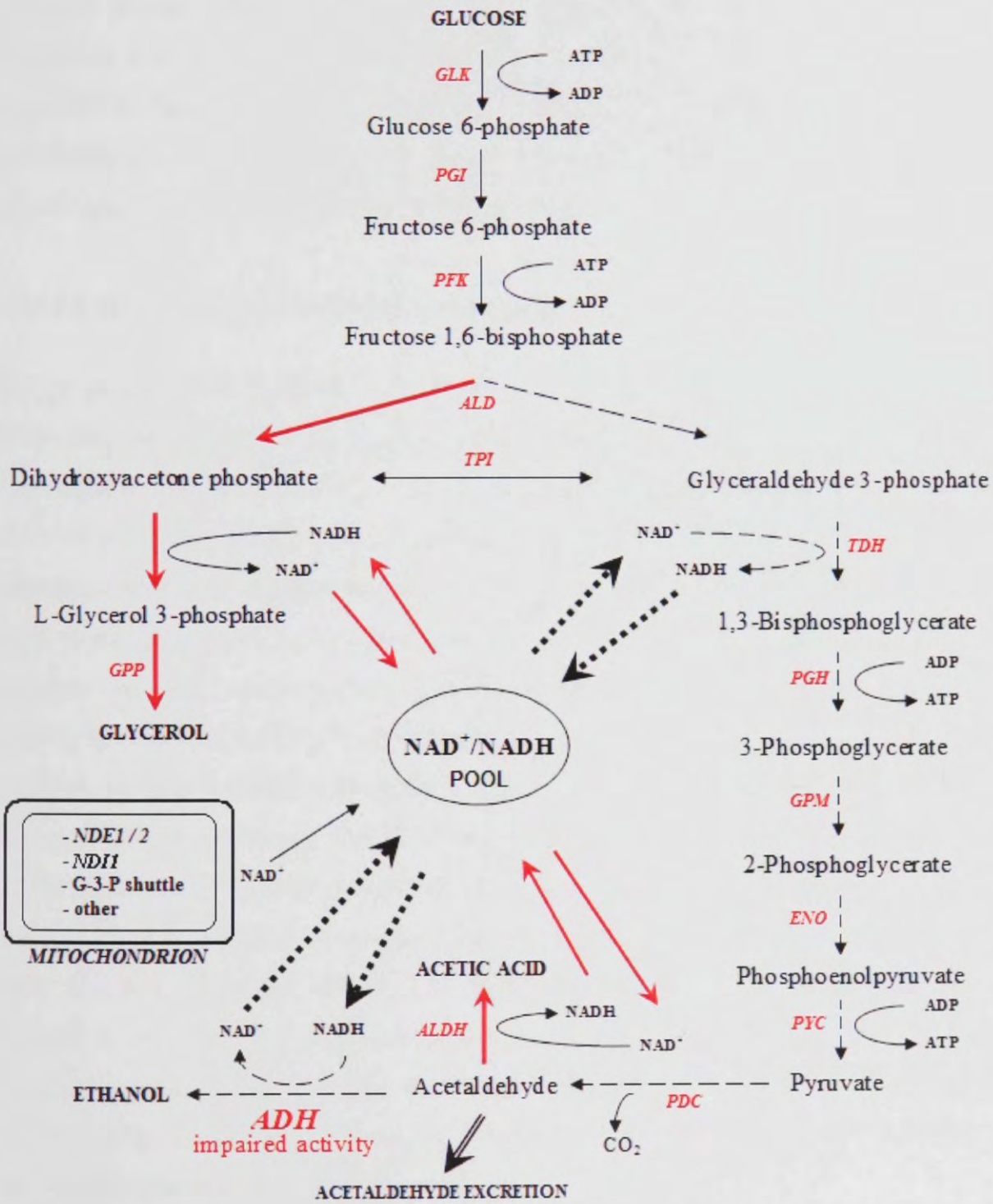
PDC-null mutants, a *PDC2* deletion mutant still possessed 19% residual Pdc activity which is sufficient to support growth on glucose (Nevoigt and Stahl 1996). Yeast with *PDC2* deletion have been observed to produce 366% more glycerol and 28% less ethanol, underlining the importance of the *PDC2* gene in central carbon metabolism (Nevoigt and Stahl 1996). Redirection of carbon metabolism away from ethanol synthesis in a *PDC2* null mutant was further enhanced by overexpression of *GPD1*, leading to a higher glycerol production rate at the expense of fermentation pathway products. This modified strain had an increase in glycerol yield of 707%, which was accompanied by a reduction in ethanol yield of 45% (Nevoigt and Stahl 1996). It is noteworthy that a deletion of *PDC2* only resulted in a small increase in acetic acid production (6%), while *GPD1* overexpression resulted in a 294% increase in acetate yield. A combination of both modifications resulted in acetate yield increase of 203%, which is lower than the acetate yield increase observed for the strain with *GPD1* overexpression only; this is most likely due to the lower acetaldehyde production resulting from the *PDC2* deletion (Nevoiht and Stahl 1996).

1.3.3.3.3 Alcohol dehydrogenase (*ADH*) mutants

Alcohol dehydrogenases (encoded by *ADH1*, *AHD3*, *ADH4* and *ADH5*) play an important role in yeast fermentation, catalyzing the reduction of acetaldehyde to ethanol (Figure 1.4). This reaction uses NADH as a cofactor, oxidising it to NAD^+; this plays an essential role in maintaining cellular redox balance during growth on glucose. Reducing ethanol formation by the impairment or deletion of *ADH* genes in the cell significantly reduces fermentative oxidation of NADH. Under these circumstances, waning NAD^+ levels (due to its glycolytic consumption), or increasing NADH levels, stimulate an increase in glycerol production, which regenerates NAD^+. Increased glycerol production may also cause a reduction in carbon flow via glycolysis and, as a result, a decrease in glycolytic-based energy production. Increased glycerol production, leading to partial restoration of NAD^+ levels, and decreased rate of conversion of acetaldehyde to ethanol stimulate a subsequent increase in acetate formation (Figure 1.5).

Yeast lacking the major isoform of alcohol dehydrogenase, *ADH1*, have impaired ethanol synthesis and increased production of glycerol (Ciriacy 1975;

Figure 1.6 Diagram of central metabolism in *S. cerevisiae* with impaired activity of *ADH* illustrating the main contributions to the NAD^+/NADH redox equilibrium during growth on excess glucose. Broken lines represent reduced carbon flow via glycolysis and fermentation, and reduction in contributions to NAD^+/NADH pool. Red lines indicate increased carbon flow towards glycerol and acetic acid production, and increased contributions to NAD^+/NADH pool. The enzymes are marked in red and they are: Glucokinase (GLK), Phosphoglucose isomerase (PGI), Phosphofructokinase (PFK), Aldolase (ALD), Triosephosphate isomerase (TPI), Glycerol-3-phosphate dehydrogenase (GPD), Glycerol-3-phosphatase (GPP), Triosephosphate dehydrogenase (TDH), Phosphoglycerate kinase (PGK), Phosphoglycerate mutase (GPM), Enolase (ENO), Pyruvate decarboxylase (PDC), Aldehyde dehydrogenase (AlDH), Alcohol dehydrogenase (ADH).


GLUCOSE
GLK
ATP
ADP
Glucose 6-phosphate
PGI
Fructose 6-phosphate
PFK
ATP
ADP
Fructose 1,6-bisphosphate
ALD
Dihydroxyacetone phosphate
TPI
Glyceraldehyde 3-phosphate
NADH
NAD+
L-Glycerol 3-phosphate
GPP
GLYCEROL
NAD+
NADH
TDH
1,3-Bisphosphoglycerate
PGH
ADP
ATP
3-Phosphoglycerate
GPM
2-Phosphoglycerate
ENO
Phosphoenolpyruvate
PYC
ADP
ATP
Pyruvate
NAD+/NADH
POOL
- NDE1 / 2
- NDI1
- G-3-P shuttle
- other
MITOCHONDRION
NAD+
ACETIC ACID
ALDH
NADH
NAD+
NAD+
NADH
ETHANOL
Acetaldehyde
PDC
CO2
ADH
impaired activity
ACETALDEHYDE EXCRETION

Johansson and Sjörström 1984). Similarly, the introduction of irreversible defects in four ADH isoenzymes (*ADH1-4*) causes significant diversion of metabolism toward glycerol synthesis, but the yeast are still able to produce up to one third of the theoretical ethanol yield (Drewke et al. 1990). An *S. cerevisiae* strain containing mutations in *ADH1-4* genes, (adh^{o}), was compared to the parent strain which expressed wild-type *ADH1*. Compared to the latter, the adh^{o} strain had significantly impaired growth, which was accompanied by an increase of 1433% in glycerol production and a reduction of 63% in ethanol yield (Drewke et al. 1990). Yeast impaired in functional *AHD1-4* isoforms also showed increased accumulation of acetaldehyde and acetic acid, with acetate yields reaching 11% (w/v); acetic acid accumulation was not observed in the parent strain.

1.3.3.3.4 Triose phosphate isomerase (*TPI*) mutants

Triose phosphate isomerase, *TPI*, is located at the branch point of glycolysis, catalyzing the conversion of dihydroxyacetone phosphate (DHAP) to glyceraldehyde 3-phosphate (GAP) (Figure 1.4). *S. cerevisiae* mutants lacking *TPI* produced glycerol at levels as high as 80-90% of the theoretical yield on glucose (0.51g/g), and this was associated with a substantial decrease in ethanol production (Compagno et al. 1996). Growth on glucose as a sole carbon source was not possible for *TPI* null strains, possibly due to an undersupply of NADH which would restrict glycerol production leading to DHAP accumulation in the cell. The restoration of growth in glucose-based medium was made possible by adding ethanol to the culture, supporting the concept that such strains are lacking in NADH supply (Campagno et al. 2001, Overkamp et al. 2002). Glucose-based growth defects in *TPI* null mutants could also be partially restored by deleting genes encoding NADH dehydrogenases, *NDE1* and *NDE2*, and gene encoding glycerol-3-phosphate shuttle protein - *GUT2*, all of which are associated with cytosolic NADH oxidation (Larsson et al. 1998, Luttik et al. 1998, Overkamp et al. 2000). Deleting these genes reduced the consumption of NADH, increasing the availability of cytosolic NADH for glycerol synthesis in *TPI* deletion mutants (Overkamp et al. 2002).

Total inactivation of *TPI* in yeast is not desirable for low-alcohol wine production because of the severe growth side-effects. However, partial or regulated inactivation of *TPI* may result in lower ethanol production and acceptable yeast growth. Mutations

in regulatory genes, such as *GCR1* and *GCR2*, (both transcriptional activators of genes involved in glycolysis), have been shown to considerably reduce the activity of *TPI*. However, the deletions of *GCR1* or *GCR2* genes were non specific to this gene, reducing activity of number of other glycolytic enzymes (Clifton and Fraenkel 1981, Uemura and Fraenkel 1990 and 1999). Also, mutations in the binding sites of the regulatory genes *REB1*, *RAP1* and *GRC1* have been shown to decrease *TPI* activity. Mutations within *REB1* or *RAP1* binding sites reduced *TPI* activity by 82% and 91% respectively, while mutation of both the *GRC1* binding sites caused almost 99% reduction in *TPI* activity (Scott and Baker 1993). Considering these observations, it is foreseeable that manipulations of the *TPI* transcription level could lead to the development of low ethanol-producing GM yeast.

1.3.3.3.5 Fps1p contributes to glycerol biosynthesis in yeast

Fps1p is a member of the Major Intrinsic Protein (MIP) family of channel proteins and facilitates glycerol export/import in *S. cerevisiae* cells. The main function of this protein is regulation of intracellular glycerol levels, and it is regulated by the osmolarity of the surrounding environment (Luyten et al. 1995, Tamas et al. 1999). Deregulation of the Fps1s channel in yeast, by expression of a constantly 'open' form of the protein, lead to an overproduction of glycerol by up to 2.7 fold, depending on the type of modifications to the channel (Remize et al. 2001). Remize et al. (2001) demonstrated that a truncated form of Fps1p, lacking the N-terminal domain, resulted in continuous leakage of glycerol out of the cell, which was compensated for via increased glycerol production. This Fps1p modification, however, resulted in impaired growth on glucose and a corresponding decrease of up to 30% in biomass formation. Further improvements in glycerol yield were obtained by *GPD* over-expression in a *FPS1* modified background, but this also led to even greater growth inhibition (Remize et al. 2001). The effect of these modifications on acetic acid production was not investigated in the study conducted by Remize et al. (2001). Genetic manipulation of the Fps1p channel may offer an alternative approach to diverting metabolism toward glycerol production, and potentially away from ethanolic fermentation.

1.3.3.3.6 Introduction of glucose oxidase *(GOX)* from *Aspergillus niger* into *S. cerevisiae*

Ethanol production in wine fermentations may be decreased by reducing the amount of available sugar in grape juice (see Section 1.3.3.1). One way this has been achieved is by introducing a foreign glucose oxidase gene (GOX) into *S. cerevisiae*. Glucose oxidase converts glucose to gluconic acid, reducing the amount of glucose available for glycolysis and ethanolic fermentation. *GOX1* from *Aspergillus niger*, expressed as a secretion product in yeast, has been shown to reduce final ethanol concentrations by up to 2% during trial fermentations (Malherbe et al. 2003). Despite the successful introduction of *GOX1* into yeast, the authors did not observe a significant increase in culture acidity during fermentation. This raises some questions about this study since the action of Gox1p should result in excess gluconic acid production and therefore contribute to elevated acidity in the culture. Such an effect was reported when GOX was used to pre-treat grape juice prior to wine fermentation (Pickering 1999a).

1.3.3.3.7 A combination of different genetic engineering approaches and ethanol biosynthesis.

In an elegant study, Cordier et al. (2007) recently investigated the impact of a combination of GM approaches in a single strain for increasing glycerol production. The genes investigated are involved in glycerol production and transport (*GPD1* and *FPS1*), glycolytic branch point conversion of DHAP to GAP (*TPI*), and the reduction or oxidation of acetaldehyde to ethanol and acetic acid (*ADH1* and *ALD3*), respectively. The combination of genetic modifications made in *S. cerevisiae* and their effect on central metabolism are shown in Table 1.2.

Although the engineered strains were designed to achieve increased glycerol production, it is evident that in each case this also resulted in a significant, and somewhat proportional, decrease in ethanol yields. For example, strain HC42, with *GPD1* and *ALD3* overexpression, and *ADH1* and *TPI1* deletions, produced 80.5% less ethanol and the glycerol yield increased by 2,200%. The shift away from ethanol metabolism in this strain, however, resulted in an increase in acetic acid yield and a large reduction in growth rate (Cordier et al. 2007). Cordier et al. (2007) also determined the levels of NAD^+ and NADH in the various stains during incubation and

Table 1.2 Growth rate, biomass and fermentation products in *S. cerevisiae* strains engineered for glycerol production (from Cordier et al. 2007). Values are expressed as 'g product per 100 g glucose', nd=not determined

Yeast Strains	Genetic modification	Growth rate (h^{-1})	Biomass yield	Ethanol	Glycerol	Acetate
CEN.PK2	Wild type	0.44±0.04	12±0.5	41±3.0	2.0±0.4	4±0.5
HC13	*GPD1* overexpression	0.44±0.04	10±0.3	32±3.0	19.3±1.4	5±0.5
HC16	*GPD1* overexpression and *TPI1* deletion	0.09±0.01	9±0.4	19±2.0	36±3.6	9±0.4
HC17	*ADH1* deletion	0.26±0.03	11±0.5	29±2.0	19±2.5	13±0.8
HC23	*GPD1* and *ALD3* overexpression	0.39±0.03	10±1.3	33±1.0	18.5± 2.0	5±0.6
HC30	*GPD1* and *ALD3* overexpression and *TPI1* deletion	0.13±0.03	9±1.4	19±2.0	42±2.5	8±0.4
HC32	*GPD1* overexpression and *TPI1*+*ADH1* deletion	0.10±0.01	9.1±0.5	8±0.4	46±2.5	nd
HC42	*GPD1* and *ALD3* overexpression and *ADH1*+*TPI1* deletion	0.13±0.01	8.1±1.3	8±1.4	46±2.7	6±0.3
FM62	*GPD1*, *ALD3* and *FPS1* overexpression, *TPI1*+*ADH1* deletion	0.12±0.02	9±1.4	12±2.8	46±1.7	nd

found that glycerol overproducing strains also had a significant increase in NAD^+/NADH ratio, providing evidence supporting the importance of this redox couple in glycerol and ethanol metabolism in *S. cerevisiae*. For instance, strains HC16 and HC42 had increases in their NAD^+/NADH ratios by 373% and 361% respectively, which was accompanied by a decrease in ATP levels. The sensitivity of ATP levels to intracellular NADH production led to the suggestion that glycolysis in wild-type *S. cerevisiae* is limited in its capacity to produce NADH; the low availability of NADH subsequently inhibiting glycerol production (Cordier et al. 2007). These results may lead to the development of new strategies (based on the limited capacity of yeast to regenerate NADH) for genetically engineering 'low-ethanol' producing yeast strains.

Geertman et al. (2006) disrupted metabolic pathways, other than glycerol synthesis, that compete for cytosolic NADH as a cofactor, increasing its availability for glycerol production. These scientists deleted the three *PDC* isoforms (*PDC1, 5* and *6)*, genes encoding the NADH dehydrogenases (*NDE1* and *NDE2*), and mitochondrial G-3-P dehydrogenase (*GUT2)*. The theoretical glycerol production for this engineered strain was calculated using a model of *S. cerevisiae* metabolic networks published by Lange (2002). This GM strain produced 0.9 mol glycerol (mol glucose)$^{-1}$ when grown in a chemostat under aerobic conditions, which was slightly higher than the theoretical estimation obtained using metabolic modeling [0.86 mol glycerol (mol glucose)$^{-1}$] (Geertman et al. 2006). The effect of these modifications on ethanol and acetic acid yields were not reported.

In light of the work presented in this section, it is evident that engineering GM yeast using a judicious combination of gene modifications, should enable the development of yeast strains with higher glycerol production and lower ethanol yields.

1.3.3.3.8 Issues associated with genetically modified yeast in wine production

Modern genetic engineering technologies coupled with our knowledge of central metabolism makes it possible to design and genetically manipulate wine yeast that produce less ethanol. However, public attitudes regarding the use of GMOs in food and beverages are often less then positive, rather in some countries consumers can be hostile towards companies that produce GMO-based products. In addition to this,

there is a range of legislation in various countries that govern the sale of products either containing GMOs or that used GMOs in their manufacture.

The Australian wine industry's position on the application of gene technology in grape and wine production is that no genetically modified organisms be used in the production of Australian wine. The reason for this is not that the industry is anti-GM, rather it acknowledges the importance of safety and public acceptance before adopting any new technology in wine production. The wine industry is of the view that there are potentially great benefits in employing recombinant gene technology, however, the industry is also conscious of the need for safety, openness and quality assurance in any use of gene technology .
(http://www.awri.com.au/information_services/media/releases/nogogmo.asp).

With this in mind it is unlikely that the Australian wine industry will adopt GM wine yeast in the near future creating a need to develop non-GMO approaches for the generation of wine yeasts that produce less ethanol.

1.3.4 Non-GMO strategies used to generate yeast with lower ethanol production

Screening wine yeasts for variants that naturally have lower ethanol production is one way to find yeast that produce low ethanol wine. However, this approach is not efficient and usually does not bring desired results. Perhaps the best option to generate low-ethanol producing wine yeast is to use the evolutionary force of natural selection. Selection (domestication) of yeast has been occurring inadvertently for millennia based on choice of fermentations that display the most desired outcomes. As is the case for natural selection, domestication requires genetic variation for the selection of desired phenotypes; variation acts as the raw material from which adaptations are shaped. A number of approaches can be used to increase genetic variation in populations and thereby enhance the possibility of generating desired traits (Chambers et al. 2007). The most common and widely used strategy is induced mutagenesis using mutagenic agents such as ethylmethane sulfonate (EMS), nitrosoguanidine (NTG), diethyl sulfonate (DES), and UV or X-ray irradiation (Lawrence 1991, Chambers et al. 2007). Alternatively, a selection strategy might be utilized that does not rely on enhancing the rate of genetic variation, but uses existing

and spontaneously emerging genetic diversity in the population, otherwise known as Adaptive Evolution or Evolutionary Engineering.

Adaptive evolution may be achieved by introducing culturing conditions that act as a selection pressure, favoring a higher rate of survival for those individuals in the evolving population that have a desired trait. Culturing populations in a specific selective environment, (e.g. high ethanol medium), may direct adaptation(s) towards desired phenotypes (e.g. ethanol-tolerant variants) (see Cakar et al. 2005, Zeyl 2005, Chambers et al. 2007). There are a number of examples illustrating the power of this approach, such as the creation of yeast strains that can anaerobically utilize xylose (Sonderegger and Sauer 2003, Kuyper 2005), yeast with enhanced maltose utilization and osmotolerance (Higgins et al. 2001) and yeast with enhanced ethanol tolerance (Brown and Oliver 1982). More recently, McBryde et al. (2006) used adaptive evolution as a tool to improve the fermentation properties of industrial wine yeast strains. Diploid wine yeast strain, L-2056, and its haploid derivative, C9, were serially transferred batchwise in chemically defined grape juice medium for 350 and 250 generations respectively. The fermentation medium itself was the only form of selection pressure in this study. Variants isolated at the end of experiment differed in mean levels of metabolite production and were able to more rapidly consume the sugars provided in the medium. The mixed population of C9 haploid strain variants produced 10.3% more glycerol, 13.5% less ethanol and 9.1% more acetic acid after 250 generations. In comparison, variant populations of the diploid strain L-2056 changed their metabolic pattern after 350 generations in a similar way to C9 with a 12% increase in glycerol production, a 3.5% reduction in ethanol yield and a 5.8% increase in acetic acid production (McBryde at al. 2006). It appears that strain competitiveness favored those variants that had a reduction in ethanol production.

Adaptive evolution is a promising approach for the improvement of fermentative performance by wine yeast. Metabolic redirection using the above method, however, led to an overall increase in acetic acid production. To create 'low-ethanol' producing variants using adaptive evolution, a selection pressure would be required that can specifically redirect yeast metabolism away from ethanol formation in favor of enhanced production of alternative metabolites, such as glycerol.

1.3.5 Agents that select for variants with reduced ethanol production

From an evolutionary perspective, creating selective conditions that force the evolution of a population toward increased tolerance/resistance to a certain stressor is relatively easy. Individuals in a yeast population that are exposed over time to a sub-lethal concentration of a stressing agent, such as ethanol, will most likely acquire adaptations (mutations) that will make them better suited (fitter) to these conditions. Over time, the evolving population will be dominated by fitter individuals that have adopted to the stressor. Finding a selection pressure that specifically targets the redirection of metabolism away from ethanol production is, however, more challenging. Since ethanol synthesis is used by yeast to in the generation of energy, it is extremely difficult to create selection conditions that will favor the survival of mutants with impaired ethanol production. Thus, to create 'low-ethanol-producing' variants using adaptive evolution, strategies need to be developed based on existing knowledge of yeast metabolism and how it responds, either from previous experimental work or based on a theoretical model, to environmental perturbations.

In this context, it has previously been shown that an increase in glycerol production, by genetic modifications to yeast, has led in a majority of cases to reduced ethanol production (Section 1.3.3.2). It is therefore possible that the introduction of selection pressure which favors the survival/biomass productivity of individuals with enhanced glycerol production will also lead to adaptations resulting in lower ethanol production. An added benefit of this approach is that enhanced glycerol levels are recognised to have a favorable influence on wine by enhancing its sweetness, smoothness and overall body (Noble and Bursick 1984, Eustace and Thornton 1987, Pretorius 2000). A variety of environmental conditions have been studied for the redirection of *S. cerevisiae* metabolism away from ethanol synthesis. Most of these conditions rely on the intracellular and/or extracellular action of chemical agents (such as salts, alkalizing agents or sulfites) on the yeast cell in triggering a variety of cellular responses, leading in most cases to direct or indirect alterations in central carbon metabolism. The most common agents that can be used to impair ethanol production in yeast are discussed below.

1.3.5.1 Glycerol overproduction using osmotic stress

Exposure of yeast to hyper-osmotic stress is detrimental to the cell; their growth rate is decreased and viability can be compromised. To survive high extracellular osmotic pressure, yeast need to maintain cellular turgor, which is essential for structural and metabolic integrity (Blomberg and Alder 1992). In *S. cerevisiae* and filamentous fungi, glycerol is a key osmolyte. When *S. cerevisiae* is exposed to high osmotic pressure (e.g. 0.9M NaCl), the intracellular concentration of glycerol may increase from below 0.05M (non stress conditions) to 1.2M (Olz et al. 1993). The most common agents that trigger osmotic stress response in *S. cerevisiae* are salts, such as sodium chloride (NaCl) and potassium chloride (KCl), but other agents at high concentrations can also cause cellular stress. The levels of glycerol produced during osmotic stress depend on many variables including the concentration of the osmotic stress agent, yeast species, medium composition, temperature, aeration and pH (Modig et al. 2007). Rapin et al. (1994) observed that during the exposure of different yeast species to hyperosmotic stress, the highest glycerol yields (up to 50 %) were reported for osmotolerant yeast species. *S. cerevisiae* grown anaerobically in the presence of 40 g/l NaCl and 50g/l glucose was found to increase glycerol yield by 66 - 105% during fermentation. At the same time ethanol yields decreased by 7.1 - 15.9 %. In another study, *S. cerevisiae* grown aerobically in medium containing 100 g/l glucose, produced 140% more glycerol when 40 g/l NaCl was added to the medium, a 33% reduction in ethanol yield was also observed (Petrovska et al. 1999).

Although osmotic stress induces redirection of metabolism away from ethanol formation and towards increased glycerol yields, acetic acid is produced in excessive amounts under these stress conditions (Modig et al. 2007). This could be problematic if using osmotic stress as a selection pressure in adaptive evolution experiments for generating 'low-ethanol' producing yeast. Yeast evolving in the presence of osmotic stress could yield variants that produce excessive amounts of acetate, as it plays a crucial role in regulating the redox balance of yeast cells under such conditions.

1.3.5.2 Use of alkalizing agents (alkalis) to increase glycerol production

Alkalis are substances that dissolve in water creating solutions with alkaline pH, i.e. pH greater than 7. Sodium carbonate (Na_2CO_3) is an alkalizing salt of carbonic acid, widely used in industry for the production of glass, caustic soda, aluminum, detergents and paints. Hydrolysing carbonate salts gives rise to alkaline reactions, thus significantly increasing the pH of such solutions (Hinkamp 1998).

The presence of alkalizing agents, such as sodium carbonate, during yeast fermentation gives rise to elevated yields of glycerol and acetic acid, and a reduction in ethanol yield (Vijaikishore and Karanth 1984). The extent of metabolic redirection is dependent on the dosage of alkalizing agent used during fermentation. Freeman and Donald (1957) studied the action of various concentrations of Na_2CO_3 during yeast fermentation. According to their findings, the addition of 30% sodium carbonate, in terms of total fermentable sugar, raised the medium pH to 9, and irreversibly inhibited yeast growth. To avoid total growth inhibition, the Na_2CO_3 was divided into smaller portions and added progressively during the course of fermentation. It was found that Na_2CO_3 dosage at 5% of the total fermentable sugar resulted in a glycerol yield increase of 196% and a 6.5% decrease in ethanol yield; Na_2CO_3 addition at 45% of the total fermentable sugar resulted in a 357% increase in glycerol yield and a 30% reduction in ethanol yields (Freeman and Donald, 1957). With progressively increasing amounts of Na_2CO_3, the acetic acid yield also gradually increased. Higher Na_2CO_3 levels (45% of the total fermentable sugar in the fermentation medium) also greatly increased total fermentation time, elongating sugar utilization time by more than 2-fold (Freeman and Donald 1957).

1.3.5.3 Use of sulfite for reducing ethanol production

The term "sulfite" is often used for all of the species and salts of sulfurous acid, including its anhydrate, sulfur dioxide (SO_2). Presence of the various chemical forms of sulfite are entirely dependant on pH and major forms can dissociate in water e.g. SO_2 (sulfur dioxide), HSO_3^- (bisulfite ion), SO_3^{2-} (sulfite ion) and $S_2O_5^{2-}$ (pyrosulfite ion) (Snayd et al. 1993). At grape juice and wine pH, usually somewhere in range of 3 - 4, the predominant form of sulfite is the bisulfite ion (HSO_3^-). This will change rapidly however in response to changing pH and thermodynamic conditions. The

dissociation equilibrium and the coexistence of the different species of sulfite are described by the following reactions:

1. $SO_2 + H_2O \leftrightarrow HSO_3^- + H_3O^+$
2. $HSO_3^- + H_2O \leftrightarrow SO_3^{2-} + H_3O^+$

with corresponding pK values of $pK_1 = 1.81$ and $pK_2 = 6.91$ (Schimz 1980).

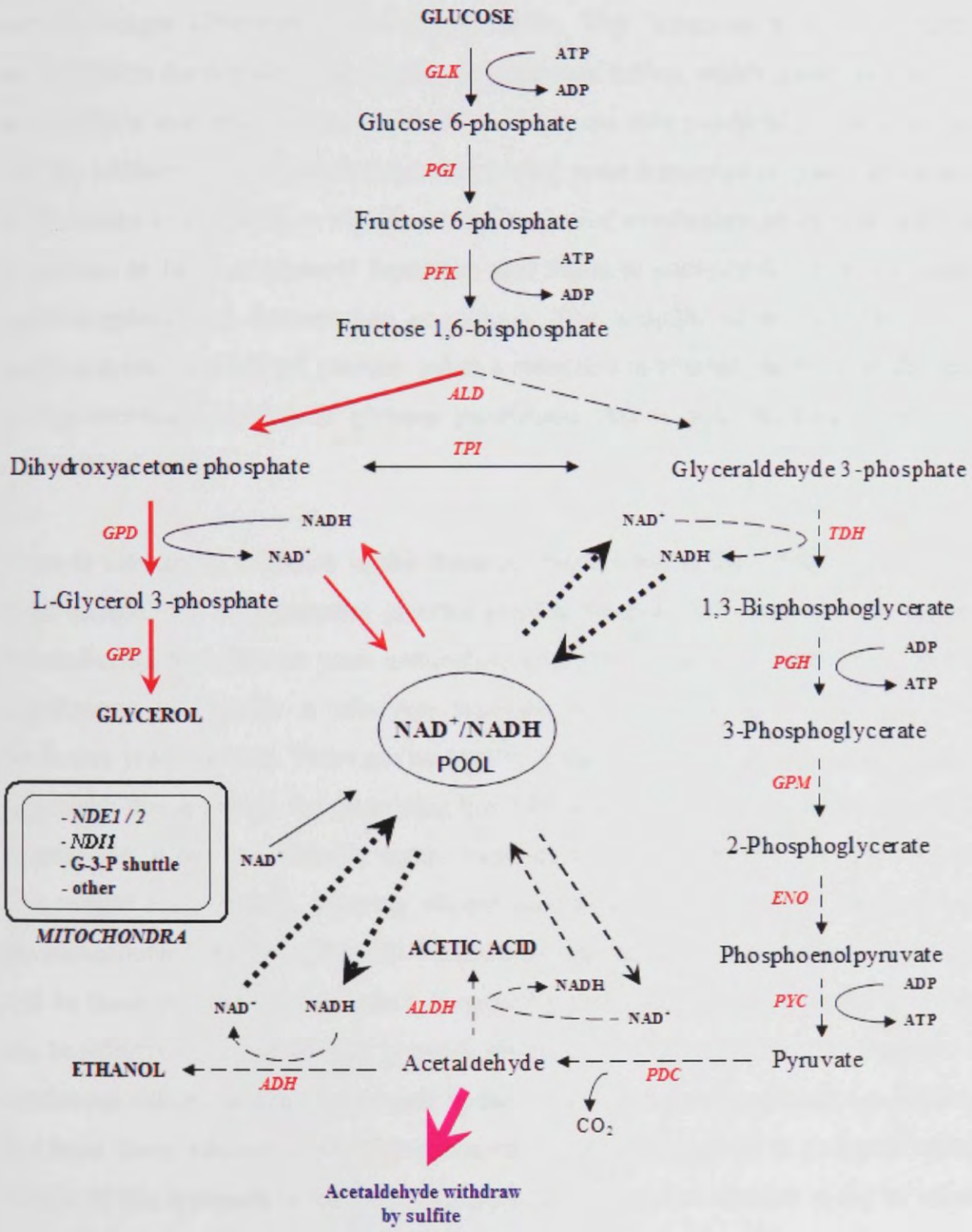
The sulfite fractions can dissociate almost instantaneously and be present in different proportions depending on the pH and thermodynamics of the environment. The existence of individual forms of sulfite at different pH are shown in Fig. 1.7

In the fermentation industry, sulfite is added to yeast cultures to increase glycerol production (Kalle and Naik 1987, Petrovska et al. 1999, Barnett 2003). This approach is known as the 'Sulfite' or 'Protol' process and was used for mass glycerol production in Germany during the 1st and 2nd World Wars (Freeman and Donald 1957, Taherzadeh 2002). The mechanism underpinning the sulfite process is principally the reaction between sulfite and acetaldehyde, which forms a stable compound known as 1-hydroxyethanesulfonate (Taylor et al. 1986). In yeast, this binding of sulfite to acetaldehyde significantly decreases the concentration of the latter, consequently reducing the amount of ethanol formed by ADH (Figure 1.8). This leads to intracellular NADH accumulation (which is a cofactor for ADH) and consequently a decrease in intracellular availability of NAD^+. The NAD^+ shortage in the cell is thought to affect glyceraldehyde 3-phosphate dehydrogenase activity, for which it is a cofactor, consequently reducing carbon flux in the glycolytic pathway. The cell can partially restore glycolytic flux by redirecting a portion of its carbon (as dihydroxyacetone-phosphate) through the glycerol synthesis pathway, which uses NADH as a cofactor, regenerating NAD^+. Under such conditions, glycerol synthesis produces an alternative pathway in the cell for restoring the intracellular NAD^+/NADH balance. Based on this mechanism, the addition of sulfite to fermentation cultures causes an imbalance in the NAD^+/NADH ratio, redirecting metabolism away from ethanol production in favor of glycerol synthesis (Figure 1.8).

Figure 1.7 The impact of pH on the dissociation and ratio of different sulfite species.


Percent in given form (%)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
pH
SO2
HSO3-
SO3 2-

Figure 1.8 Diagram of central metabolism and fermentation in *S. cerevisiae* exposed to sulfite stress during growth on excess glucose. Broken lines represent reduced carbon flow via glycolysis and fermentation, and reduction in contributions to NAD^+/NADH pool. Red lines indicate increased carbon flow towards glycerol and acetic acid production, and increased contributions to NAD^+/NADH pool. Pink arrow symbolizes acetaldehyde being trapped by sulfite. The enzymes are marked in red and they are: Glucokinase (GLK), Phosphoglucose isomerase (PGI), Phosphofructokinase (PFK), Aldolase (ALD), Triosephosphate isomerase (TPI), Glycerol-3-phosphate dehydrogenase (GPD), Glycerol-3-phosphatase (GPP), Triosephosphate dehydrogenase (TDH), Phosphoglycerate kinase (PGK), Phosphoglycerate mutase (GPM), Enolase (ENO), Pyruvate decarboxylase (PDC), Aldehyde dehydrogenase (AlDH), Alcohol dehydrogenase (ADH).


GLUCOSE
GLK
ATP
ADP
Glucose 6-phosphate
PGI
Fructose 6-phosphate
PFK
ATP
ADP
Fructose 1,6-bisphosphate
ALD
Dihydroxyacetone phosphate
TPI
Glyceraldehyde 3-phosphate
GPD
NADH
NAD+
L-Glycerol 3-phosphate
GPP
GLYCEROL
NAD+
NADH
TDH
1,3-Bisphosphoglycerate
PGH
ADP
ATP
3-Phosphoglycerate
GPM
2-Phosphoglycerate
ENO
Phosphoenolpyruvate
PYC
ADP
ATP
Pyruvate
NAD+/NADH
POOL
- NDE1 / 2
- NDI1
- G-3-P shuttle
- other
MITOCHONDRA
NAD+
ACETIC ACID
NAD+
NADH
ALDH
NADH
NAD+
ETHANOL
ADH
Acetaldehyde
PDC
CO2
Acetaldehyde withdraw
by sulfite

Freeman and Donald (1957) studied the effect of a range of sulfite dosages on yeast by-product formation. An increase in sulfite concentration from 5% to 50% (based on fermentable sugar) resulted in a glycerol yield increase of 120% and corresponding decrease in ethanol yield of approximately 52%. It was also observed that increasing sulfite levels did not increase acetic acid yield, rather, acetate levels declined at higher sulfite dosages (Freeman and Donald 1957). This observation is most likely attributable to the acetaldehyde-binding properties of sulfite, which results in a loss of acetaldehyde available as a substrate for ALD acetic acid production. The effect of Na_2SO_3 addition on by-product formation during yeast fermentation was also studied by Petrovska et al (1999). A significant redirection of metabolism away from ethanol production in favor of glycerol formation was found to occur in *S. cerevisiae* under sulfite-supplemented fermentation conditions. The addition of 40 g/l Na_2SO_3 to medium containing 100 g/l glucose, led to a reduction in ethanol yields of 56.6% and a large increase (1174%) in glycerol production. Acetic acid production was not reported in this study.

There is substantial evidence in the literature demonstrating the effect of sulfite on yeast metabolism, i.e. increasing glycerol yield at the expense of ethanol production. This influence by sulfite on yeast metabolism could be exploited in adaptive evolution experiments to provide a selection pressure for the creation of 'low-ethanol' producing yeast variants. There are no reports in the literature that have used, or even suggested, this approach for generating non-GM yeast with lower ethanol yields yet, in principle, it has considerable merit. Yeast exposed to sulfite will be deprived of intracellular acetaldehyde, reducing ethanol production and causing an imbalance in the intracellular NAD^+/NADH ratio. In this environment, the most competitive strains will be those that are more effective in restoring the redox balance and one way this can be achieved is by increasing glycerol production. Applying these conditions in a continuous culture setting could result in the evolution of stable spontaneous mutants that have lower ethanol yields and increased glycerol production. A potential added benefit of this approach is that acetic acid production in such mutants could be lower than the parent strain due to the intracellular acetaldehyde shortage; this is very desirable in wine yeast strains. To conclude, it is hypothetically possible that the use of sulfite stress in adaptive evolution experiments would result in the evolution of stable yeast variants with higher glycerol yields and lower yields of ethanol and acetic

acid. Such non-GM strains would be highly suitable for the production of quality wine with moderate ethanol concentrations.

1.4 The main objective of this project

The main objective of this project was to develop a non-genetic engineering approach for generating *S. cerevisiae* strains that produce less ethanol compared to their parent strains. To achieve this, adaptive evolution was used with sub-lethal concentrations of sodium sulfite (Na_2SO_3) providing the selection pressure. The rationale being that sulfite sequesters acetaldehyde, thereby limiting NAD^+ regeneration associated with acetaldehyde reduction to ethanol. In such an environment, the process of evolution will favor spontaneous variants that increase glycerol production as a means of maintaining intracellular redox balance; the intracellular acetaldehyde shortage should also result in reduced acetic acid production. Demonstrating in principle that this approach can generate non-GM yeast strains with lower ethanol production could lead to its application by the wine industry for producing wines with moderate ethanol concentrations.

CHAPTER 2

MATERIALS AND METHODS

2.1 Microorganisms and microbiological methods

2.1.1 Organisms

Saccharomyces cerevisiae BY4742 haploid (*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*) and *Saccharomyces cerevisiae* AWRI1628 haploid (*MAT*a), were used in this study. *S. cerevisiae* BY4742 is well characterized laboratory strain, derived from S288a. *S. cerevisiae* AWRI1628 is a haploid wine yeast. It was derived from industrial diploid N96, which is marketed by Anchor Bio-Technologies and commonly used by winemakers around the world. Both strains used in this study were obtained from culture collection maintained by the Australian Wine Research Institute (AWRI).

2.1.2 Media

Media used in this study was prepared by dissolving adequate medium components in distilled, de-ionized MiliQ^{TM} water and sterilized at 121°C for 20 minutes. A solution of glucose was prepared and sterilized separately from the other components of the medium. After sterilization, proportional volumes of medium components were measured using a measuring cylinder, mixed together and the final volume was adjusted to the appropriate level with sterile, de-ionized MiliQ^{TM} water. The types of media used in this study are listed and described below.

YPD contained 1 % (w/v) yeast extract (Amyl Media), 2 % (w/v) bacteriological peptone (Amyl Media), 2 % (w/v) D-glucose (Sigma), dissolved in de-ionized MiliQ^{TM} water and sterilized as describe above. YPD solid medium, used for viable plate preparation, was prepared similarly to YPD but with the addition of 1.5 % (w/v) of bacteriological agar prior to autoclaving.

2xYPD was prepared similarly to YPD but using double the amount of each ingredient.

YPD-10 contained 1 % (w/v) yeast extract (Amyl Media), 2 % (w/v) bacteriological peptone (Amyl Media) and 10 % (w/v) D-glucose (Sigma), dissolved in distilled, de-ionized water and autoclaved. In most cases the medium was adjusted to pH of 4.5 with HCl unless otherwise specified. This medium was used mainly for batch incubations designed to analyse glycerol, ethanol and acetic acid formation.

YPD-10S contained 1 % (w/v) yeast extract (Amyl Media), 2 % (w/v) bacteriological peptone (Amyl Media), 10 % (w/v) D-glucose (Sigma), dissolved in MiliQ™ de-ionized water and autoclaved. The medium was supplemented with various levels of sodium sulfite (Na_2SO_3) (BDH AnalaR®), depending on the experiment. pH was adjusted either to 4.5 with HCl or 8.0 with NaOH. The dosages of sodium sulfite and pH are specified in the text describing particular experiments.

YPD-10K contained 1 % (w/v) yeast extract (Amyl Media), 2 % (w/v) bacteriological peptone (Amyl Media), 10 % (w/v) D-glucose (glucose). All of the components were dissolved in MiliQ™ de-ionized water and autoclaved. Medium was supplemented with various dosages of potassium chloride (KCl) depending on the experiment. pH of the medium was adjusted to 4.5 with HCl, unless otherwise stated. The dosages of potassium chloride are specified in the text describing particular experiments.

2.1.3 Solutions and buffers

A list of recipes for commonly used solutions and buffers is given in Appendix A. All solutions and buffers were prepared from analytical grade reagents unless otherwise stated. Chemicals were dissolved in MiliQ™ de-ionized water in glass containers and pH was adjusted to a desired level using a pH meter (Radiometer Analytical SAS, MeterLab® DHM210) calibrated with standards purchased from the same company. Solutions and buffers were sterilized by autoclaving at 121°C for 20 minutes or by filtration using 0.22 μm Millipore® membrane filters.

2.1.4 Glassware and non-glass vessels preparation

All glassware used in the experiments was washed and acid rinsed using a dishwasher. It was then sterilized at 121°C for 5 minutes and dried at 70°C until water drops and/or

vapor was no longer visible inside the glass vessels. Most of the non-glass vessels, such as polyester tubes etc., were purchased sterile and ready for aseptic use.

2.1.5 Cultures

2.1.5.1 Maintenance of yeast cultures

Yeast strains designated for storage were grown overnight in YPD medium. Cells were harvested by transferring 10 ml aliquots to 10 ml sterile polyester tubes and spinning down at 1780 g for 2 min. After discarding of supernatant, cell pellets were re-suspended in cryoprotective fluid from Protect vials, transferred into the same vials and gently shaken to redistribute cells inside and around internal beads. After 30-60 seconds, excess cell-containing cryoprotective fluid was withdrawn using a sterile pipette. The prepared stock cultures were immediately frozen and stored at -80°C. All these operations were performed using barrier sterile tips to avoid cross contamination of the cultures. For short term storage, yeast stocks were streaked out on YPD agar plates, incubated at 30°C for 48 hours and stored at 4°C.

2.1.5.2 Inoculum preparation

Cells were transferred from stocks kept at -80°C into 20 ml of YPD in 50 ml polyester tubes and incubated overnight at 30°C/140 rpm. Culture tubes were placed and shaken at approximately a 45° angle to allowed adequate mixing of the culture during the incubation period. When the parent cultures reached late exponential phase, the culture OD_{600} was used to determine the inoculum volume needed to give an initial OD_{600} of 0.1 in the experimental culture. Microscopic examination of the parent culture was conducted prior to experimental culture inoculation to check for contamination. The appropriate volume of parent culture was transferred to 10 ml sterile tubes, centrifuged for 2 minutes at 1780 g and the supernatant discarded, leaving behind the cell pellet. Approximately 1 ml of fresh medium was aseptically removed from total volume of experimental culture medium and used to re-suspend the inoculum cell pellet in the polyester tube. The resuspended inoculum was transferred into the experimental culture medium giving an intial OD_{600} of 0.1. The inoculation of all experiments described in this thesis was performed using barrier sterile tips to avoid contamination of the cultures.

For the purposes of experiments described in Chapter 6, the inocula were prepared similarly as described above, however, the parent cultures were grown in sterile 250 ml Erlenmyer flasks covered with aluminum foil and containing 100 ml of YPD medium. Flasks with air locks were used to prepare inocula for anaerobic experiments.

2.1.5.3 Aerobic growth

For each set of experimental conditions, triplicate cultures were conducted. Cultures for aerobic experiments were inoculated at an initial OD_{600} of 0.1 using inocula taken from the same parent culture at the same time. Inocula were prepared as described above (Section 2.1.5.2). These incubations were conducted using 250 ml Erlenmeyer flasks with a working medium volume of 100 ml, unless otherwise stated. Flasks were covered with aluminum foil that allowed free gas exchange between external and internal flask space. Aerobic experiments were carried out in shaking water baths at 30°C/140 rpm unless otherwise stated. The temperature of all water baths was adjusted and monitored using one thermometer for all of the experiments. This practice ensured a consistent temperature was used for all incubations.

The incubations described in Chapter 6 were conducted in 1000 ml Erlenmyer flasks covered with aluminum foil, and with a working medium volume of 500 ml. The cultures were incubated in shaking incubators at 30°C/140 rpm.

2.1.5.4 Semi-anaerobic growth

Semi-anaerobic incubations used in this study refer to growth conditions in which there was limited access to oxygen during incubation. For each set of experimental conditions, triplicate cultures were conducted. For all semi-anaerobic experiments, cultures were inoculated at an OD_{600} of 0.1 using inocula taken from the same parent culture at the same time. Inocula were prepared as described in Section 2.1.5.2. Incubations were conducted in 250 ml Erlenmeyer flasks with air-locks, which did not permit free gas exchange during incubation. Oxygen that was initially present in the incubation flasks was metabolised in the early incubation stages and CO_2 production by the cells ensured that the cultivation became anaerobic. The fermentations were conducted with a working volume of 100 ml of medium in shaking water baths at

30°C/140 rpm. The temperature of all water baths was adjusted and monitored using one thermometer for all of the experiments.

2.1.5.5 Anaerobic growth

For each set of experimental conditions, triplicate cultures were conducted. Medium used for anaerobic incubations was transferred (500 ml) into sterile 1000 ml Erlenmyer flasks supplemented with magnetic stirrers and covered with aluminum foil. These flasks were subsequently transferred into an anaerobic hood, placed on a magnetic stirrer and left stirring at 140 rpm for 24 hours to de-oxygenate the medium. Parent cultures used for the anaerobic experiments were incubated overnight under semi-anaerobic conditions as described in Section 2.1.5.4. Subsequently, pre-determined volumes of parent cultures, (inocula), needed to provided an initial OD_{600} of 0.1 in the anaerobic experimental cultures, was measured into sterile tubes, and centrifuged for two minutes at 1780 g. The supernatant was discarded and the pellets in the tubes de-oxygenized in the anaerobic pre-chamber, and subsequently transferred into the anaerobic hood. The cell pellets were then re-suspended in the anaerobic experimental medium. Each triplicate culture was inoculated using inocula taken from the same parent culture at the same time. The cultures were incubated anaerobically at 30°C/140 rpm. Samples of the cultures were taken anaerobically at regular intervals.

2.1.5.6 Microplate growth

Parent cultures for microplate experiments were prepared as described in Section 2.1.5.2. The volume of the parent culture used for the inoculum was calculated so that the inoculum preparation had an initial OD_{600} of 0.4. The inoculum was prepared in 10 ml of experimental medium in polyester tubes and 50 µl of inoculum was transferred to wells in a 96-well micro-plate, of which every well was filled with 150µl of experimental medium. The microplate was sealed with Breathe-Easy® (U.S. Patent No. 5,858,770) gas permeable sealing membrane for microplates, and incubated at 30°C in a Therumo Multiscan Ascent plate reader. An OD_{630} of the culture was measured every 30 minutes. Data from microplate experiments were processed using Microsoft® Office Excel 2003.

2.2 Analytical methods

2.2.1 OD_{600} measurement

Measurements of culture absorbance were conducted at 600 nm using a Beckman Coulter™ DU® 530 UV/Vis Spectrophotometer. Absorbance measurements were conducted in Greiner Bio-One 3ml Semi-Micro-Cuvettes, (10 x 10 x 45 mm), manufactured from crystal clear polystyrene. The spectrophotometer was calibrated against sterile medium of the same composition as was used for yeast incubations. At high culture densities, samples were diluted with filtered water (0.2 μm filter MiliQ™) to give OD_{600} readings in range of 0.1 - 0.5. Medium used for spectrophotometer calibration was diluted with filtered MiliQ™ water according to the dilution factor used for sample preparation. Data was plotted and analyzed using Microsoft® Office Excel 2003.

2.2.2 Cell population

A hemocytometer (Precicolor, HBG, Germany) was used to calculate cell number at particular stages of yeast cultivation. Prior to use, the hemocytometer and cover slip were cleaned with ethanol, wipe-dried, and the cover slip placed on top of the hemocytometer. A cell suspension (diluted in to be in the OD_{600}range of 0.1 - 0.5) was mixed by vortexing and loaded between hemocytometer and cover slip, filling entirely both hemocytometer chambers. The loaded hemocytometer was left for approximately 1 minute to allow the cells to settle. It was then placed under a microscope (40x magnification) and the middle 1 mm^2 square was counted. Samples were calculated in both chambers of hemocytometer. The cell population was calculated taking into account the sample dilution and number of counted fields.

2.2.3 Biomass measurement

Biomass of cultures was estimated based on the dry cell weight (g/l). A suspension of 10 ml of the thoroughly mixed cells was filtered through 0.22 μm membrane filters. The cells were collected on the surface of filter, dried and weighed on an Adam AMB 50 drying balance. The total mass of the dried cells was estimated by subtracting the initial weight of the dried filter.

2.2.4 Analysis of chemical composition of samples using HPLC

2.2.4.1 Preparation of HPLC standards

All of the chemicals used to prepare standard solutions were high purity, analytical grade reagents. Each compound used in standard solutions was weighed on a calibrated analytical balance in the amounts shown in Table 1. The compounds were dissolved in approximately 70-80 ml of de-ionized MiliQ™ water in a 100 ml volumetric flask. When all of the compounds were added, the final volume was adjusted to 100 ml with de-ionized MiliQ™ water, mixed and filter-sterilized. Two separate fractions of the compounds were prepared in 2X concentrated solutions and used later for preparation of standard dilutions. The precise weight and purity of each compound used for stock solutions were noted and, using these values, actual concentration of each compound in the standard was calculated and used for calibration in accordance with the analytical software. Standards containing volatiles, such as ethanol, were aliquoted in appropriate amounts into polyester tubes and kept frozen at -20°C to prevent compound loss via evaporation.

Standard dilutions were prepared from 2X stock solutions by mixing 2 ml of each stock solution. The mixture was subsequently diluted 2-, 3-, 4- and 5-times in de-ionized MiliQ™ water giving 5 serial dilutions of the standards. Freshly prepared standards were used with every HPLC run, and values obtained from the standard analysis were used to calibrate the analytical software.

2.2.4.2 Sample preparation for HPLC analysis

Fermentation samples were transferred to 2 ml polyester tubes and centrifuged at 16000 g for 1 minute. The supernatant was then transferred to fresh tubes and either kept frozen at -20°C, or directly used for HPLC analysis. Samples were prepared for HPLC analysis by diluting the original samples according to the expected concentration range of the compounds to be measured. If not otherwise stated, samples were diluted 5x in de-ionized MiliQ™ water; aliquots of 400 µl of the original samples were transferred to 2 ml Eppendorf tubes containing 1.6 ml of water, mixed gently and centrifuged at 16000 g for 5 minutes. Samples were then transferred to Agilent™ HPLC vials and sealed with Agilent™ screw caps.

2.2.4.3 HPLC system

A high performance liquid chromatography (HPLC) system (Agilent/HP 1100 series) was used comprising: degasser (G1322A), quaternary pump (G1311A), autosampler (G1313A), column compartment (G1316A), DAD (G1315A) and RID (1362A). The column used was an Aminex® HPX-87H organic acid ion exchange column (300 x 7.8 mm) (Bio-Rad Laboratories Inc. Hercules, CA), operating at 65°C. The mobile phase was 5 mM sulfuric acid (H_2SO_4) and the flow rate during analysis was 0.5 ml/min. 10 µl of each sample was injected into the column, and each analysis run lasted 35 minutes.

2.2.4.4 Chemical analysis

Concentrations (g/l) of particular compounds from samples were analyzed using Agilent ChemStation for LC 3D software (Copyright® Agilent Technologies 1990-2002). Before analyzing sample content, the software was calibrated using the standards prepared as described above (Section 2.2.1). After calibration, each peak representing a particular compound was manually integrated, i.e. a base line was added that best encompassed peak area. Similar integration principals were used for every peak. After manual integration of all peaks, compound concentrations were automatically quantified against calibration curves, and results exported to Microsoft® Office Excel 2003. This software was used for data analysis, creation of charts and statistical analysis of the HPLC results.

2.2.5 Estimation of glucose concentration using CliniTest

Fermentations were generally run to completion (i.e. dryness) and this was confirmed using CliniTest®, (Bayer HealthCare Mgf., Ltd.) tablets. Approximately 1 ml sample of ferments were spun at 16,000 g for 60 seconds, and the supernatants poured into fresh tubes. Aliquots of 200 µl of supernatant were subsequently transferred to heat resistant polyester tube and diluted with 400 µl de-ionized MiliQ™ water. One CliniTest® tablet was dropped into the diluted sample, left for approximately 15 seconds and color compared to an indicator chart supplied by the manufacturer.

2.2.6 Determination of osmotic strength of growth media

When necessary, osmotic strength of media was determined using a Vapor Pressure Osmometer, Wescor 5500. Prior to use, the osmometer was calibrated using osmolarity standards 290 mmol/kg and 1000 mmol/kg (Helena Laboratories). All samples to be analyzed were filtered through 0.22 μm Milipore™ membrane filters. Preliminary experiments revealed, some media had an osmotic strength greater than the maximum measurable threshold for the osmometer used. Thus, all samples were diluted 2X with de-ionized MiliQ™ water prior to measurement. Osmotic strength determinations were performed on triplicate 10 μl samples of media.

2.2.7 Tetrazolium overlay

The Tetrazolium overlay method was used to assess petite frequency as described by Ogur et al (1953).

2.3 Genetic and Molecular biology methods

2.3.1 Genomic DNA isolation from yeast

Strains from which DNA was to be isolated were grown overnight in YPD at 30°C/140 rpm. Cells were harvested from these cultures by centrifugation for 5 min at 1780 g. Pellets were washed in sterile de-ionized MiliQ™ water, spun down for 5 min at 1780 g, and the supernatant was discarded. Isolation of genomic DNA was conducted as described in (Ausubel 2001), using acid washed, sterile glass beads and breaking buffer to fragment yeast cell walls. However, a Mini-Beadbeater 8 (Biospec Products) was used to shake the cell and glass beads mixture instead of vortexing.

2.3.2 Yeast transformation

All transformations of *S. cerevisiae* were conducted using the lithium acetate/polyethylene glycol method, as described in (Ausubel 2001).

2.3.3 Mating type interconversion

Strains designated for mating type inter-conversion were inoculated from frozen stocks into YPD medium and grown overnight at 30°C/140rpm (see Section 2.1.5.2). Overnight cultures were harvested by centrifugation (5 min/1780 g), washed in sterile MiliQ™ water and transformed with pHO-NAT plasmid using the lithium acetate/polyethylene glycol transformation method (see Section 2.3.2). Transformed cultures were plated onto YPD, incubated for approximately 24 hours at 30°C, than replica-plated onto selective YPD medium containing 100 µg/l of CloNAT and incubated for 48 hours at 30°C. Colonies that grew on selective plates were derived from cells that had been successfully transformed, thus gaining CloNAT resistance. Mating type switching and subsequent diploid formation in transformed haploids occurs in two steps: first, individual cells switch mating type due to mating type cassette substitution, catalyzed by HOp activity; second, cells of opposite mating types fuse and form α/a diploids. Colonies resistant to CloNAT, that were expected to be diploids[1], were inoculated into 50 ml polyester tubes in 15 ml of non-selective YPD and grown overnight at 30°C/140 rpm; growth in these non-selective conditions leads to loss of the plasmid. Overnight cultures were diluted in sterile MiliQ™ water and plated onto YPD agar plates to yield 50 - 100 colonies per plate, which were replica-plated onto YPD agar plates containing 100 µg/l of CloNAT and incubated for 24 hours at 30°C. Colonies sensitive to CloNAT were selected as sensitivity to this drug meant that the plasmid was lost. Several CloNAT-sensitive colonies were dissected to single cells using a micromanipulator (Singer MSM System 300) and subsequent colonies were screened for ploidy using mating type PCR.

2.3.4 Mating of haploid yeast strains

Mating was conducted on YPD agar plates. Strains to be mated were transferred from frozen stocks onto YPD agar plates and incubated at 30°C for 24 hours. Similar amounts of cells were than loop-transferred onto fresh YPD plates, so to create two patches in close proximity. These were subsequently mixed with each other on the surface of the plate using a sterile loop, and spread as an even layer. The mixed cultures were incubated for 24 hours at 30°C, after which the mixture was harvested

[1] Diploid colonies can generally be distinguished from haploids because of size difference; diploid colonies are typically larger than haploids, but this has to be confirmed.

with a sterile loop, diluted in YPD, and the density of diluted culture adjusted to OD_{600} of 0.1 using sterile de-ionized MiliQ™ water. Subsequently, diluted cultures were spread onto a fresh YPD agar plates using gradually increasing volumes of the diluted culture to obtain plates containing 20-50 colonies. From these plates, the biggest colonies (putative diploids) were dissected on YPD plates to obtain single cells. After dissection, random colonies were tested for ploidy using mating-type PCR (see Section 2.3.7.2). Frozen stocks of confirmed diploids were prepared as describe in Section 2.1.5.1.

2.3.5 Sporulation of diploids

Diploids to be sporulated were inoculated from frozen stocks into YPD and incubated overnight at 30°C/140 rpm. 10 ml of each overnight culture was harvested by centrifugation at 1780 g for 5 min, washed in sterile MiliQ™ water and plated onto agar plates containing 2% (w/v) potassium acetate medium. Plates were sealed with Parafilm® and incubated at room temperature. After 5 to 7 days a proportion of diploid cells had undergone sporulation; this was confirmed by microscopic examination of samples of the cultures collected from potassium acetate supplemented agar plates.

2.3.6 Dissection of tetrads

Spores were dissected using a micromanipulator. Prior to dissection, the sporulation suspension was incubated in Zymolase solution at 37°C for 5 minutes, to partially digest the ascus wall. After incubation, a sample of asci suspension was diluted with chilled de-ionized MiliQ™ water and transferred onto YPD agar plates. Tetrads were dissected on YPD plates to generate groups of four spores using micromanipulator. Plates containing dissected tetrads were incubated at 30°C for 48 h. Spores from tetrads that yielded four even colonies were tested for ploidy using mating-type PCR. Groups of spores that displayed 2:2 ('α':'a') segregation pattern of the mating-type PCR were used for further studies. Frozen stocks of these were prepared as described in Section 2.1.5.1.

2.3.7. Polymerase Chain Reaction (PCR)

A Master MIX of template DNA, PCR reaction buffer, magnesium chloride ($MgCl_2$), nucleotides (dNTPs), was prepared as required for each set of PCR reactions. Ingredients of PCR Master MIX were thawed and kept on ice during preparation. Master MIX was prepared in sterilized, 1.5 ml polyester tubes, mixed throughoutly and aliquoted into 200 µl PCR tubes at 24 µl, and 1µl of template was subsequently added to each tube. The list of PCR primers, reagents and PCR programs used for particular PCR reactions are shown in Section 2.3.5.1 and 2.3.5.2.

iProof™ High Fidelity DNA Polymerase was used to prepare fragments to be sequenced. Primers, reagents and protocols used for amplification of DNA to be sequenced are described in Appendix B.

2.3.7.1 Ty1 Transposon PCR

Ty1 transposon PCR was used to confirm the parentage of strains generated in work described in this thesis; the number and location of Ty1 transposons are specific to particular yeast strains. The PCR protocol including description of primers, is given in Table 2.1

2.3.7.2 Mating-type PCR

Mating-type PCR was used in this study for ploidy assessment and to determine the mating type of the haploids to be crossed. The PCR protocol including description of primers, is given in Table 2.2.

2.3.8 Gel electrophoresis and DNA visualization

Electrophoresis of DNA was performed in 1% (w/v) Molecular Biology Grade Agarose, (ScientifiX™) gels in 1 x TBE buffer (Appendix A). Gels were prepared by suspending agarose in TBE buffer, and subsequently heating in a microwave oven until agarose was fully dissolved. Dissolved agarose was left to partially cool before pouring. When the gel was fully solidized, it was transferred into electrophoresis container filled with TBE buffer. PCR products were mixed with PCR loading buffer

Table 2.1 Details of reagents and PCR products for Ty1 transposon DNA fingerprinting.

[A]) 10x reaction buffer for Taq polymerase was supplied by the manufacturer of polymerase, Astral Scientific.

[B]) The mix of dNTPs was prepared from Deoxynucleoside Triphosphate Set, Roche, which contained dATP, dCTP, dGTP and dTTP (10 mM, each). To prepare 100 µl of the mix, 10 µl of each nucleotide (100 mM) was added into 60 µl sterile de-ionized MiliQ™ water.

[C]) DNA used for the reaction was isolated from yeast as described in Section 2.3.1

Ty1 transposon primers:

MLD1 (forward primer) 5'-CAAAATTCACCTATA/TTCTCA-3'
MLD2 (reverse primer) 5'-GTGGATTTTTATTCCAACA-3'

Concentration of the components used for Ty1 transposon PCR reaction

Master MIX Components	Amount used for single PCR reaction
10x reaction buffer [A]	2.5 µl
$MgCl_2$ (25 mM)	2.5 µl
dNTP's (10 mM) [B]	1.0 µl
Forward primer (10 µM)	1.0 µl
Reverse primer (10 µM)	1.0 µl
H_2O	15.8 µl
Taq polymerase (5 U/µl)	0.2 µl
DNA [C]	1.0 µl
	Total volume 25 µl

PCR program

		5.0 min	95°C
3 X		0.5 min	95°C
		0.5 min	42°C
		2.0 min	72°C
29 X		0.5 min	95°C
		0.5 min	45°C
		2.0 min	72°C
		10 min	72°C
		hold at	20°C

Table 2.2 Details of reagents and PCR products for mating-type PCR.

[A]) 10x reaction buffer for Taq polymerase was supplied by the manufacturer of polymerase, Astral Scientific.

[B]) The mix of dNTPs was prepared from Deoxynucleoside Triphosphate Set, Roche, which contained dATP, dCTP, dGTP and dTTP (10 mM, each). To prepare 100 µl of the mix, 10 µl of each nucleotide (100 mM) was added into 60 µl sterile de-ionized MiliQ™ water.

[C]) Template for mating-type PCR reactions were obtained directly from yeast cells. Cells were picked from colonies with a sterile pipette tip, transferred to PCR tubes and microwaved at max for 2 minutes prior to Master MIX addition.

Mating Type primers:

MAT locus primer	5'-AGTCACATCAAGATCGTTTATGG-3'
'α' specific primer	5'-GCACGGAATATGGGACTACTTCG-3'
'a' specific primer	5'-ACTCCACTTCAAGTAAGAGTTTG-3'

Concentration of the components used for mating-type PCR reaction

Master MIX Components	**Amount used for single PCR reaction**
10x reaction buffer [A]	2.5 µl
$MgCl_2$ (25 mM)	2.5 µl
dNTP's (10 mM) [B]	1.0 µl
MAT specific primer (10 µM)	0.5 µl
'α' specific primer (10 µM)	0.5 µl
'a' specific primer (10 µM)	0.5 µl
H_2O	15.8 µl
Taq polymerase (5 U/µl)	0.2 µl
DNA [C]	
	Total volume 24.5 µl

PCR program

		2 min	92°C
30 X		1 min	92°C
		2 min	58°C
		2 min	72°C
		10 min	72°C
		hold at	20°C

(6X Ficoll), loaded into wells in the gel, and run at 90 V for the requisite time. Gels were than stained in 0.001 mg/ml ethidium bromide for 15 minutes, and visualized under UV light.

2.3.9 Purification DNA to be sequenced

Amplified DNA fragments to be sequenced were purified using UltraClean™ PCR Clean-up DNA Purification Kit (MO BIO Laboratories, Inc.). DNA samples were re-suspendedvolumes of SpinBind buffer (supplied with the kit), transferred to spin filter units and centrifuged at 16,000 g for 30 seconds. The spin filter basket was removed and liquid flow-through discarded from the collection tube by decanting. Next, the spin filter basket was replaced in the same tube, 300 µl of 80% (v/v) ethanol was added and spun for 60 seconds at 16,000 g. The flow-through was discarded by decanting, spin filter basket placed back into the same collection tube and spun again for 60 seconds at 16,000 g to remove residues of ethanol. Subsequently, spin filter baskets were transferred to new collection tubes and 50 µl of sterile MiliQ™ water was added and left for 60 seconds. Tubes were spun for 60 seconds at 16,000 g and spin filter baskets discarded. The solution containing DNA to be sequenced was collected in collection tubes.

2.3.10 DNA sequencing and analysis of sequencing data

DNA sequencing was conducted at The Australian Genome Research Facility, Ltd. (AGRF) (Brisbane). Purified DNA, sterile MiliQ™ water and sequencing primers were aliquoted into 1.5 µl sterilized micro-centrifuge tubes. The final concentrations of DNA and primers were prepared according to the requirements of AGRF as follows: 10 - 40 ng DNA, 6.4 pmol primer. Quality of sequencing data gradually worsen along with increasing nucleotide positions on sequenced DNA fragment. Standard sequencing may give good resolution data up to approximately 700 bp. As all of the fragments to be sequenced were longer than 1kb, sequencing primers were designed that anneal every approximately 450 - 500 bp, starting from 5'- end of each amplified fragment. Thus, it was possible to obtain overlapping sequences along the total length of the DNA fragments. Sequencing data obtained from AGRF was processed using Vector NTI software. Chromatograms of each sequence were examined manually for quality and only unambiguous, clear sequencing data was used

to assemble the fragments. Prior to assembling, each sequence was trimmed off up- and down- stream for low quality sequences. Fragments sequenced for 'low-ethanol' variants derived from BY4742, B2-c3-1s and B2/1-c4-2s, were compared to *S. cerevisiae* genome sequence data (http://www.yeastgenome.org) (data not shown). Fragments sequenced from 'low-ethanol' variant A3-c2-1s were compared to its parental strain AWRI1628 (sequence lodged at The Australian Wine Research Institute). Primers used to amplify DNA fragments of interest as well as sequencing primers were designed using primer designing tools at http://www.yeastgenome.org (see Appendix B).

CHAPTER 3

INTRODUCING SULFITE AS A SELECTION PRESSURE TO GENERATE 'LOW-ETHANOL' YEAST

3.1 Aim

To determine the optimal concentration of the selection pressure, sodium sulfite, for the generation of 'low-ethanol' variants of *S. cerevisiae* using adaptive evolution.

3.2 Introduction

The primary objective of this project was to generate low ethanol-producing strains of *S. cerevisiae* by redirecting carbon metabolism towards glycerol synthesis at the expense of ethanol production. It was hypothesized in Chapter 1 that this could be achieved using an evolutionary engineering approach, with sulfite providing the selection pressure. It is argued in Section 1.3.5.3 that the ability of sulfite to shift central metabolism away from ethanol production and towards glycerol biosynthesis could be applied in a growth competitive environment to select for stable spontaneous mutants that have improved capacity for producing glycerol. Their higher glycerol production rates should improve their energetics in a sulfite-stress environment and provide the variants with a growth competitive edge compared to the rest of the cell population.

Before evolutionary engineering experiments could be conducted, it was necessary to characterize the growth profile and kinetics in the presence of sulfite of the *S. cerevisiae* strains to be used. This was important since the amount of sulfite to be added to the cultures needed to provide an appropriate sulfite-induced stress without being lethal; this amount needed to be determined experimentally for each strain.

The purpose of the work described in this chapter was to characterize the growth profiles, and glycerol/ethanol yields of *S. cerevisiae* strains BY4742 and AWRI1628, in the presence of various amounts of sulfite. This information was then used to determine the sulfite concentrations to be applied in the evolutionary engineering experiments (in Chapter 4 and 5) such that sulfite provided a non-lethal, but growth

restricting, selection pressure for the selection and isolation of 'high-glycerol', but 'low-ethanol' producing variants.

3.3 Results

3.3.1 Determination of optimum pH and sulfite concentrations for adaptive evolution selection

Preliminary results were performed to determine the broad parameters for future experiments. Sulfite could not be used below pH 4.5 because it generated precipitates of unknown origin in the medium. At pH 4.5 (where sulfite is present predominantly as HSO_3^-), Na_2SO_3 concentrations above 3 g/l were found to be lethal to both strains. At pH 8 (where sulfite is present predominantly as SO_3^{2-}), however, much higher concentrations of Na_2SO_3 could be used such that 40 g/l, although not lethal, significantly inhibited yeast growth. With these preliminary findings in mind, and knowledge of the pH dissociation profiles for sulfite (see Section 1.3.5.3), it was decided to focus on pH 4.5 and 8 as shown in Table 1. The reason for choosing pH 4.5 and 8 is that at each of these two pH conditions, sulfite occurs in dissimilar forms, which more than likely contribute to its different toxic effect on yeast, and most importantly, metabolic redirection away from ethanol production.

To investigate the influence of sulfite concentration on yeast growth, strains BY4742 and AWRI1628 were incubated separately in YPD medium until they reached the late exponential stage. The strains were then inoculated into pre-warmed (at 30°C) 'sulfite selection medium' (YPD-10S) to an initial OD of 0.1, which was equivalent to around 6×10^6 cells/ml for both strains (Section 2.1.5.2). The YPD-10S was supplemented with a range of Na_2SO_3 concentrations depending on pH and yeast strain (Table 3.1). Incubations were performed under aerobic conditions at 30°C/140 rpm until entire exhaustion of glucose, and all experiments were performed in triplicate as described in Section 2.1.5.3. Growth was monitored by OD_{600} readings and samples were taken for measurement of glycerol, ethanol, acetic acid and glucose as described in Sections 2.2.1 and 2.2.4 respectively.

Table 3.1 pH and sulfite concentrations used to test their effect on *S. cerevisiae* growth.

S. cerevisiae strain	pH	Na_2SO_3 concentration (g/l)		
BY4742	4.5	0.5	1	1.5
AWRI1628	4.5	1.5	2	2.5
BY4742	8	20	30	40
AWRI1628	8	20	30	40

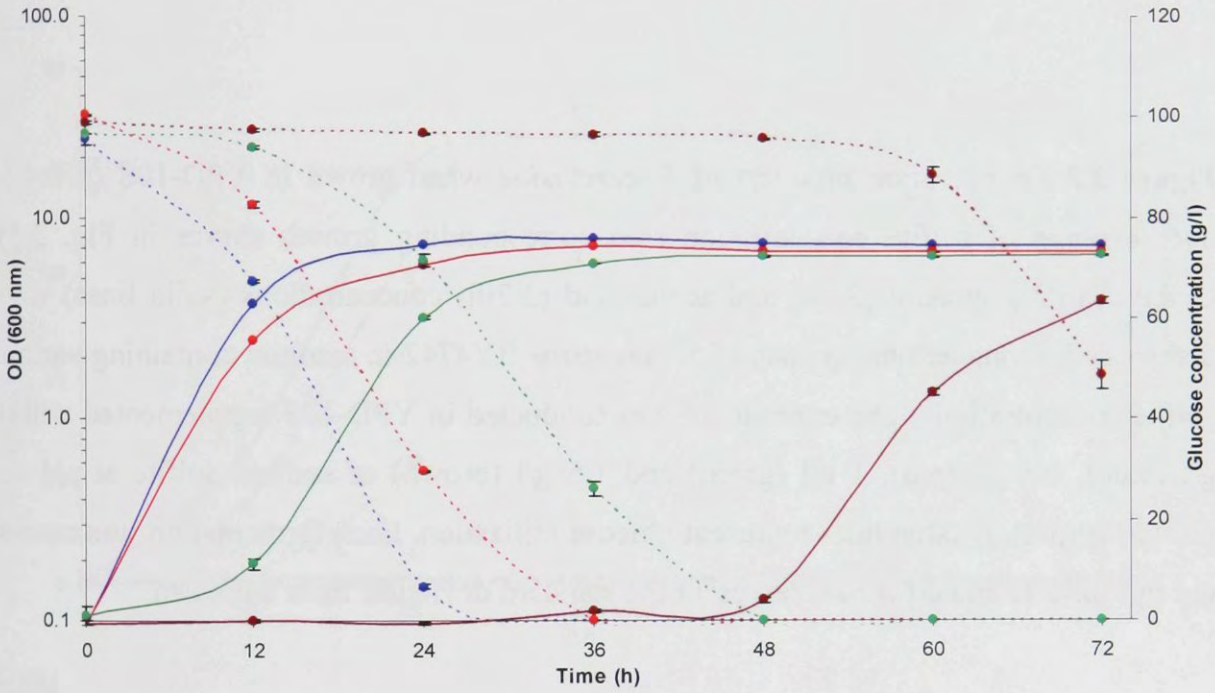
3.3.2 Effect of sulfite on yeast metabolism at pH 4.5

Fermentation experiments in YPD-10S containing sub-lethal concentrations of sulfite at pH 4.5 were conducted using the BY4742 strain. The growth profiles show that sulfite has a high toxicity at this pH. The introduction of 1 g/l sodium sulfite almost halved the sugar consumption rate compared to cultures with no added sulfite and the addition of 1.5 g/l sulfite inhibited the commencement of yeast growth for approximately 48 hours (Figure 3.1). The glycerol concentrations measured at the point of glucose exhaustion increased by around 1 g/l in cultures containing 1 g/l sulfite compared to cultures with no sulfite addition (Figure 3.2); surprisingly, ethanol levels were slightly higher in cultures with 1 g/l added sulfite (Figure 3.2). Acetic acid concentrations were lower in the presence of sulfite compared to cultures without added Na_2SO_3 (Figure 3.2).

The introduction of sulfite to BY4742 cultures at pH 4.5 caused an increase in glycerol yields, from 0.022 to 0.043 g glycerol/g glucose, and ethanol yields also slightly increased (Table 3.2). Overall, the glycerol to ethanol ratio increased from 0.050 to 0.098 g glycerol/g ethanol as the sulfite concentration increased from 0 to 1.5 g/l; it should be noted that only half of the glucose was consumed in the culture containing 1.5 g/l sulfite after 72 hours incubation.

The above results show that sulfite addition to BY4742 cultures at pH 4.5 redirects carbon metabolism toward glycerol production, however, not at the expense of ethanol production. The increase in glycerol yield must have been at the sacrifice of other fermentation products and there is some evidence in the above results to suggest that acetic acid is one of these products. The above outcomes defy the purpose of this project which is to produce more glycerol, but at the cost of ethanol production. Although the original experimental plan included testing the effect of sulfite addition on the growth of *S. cerevisiae* AWRI1628 cultures at pH 4.5, it was decided to terminate this work in favor of conducting such experiments at pH 8. The reasons for the change in plan were the lack of desired effect by sulfite at pH 4.5 on BY4742, and preliminary experiments indicated that sulfite had a significantly greater impact on the glycerol to ethanol ratio at pH 8 (data not shown).

Figure 3.1 Growth and sugar utilization for *S. cerevisiae* BY4742 in YPD-10 (pH 4.5) over a range of sulfite concentrations. Growth (solid lines) and glucose utilization (broken lines) of *S. cerevisiae* BY4742 in YPD-10S (pH 4.5) supplemented with increasing levels of sodium sulfite: 0 g/l (blue), 0.5 g/l (red), 1 g/l (green) and 1.5 g/l (brown) of Na_2SO_3. The incubations were conducted under aerobic conditions (30°C/140 rpm). Each fermentation was carried out in triplicate and the error bars represent the standard deviation from the mean.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1.0
0.1
OD (600 nm)
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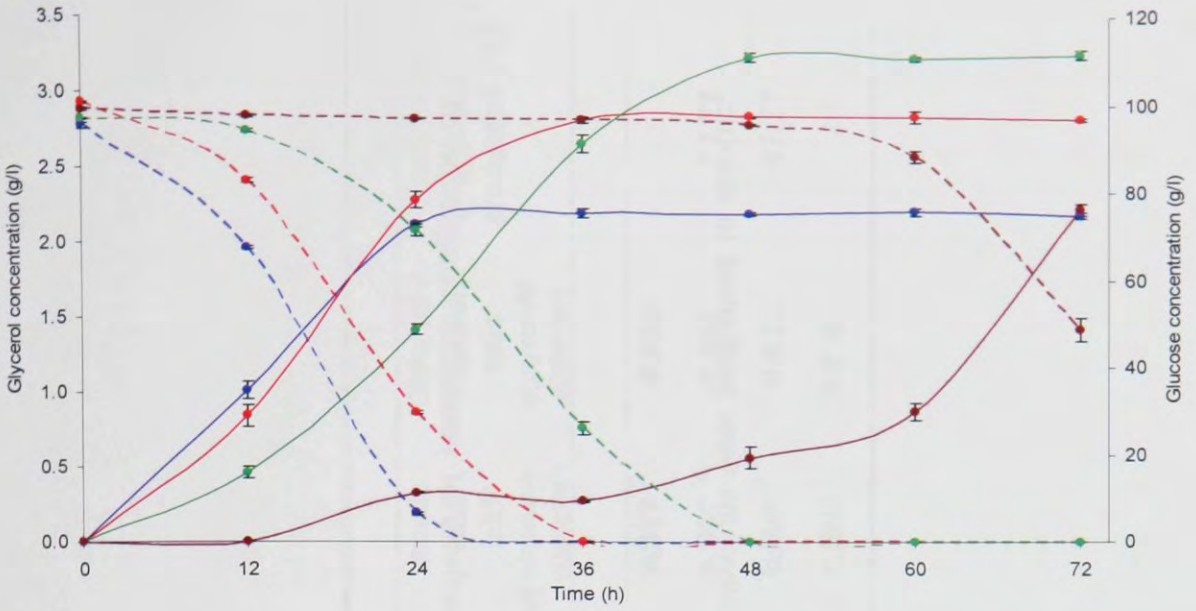
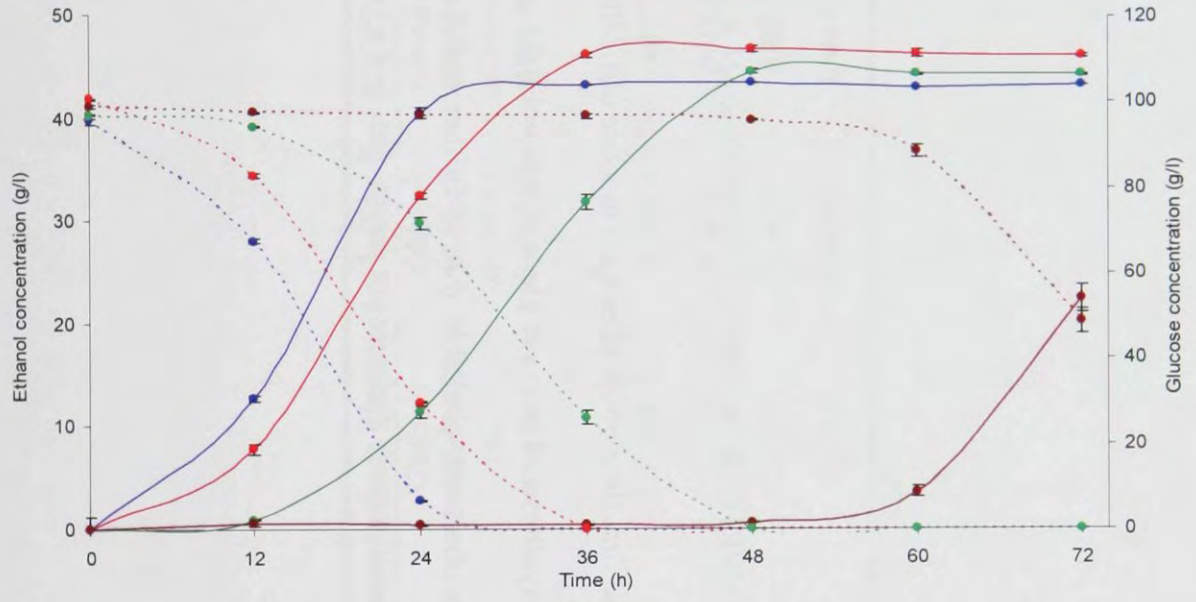
100
80
60
40
20
0
Glucose concentration (g/l)
0
12
24
36
48
60
72
Time (h)

Figure 3.2 Fermentation products of *S. cerevisiae* when grown in YPD-10S (pH 4.5) over a range of sulfite concentration (see corresponding growth curves in Fig. 3.1). Glycerol (3.2i), ethanol (3.2ii) and acetic acid (3.2iii) concentrations (solid lines) were determined during aerobic growth of *S. cerevisiae* BY4742 in medium containing various sulfite concentrations. The experiment was conducted in YPD-10S supplemented with 0 g/l (blue), 0.5 g/l (red), 1 g/l (green) and 1.5 g/l (brown) of sodium sulfite at pH 4.5 (30°C/140rpm). Broken lines represent glucose utilization. Each fermentation was carried out in triplicate and error bars represent the standard deviation from the mean.

3.2i



3.2ii



3.2iii

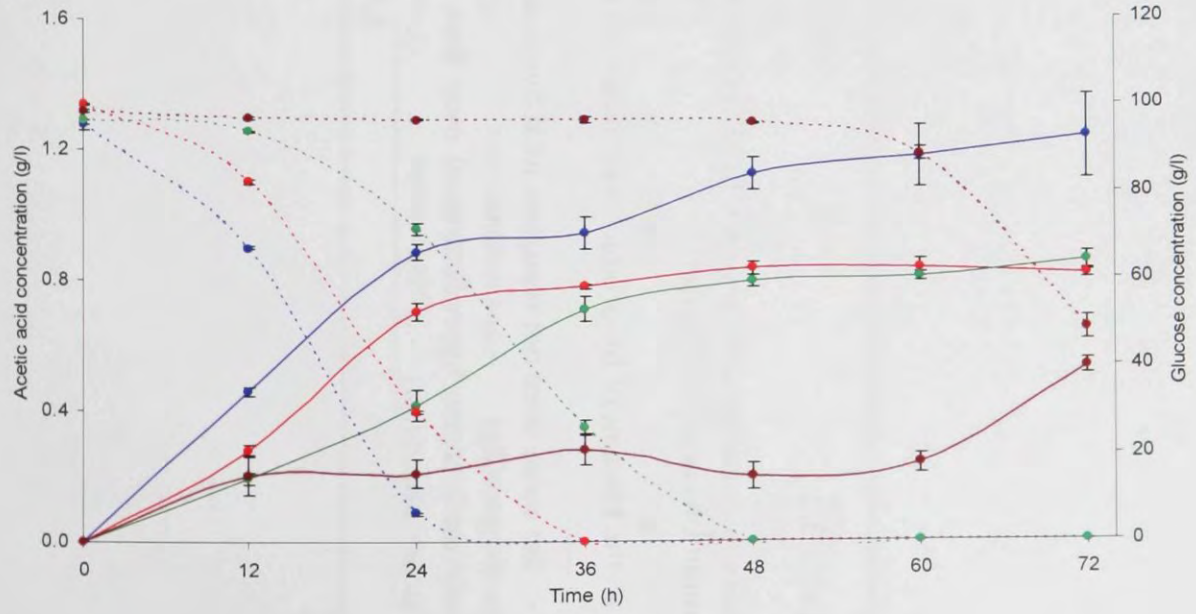


Table 3.2

Substrate and product profiles for *S. cerevisiae* AWRI1628 incubated in YPD-10 at pH 4.5. Incubations were performed in aerobic conditions at 30°C/140 rpm.

* - the amount of by-product was measured at the first time point after total glucose utilization

[1] - the value does not represent total by-product synthesized because glucose was not fully utilized

[2] - an increase byproduct level over time was observed, probably due to further metabolic activity of yeast after completion of glucose utilization

Fermentation characteristics for BY4742								
Sulfite concentration in the medium(g/l)	Approximate glucose consumption time (h)	Glucose consumed (%)	Glucose consumption rate g/lh	Ethanol yield* g(ethanol)/ g(glucose)	Glycerol yield* g(glycerol)/ g(glucose)	Acetic acid yield* g(acetate)/ g(glucose)	Glycerol / Ethanol ratio g(glycerol)/ g(glucose)	Increase in glycerol production (%)
pH 4.5								
0	**28**	**100**	**3.571**	**0.432**	**0.022**	**0.009** [2]	**0.050**	-
0.5	**36**	**100**	**2.702**	**0.469**	**0.028**	**0.007** [2]	**0.061**	**27.72**
1.0	**48**	**100**	**2.083**	**0.445**	**0.032**	**0.008** [2]	**0.072**	**47.27**
1.5	**> 72**	**51.35** [1]	**0.713**	**0.438** [1]	**0.043** [1]	**0.010** [1]	**0.098** [1]	-

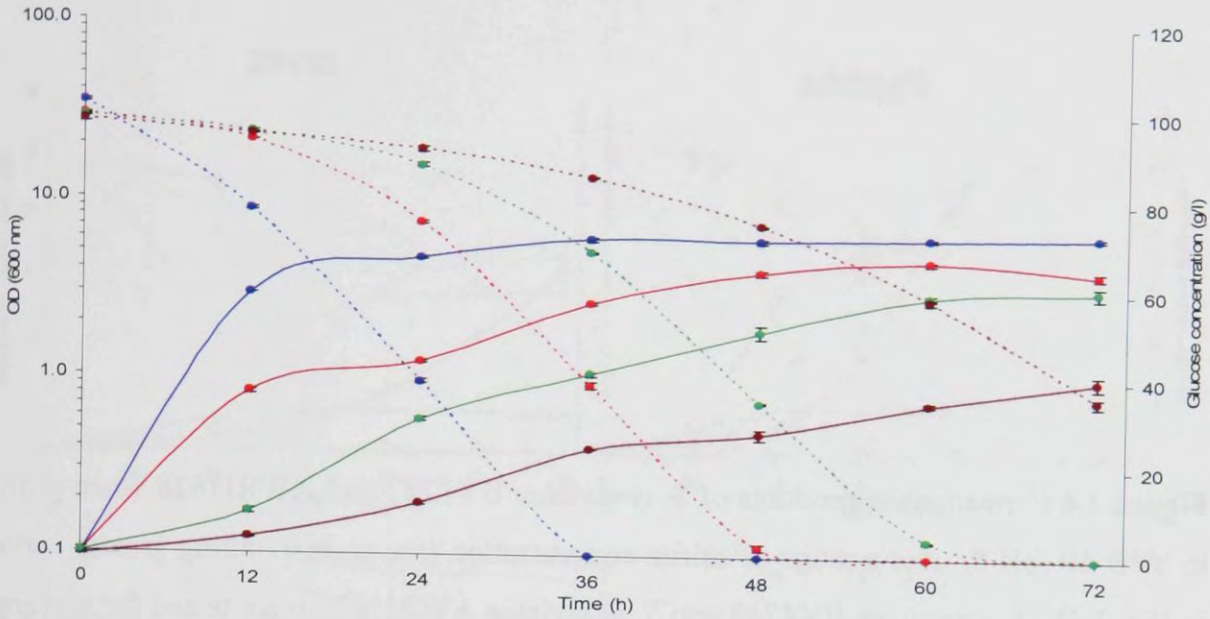
3.3.3 Effect of sulfite on yeast metabolism at pH 8

The impact of sulfite on yeast metabolism at pH 8 was investigated using Na_2SO_3 concentrations in the range 0 - 40 g/l. Growth and glucose utilization profiles for strains BY4742 and AWRI1628 are shown in Figure 3.3i and 3.3ii, respectively. Results from experiments conducted on BY4742 show, that sodium sulfite was considerably less inhibitory to the yeast at alkaline pH compared to its effect at pH 4.5, with growth occurring at up to 40 g/l Na_2SO_3 at pH 8 (Figure 3.3i), compared to the 48 hour lag caused by 1.5 g/l Na_2SO_3 at pH 4.5 (Figure 3.1). The effect of sulfite on the growth profile of BY4742 was also different with sulfite causing extended lag periods, yet having a relatively small impact on growth rate at pH 4.5, whereas at pH 8 sulfite had a much greater negative impact on growth rate. Under the environmental conditions used in these experiments, AWRI1628 was considerably more robust than BY4742 both in the presence and absence of added Na_2SO_3. In all conditions, AWRI1628 had higher growth rates and cell yields during 72 hours incubation compared to BY4742. In keeping with the growth profiles, AWRI1628 also had a higher glucose consumption rate than BY4742 under all the experimental conditions used (Figures 3.3i and 3.3ii). Notably, AWRI1628 used all of the 100 g/l glucose in the medium containing 40 g/l Na_2SO_3 after 60 hours, yet in the same conditions BY4742 had only used approximately 42% of glucose at this time point.

Based on previous studies (Petrovska et al. 1999), carbon flow was significantly redirected away from ethanol formation and toward glycerol production during fermentation under 'high sulfite' and 'high pH' conditions (Fig 3.4, Table 3.3). Supplementation of the medium with 30 g/l of Na_2SO_3 reduced final ethanol concentrations by approximately 30% and 33% for BY4742 and AWRI1628 respectively. Under the same conditions, final glycerol concentrations were significantly higher; an increase of approximately 380% in glycerol yield was observed for BY4742, and an increase in glycerol yield of 441% was measured for AWRI1628. A further increase in sulfite concentration had an even more prominent effect on metabolic flux distribution in both strains. A Na_2SO_3 concentration of 40g/l decreased the ethanol yield of AWRI1628 by 41%, and a 539% increase in glycerol yield was observed. BY4742, on the other hand, was unable to use all of the glucose after 72 hours incubation at this sulfite concentration however, based on the amount of

Figure 3.3 Growth (solid lines) and sugar utilization profiles (broken lines) of *S. cerevisiae* BY4742 (Fig. 3.3i) and *S. cerevisiae* AWRI1628 (Fig 3.3ii) were followed during their incubation in rich medium supplemented with various levels of sodium sulfite. The experiment was conducted in YPD-10 at pH 8 and the sulfite concentrations used were as follows: 0 g/l (blue), 20 g/l (red), 30 g/l (green) and 40 g/l (brown). Fermentations were performed under aerobic conditions (30°C/140 rpm), each being carried out in triplicate. Error bars represent standard deviations from means.

3.3i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10.0
1.0
0.1
OD (600 nm)
120
100
80
60
40
20
0
Glucose concentration (g/l)
0
12
24
36
48
60
72
Time (h)

3.3ii

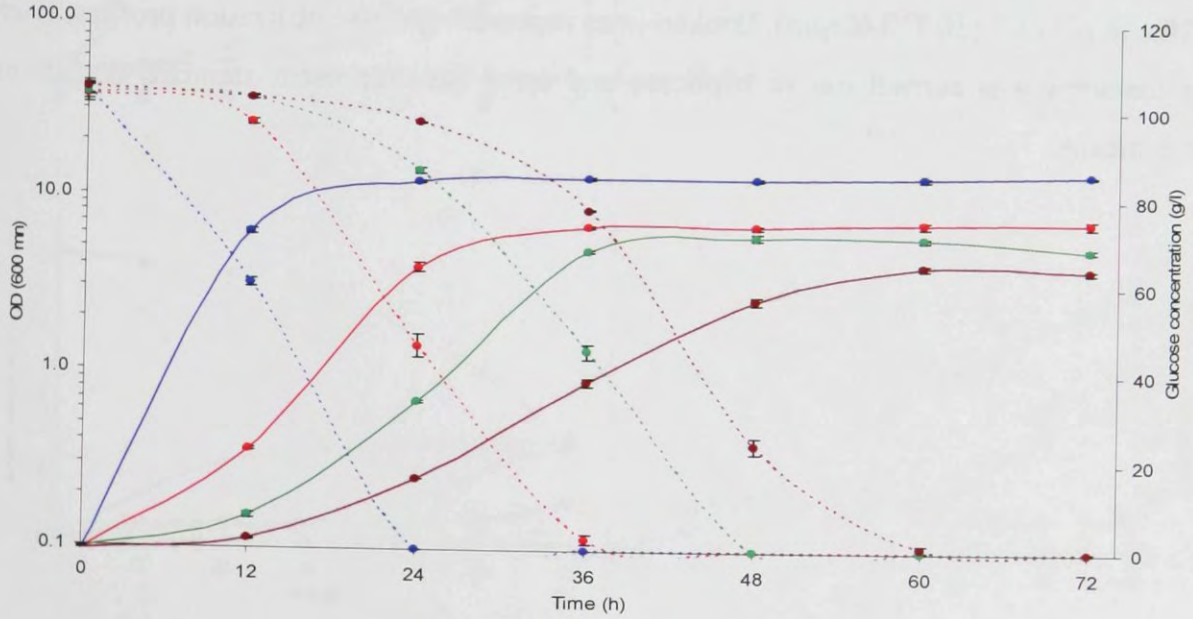

100.0
10.0
1.0
0.1
OD (600 nm)
120
100
80
60
40
20
0
Glucose concentration (g/l)
0
12
24
36
48
60
72
Time (h)

Figure 3.4 Fermentation products of *S. cerevisiae* BY4742 and AWRI1628 when grown in YPD-10 (pH 8) over a range of sulfite concentration (see corresponding growth curves in Fig. 3.3). *S. cerevisiae* BY4742 and *S. cerevisiae* AWRI1628 were tested for glycerol, ethanol and acetic acid production (solid lines) during aerobic growth in medium containing various sulfite concentrations. The incubations were conducted in YPD-10, supplemented with 0 g/l (blue), 20 g/l (red), 30 g/l (green) and 40 g/l (yellow) of sodium sulfite at pH of 8 (30°C/140rpm). Broken lines represent glucose utilization profiles. Each fermentation was carried out in triplicate and error bars represent standard deviations from means.

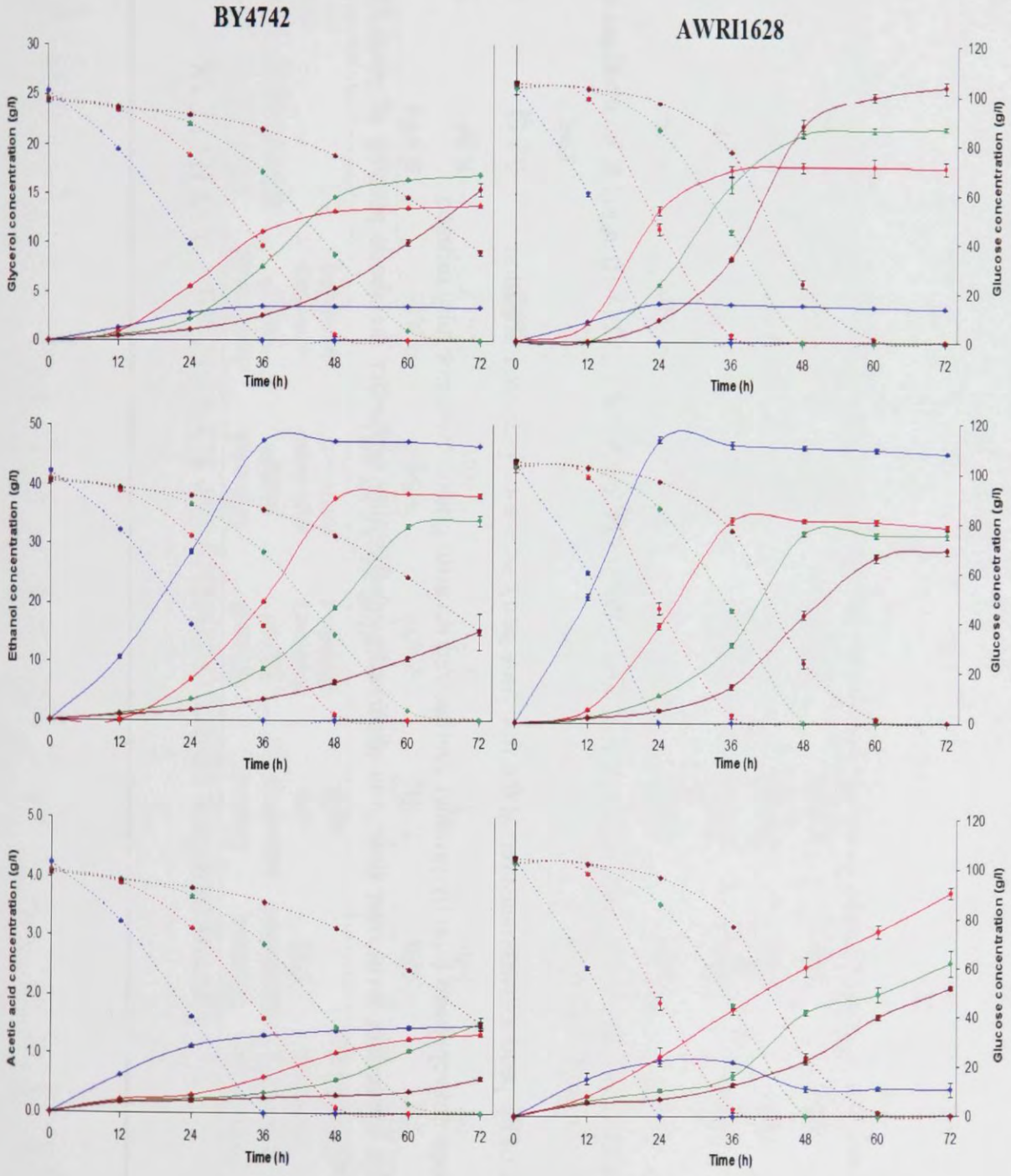

BY4742
AWRI1628
Glycerol concentration (g/l)
Ethanol concentration (g/l)
Acetic acid concentration (g/l)
Glucose concentration (g/l)
Time (h)

Table 3.3
Substrate and product profiles for *S. cerevisiae* BY4742 and AWRI1628 incubated in YPD-10 at pH 8. Incubations were performed in aerobic conditions at 30°C/140 rpm.

* - the amount of by-product was measured at the first time point after total glucose utilization

[1] - the value does not represent total by-product synthesized because glucose was not fully utilized

[2] - an increase byproduct level over time was observed, probably due to further metabolic activity of yeast after completion of glucose utilization

Fermentation characteristics for BY4742 and AWRI1628

Sulfite concentration in the medium(g/l)	Approximate glucose consumption time (h)	Glucose consumed (%)	Glucose consumption rate g/lh	Ethanol yield* g(ethanol)/ g(glucose)	Glycerol yield* g(glycerol)/ g(glucose)*	Acetic acid yield* g(acetate)/ g(glucose)	Glycerol / Ethanol ratio	Decrease in ethanol production (%)	Increase in glycerol production (%)
pH 8 (BY4742)									
0	**36**	**100**	**2.702**	**0.473**	**0.035**	**0.013** [2]	**0.074**	**-**	**-**
20	**50**	**100**	**2**	**0.375**	**0.134**	**0.012** [2]	**0.357**	**20.70**	**384.00**
30	**65**	**100**	**1.538**	**0.328**	**0.168**	**0.015**	**0.512**	**30.68**	**481.14**
40	**< 72**	**64.3**	**0.893**	**0.247** [1]	**0.239** [1]	**0.009** [1]	**1.021** [1]	**-**	**-**
pH 8 (AWRI1628)									
0	**24**	**100**	**4.160**	**0.473**	**0.039**	**0.009** [2]	**0.083**	**-**	**-**
20	**36**	**100**	**2.702**	**0.338**	**0.174**	**0.018** [2]	**0.514**	**28.51**	**438.03**
30	**48**	**100**	**2.083**	**0.316**	**0.211**	**0.017** [2]	**0.666**	**33.16**	**535.28**
40	**60**	**100**	**1.666**	**0.277**	**0.249**	**0.016** [2]	**0.896**	**41.31**	**631.98**

glucose used during this time, the ethanol yield continued to decrease with a further increase in glycerol yield. These changes in glycerol and ethanol yields were also reflected in the changes in glycerol/ethanol ratios for non-sulfite and sulfite-supplemented fermentations (Table 3.3).

For BY4742, the glycerol/ethanol ratio increased by 4.8-, 6.9- and 13.9-fold for cultures containing 20, 30 and 40 g/l Na_2SO_3 respectively. AWRI1628 had increases in glycerol/ethanol ratios of 6.2-, 8.0- and 10.8-fold in cultures containing 20, 30 and 40 g/l Na_2SO_3 respectively.

There are some interesting observations to be made about acetic acid production in these experiments. The first being that BY4742 produced either similar or lower acetic acid concentrations in the presence of added Na_2SO_3 compared to the 'non- Na_2SO_3' control, yet AWRI1628 always produced more acetic acid in the presence of added Na_2SO_3 (Figure 3.4; Table 3.3). Secondly, for both strains in the presence of added Na_2SO_3, a general observation can be made that the amount of acetic acid produced decreased with increasing amounts of Na_2SO_3 in the culture. The latter observation is probably a result of higher sulfite levels binding to greater amounts of acetaldehyde thereby removing it as a substrate for acetate production. Finally, acetate levels continued to rise after glucose exhaustion in cultures of both strains containing Na_2SO_3. This is probably due to post-fermentation metabolic activities, presumably the formation of acetaldehyde from ethanol via its oxidation, which may have made it available for acetate formation (Figure 3.4).

3.3.4 Osmotic strength of sulfite selective medium

The amount of sulfite used in the above experiments might have been expected to induce an osmotic stress, which may, in turn, have contributed to elevated glycerol production via HOG pathway activation. To estimate the possible contribution of osmotic pressure to the previously observed metabolic responses, the sulfite-containing medium was tested for its osmotic strength using a Vapor Pressure Osmometer (see Section 2.2.6). The media tested were: YPD-10, YPD-10 containing 40 g/l of Na_2SO_3 at pH 8, YPD-10 containing 40 g/l of NaCl at pH 8 (both used in this study), medium used by Petrovska et al. (1999) without Na_2SO_3 or NaCl, Petrovska et al. (1999) medium containing 40 g/l Na_2SO_3, or 40 g/l of NaCl. The

results show that NaCl-supplemented medium possesses a higher osmotic strength than that supplemented with an equivalent amount of sodium sulfite, with the osmotic strength of sulfite-supplemented YPD-10 being approximately midway between unsupplemented and NaCl-supplemented (40 g/l) YPD-10 medium (Table 3.4).

3.4 Discussion

In the experiments described in this chapter sulfite redirected yeast metabolism away from ethanol production, leading to increased glycerol yields. This effect by sulfite on *S. cerevisiae* metabolism is thought to occur because of its binding potential with acetaldehyde creating a non-toxic compound, 1-hydroxyethanesulfonate (Taylor et al. 1986); thus, it sequesters acetaldehyde. To compensate, the yeast cell must alter metabolism to maintain redox balance. One way to achieve this is by the redirection of metabolism towards glycerol synthesis, which involves the oxidation of NADH to NAD^+. However, these effects by sulfite on yeast metabolism are pH dependent. At both pH 4.5 and 8.0, increasing Na_2SO_3 concentrations increased the glycerol yields for both *S. cerevisiae* strains, but the magnitude of change in glycerol production was far greater at a medium pH of 8, mostly because greater sulfite concentrations could be used in the medium at this pH. Ethanol production in sulfite-containing medium was affected differently depending on medium pH. Na_2SO_3 addition had little effect on ethanol production at pH 4.5; only at pH 8 ethanol production was substantially decreased. Although the reasons for these observations are yet to be determined, it is reasonable to speculate that the ability of sulfite to enter the intracellular environment may have a crucial role.

At acidic pH, where the predominant form of sulfite is uncharged sulfur dioxide (SO_2) or sulfurous acid (H_2SO_3), sulfite can enter the cell via passive diffusion. When in the cell, SO_2 or H_2SO_3 molecules encounter a neutral intracellular pH (usually in range 6 - 7), which is independent of extracellular pH, causing the dissociation of these sulfite forms into positively charged ionic forms of sulfite and bisulfite ions. In these ionic forms, sulfite can react with acetaldehyde and a number of intracellular components, thus influencing intracellular function. This project demonstrated the higher toxicity of sulfite on yeast growth at acidic pH compared to alkaline pH, as

Table 3.4 Osmotic strength of media containing Na_2SO_3 or NaCl

Media	Osmotic strength (mmol/kg)
YPD-10 (pH 8) (used in this study)	**788** ± 5
YPD-10 (pH 8), 40 g/l Na_2SO_3 (used in this study)	**1443** ± 13
YPD-10 (pH 8), 40 g/l NaCl (used in this study)	**2122** ± 4
Not supplemented with Na_2SO_3 or NaCl (from Petrovska et al. 1999)	**715** ± 2
Containing 40 g/l Na_2SO_3 at pH 7 (from Petrovska et al. 1999)	**1555** ± 5.3
Containing 40 g/l NaCl at pH 7 (from Petrovska et al. 1999)	**1994** ± 6.3

previously reported by Schimz (1980). An important observation in this work is that ethanol yields did not decrease in the presence of sulfite at pH 4.5, rather they increased slightly. Ethanol productivity however did decrease substantially. This is not expected from a sulfite mechanism that acts predominantly by sequestering large amounts of acetaldehyde, in which case ethanol yield would be affected. With this in mind, it is more likely that at pH 4.5, sulfite was primarily affecting cell growth by inhibition of enzymes associated with central carbon metabolism. Enzyme inhibition by sulfite has been previously reported with glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase being particularly affected (Hinze and Holzer 1985 and 1986, Maier et al. 1985). The inhibition of glyceraldehyde-3-phosphate dehydrogenase in yeast could be expected to reduce carbon flux in glycolysis and the fermentative pathway, leading to a decrease in growth rate and ethanol productivity, but not necessarily affecting ethanol yield. The inhibition of glycolysis could also account for the observation that yeast produced elevated levels of glycerol while exposed to increasing sulfite levels at pH 4.5, since inhibition of glyceraldehyde-3-phosphate dehydrogenase could divert carbon flow towards glycerol biosynthesis, and such an effect was also observed in this study (Fig. 3.2). Sulfite inhibition of alcohol dehydrogenase at pH 4.5 could also be expected to have similar outcomes to that described above for glyceraldehyde-3-phosphate dehydrogenase. The mechanism in this case being the inhibition of alcohol dehydrogenase activity, causing a decrease in the NADH oxidation rate which could be compensated for by an increase in glycerol production.

At pH 8 sulfite predominantly occurs as a negatively charged sulfite ion (SO_3^{2-}), and this form is unlikely to freely enter the cell. Extracellular sulfite however can still bind to acetaldehyde excreted from the cell, generating and maintaining a high acetaldehyde concentration gradient across the cytoplasmic membrane. Sulfite at pH 8 should therefore maintain a high acetaldehyde diffusion rate out of the cell, depriving the fermentation pathway of intracellular acetaldehyde used for ethanol production, subsequently disrupting the redox balance. Unable to enter the cell, sulfite at pH 8 can not directly inhibit intracellular glycolytic enzymes as possibly occurs at acidic pH (Schimz 1980). Consequently, much greater concentrations of sulfite could be introduced to the medium at pH 8 before it was observed to impact on yeast growth. Given the above, it is speculated that the ionic form of sulfite, which predominates at

pH 8, may act as a redox balance disruptor from outside the cell, trapping extracellular acetaldehyde and causing redirection of metabolic carbon flow towards pathways, such as glycerol production, that yeast can use to restore the redox balance.

For both BY4742 and AWRI1628 strains at pH 8, redirection of metabolic carbon flow was observed away from ethanol biosynthesis, favoring glycerol production (Figure 3.4). Growth and by-product profile of the two strains at pH 8 were not the same however, with strain BY4742 being more affected than strain AWRI1628. This is evident even in the absence of Na_2SO_3 addition where BY4742 had a significantly lower final cell yield and growth rate than AWRI1628. Interestingly, both strains had similar ethanol yields when grown in non-stressed medium, BY4742 taking around 10 hours longer to reach peak ethanol levels.

AWRI1628 metabolism appears to be more responsive to sulfite addition than was the case for BY4742. In the presence of sulfite, AWRI1628 produced more glycerol and acetic acid, and less ethanol compared to BY47242. This may also account for the higher growth rates of AWRI1628 in the presence of sulfite, since higher glycerol synthesis presumably confers greater capacity for NAD^+ regeneration. The ability of AWRI1628 to produce more glycerol than BY4742 in the presence of sulfite most likely accounts for both the lower ethanol production of AWRI1628, (since less NADH is available) and higher acetate production, (since more NAD^+ is available for aldehyde dehydrogenase activity). Both strains produced significantly different levels of acetic acid while incubated under sulfite stress. The acetic acid profile of AWRI1628 in the presence of sulfite is unusual in that acetic acid levels continue to increase even after glucose had expired, at the same time the ethanol and glycerol concentrations were relatively constant suggesting that the source of acetate was not via aldehyde dehydrogenase. This could be related to the history of this strain as a wine producer since wine yeast strains are noted for their production of ethyl acetate and isoamyl acetate esters, which are important for wine aroma. The production of these acetates is catalyzed by acetyltransferase (Plata et al. 2003).

High Na_2SO_3 concentrations in the medium at pH 8, (20 - 40 g/l), would be expected to increase osmotic pressure on the yeast cells, and yeast exposed to osmotic pressure are known to increase glycerol production as a means of maintaining turgor pressure

(Nevoigt and Stahl 1997, Klipp 2005). It was therefore possible that the increased glycerol production observed by yeast exposed to 40 g/l sodium sulfite is due to an osmotic stress response, rather than acetaldehyde consumption by sulfite and a subsequent redox imbalance. However, Petrovska et al. (1999) found this not be the case for *Saccharomyces cerevisiae* when separately subjected to osmotic stress and sulfite-induced stress (Petrovska et al. 1999). This researcher compared the physiological response of *S. cerevisiae* TMF strain (grown in rich medium at pH 7) when subjected to 40 g/l of NaCl (osmotic stress) or 40 g/l of Na_2SO_3 (sulfite stress). Compared to cells exposed to NaCl, the authors found that cells exposed to sulfite had higher glycerol yields (430% higher), lower ethanol yields (lower by 38%) and an increase in the glycerol/ethanol ratio of 754% (2.39 g/g compared to 0.28 g/g). The authors also found that the sulfite-exposed yeast cultures consumed sulfite during incubation, presumably due to sulfite binding with acetaldehyde. It was concluded that sulfite was substantially more efficient at shifting *S. cerevisiae* metabolism toward glycerol production compared to a NaCl-induced osmotic stress and that this effect was primarily due to sulfite binding to acetaldehyde. Petrovska et al. (1999) established that the exposure of yeast to 40 g/l sulfite at pH 7 increases glycerol production, and decreases ethanol production, to a far greater extent than that caused by 40 g/l sodium chloride. It was not clear, however, whether the equivalent concentration of different stressors, sodium sulfite and sodium chloride, induce the same osmotic pressure. The results shown in Table 3.4 strongly support the conclusions of Petrovska et al. (1999) by providing evidence that the sulfite effect on yeast is not due, in large part, to osmotic stress. Rather, and as concluded by Petrovska et al. (1999), the sulfite most likely influences metabolism by binding to acetaldehyde (subsequently affecting cellular redox balance), with a minor role possibly being attributable to osmotic stress caused by the high sodium sulfite concentrations.

Although the magnitude of the responses by BY4742 and AWRI1628 to various levels of sulfite addition were slightly different, it was decided that a concentration of 30 g/l of Na_2SO_3 at pH 8 was suitable for the adaptive evolution selection experiments described in the following chapter. A sulfite concentration of 40 g/l had a substantial impact on the growth rate of BY4742 and it was felt that this would necessitate lengthy incubation periods for the serial cultures under sulfite selection pressure.

Both strains had significant growth and metabolic responses to 30 g/l of sulfite which should provide sufficient selective pressure in the adaptive evolution experiments described in the proceeding chapter.

3.5 Conclusions

- Addition of sulfite at pH 4.5 was highly toxic for *S. cerevisiae* and did not give satisfactory redirection of metabolic carbon flow away from ethanol biosynthesis.
- The significantly lower toxicity of sulfite at pH 8 allowed the use of much higher Na_2SO_3 concentrations with moderate impact on growth rate, and significant redirection of metabolism away from ethanol biosynthesis, increasing glycerol yield.

CHAPTER 4

ADAPTIVE EVOLUTION AS A STRATEGY FOR THE GENERATION OF 'LOW-ETHANOL' YEAST

4.1 Aim

The main aim of this chapter was to test the effectiveness of using sulfite as a selection agent for the generation of 'low-ethanol' variants of *S. cerevisiae* BY4742 and *S. cerevisiae* AWRI1628.

4.2 Introduction

Adaptive evolution is the Darwinian process that drives the generation of adaptations in response to various agents of natural selection including changing environmental conditions. The principals of natural selection can be applied in the laboratory to generate novel mutant organisms with desirable adaptations, and microorganisms are particularly amenable to this approach (see, for example, Chambers et al. 2007). However, finding the appropriate selection pressure to drive targeted changes in phenotypes is not necessarily straightforward, and attempting to generate mutants of *S. cerevisiae* that produce reduced amounts of ethanol is a case in point.

As discussed in Section 1.3.5, a number of chemical stressors exist that can induce redirection of metabolism of *S. cerevisiae* towards glycerol production, and, as a consequence, create depleted ethanol yields. One such chemical is sulfite and this chapter describes the application of adaptive evolution, using sulfite as a selection pressure, to drive the generation of 'low-ethanol' variants of *S. cerevisiae*.

4.3 Results

4.3.1. Establishment of adaptively evolving populations

Two sets of triplicate evolving populations were established for adaptive evolution under sulfite stress; one population was derived from *S. cerevisiae* BY4742, the other from

S. cerevisiae AWRI1628 (see Figure 4.1). To generate triplicates, both strains were cultured overnight in YPD liquid medium and subsequently diluted and transferred onto YPD agar plates to derive single colonies. Whilst this approach was adequate to obtain clonal populations of BY4742, it was noticed in previous work that AWRI 1628 forms large 'clumps' (see Figure 4.2), thus, single colonies on YPD plates were likely to be non-clonal. Therefore, a micromanipulator was used to seed single colonies from single cells for this strain. Three random, separate colonies from each parent were inoculated into YPD, grown overnight at 30°C/140 rpm, then samples of the cultures were frozen as described in Section 2.1.5.1.

Triplicate 'starter populations' of each strain (B1, B2 and B3 derived from BY4742, and A1, A2 and A3 derived from AWRI1628), were inoculated from the above frozen stocks as described in Section 2.1.5.2 (see Figure 4.1). These 'starter populations' were then used to inoculate 'founding populations' FB1, FB2, FB3 and FA1, FA2, FA3 at an OD of 0.1 in 100 ml of YPD-10S, (30 g/l Na_2SO_3, pH 8), in 250 ml Erlenmyer flasks covered with aluminum foil. For both BY4742 and AWRI1628 an OD_{600} of 0.1 was equivalent to approximately 5.5 x 10^6 cells/ml. The six founding populations were grown to an OD_{600} of approximately 3.2, equivalent to approximately 2 x 10^8 cells/ml, and were then passaged into the same volume of fresh, pre-warmed to 30°C, YPD-10S medium, to an OD_{600} of 0.1. Thus, founding populations gave rise to subsequent evolving populations; EB1, EB2 and EB3 derived from B1, B2 and B3 respectively, and EA1, EA2 and EA3 derived from A1, A2 and A3 respectively. The evolving populations were incubated in YPD-10S until they reached an OD_{600} of 3.2, (thus completing approximately five generations), when they were again passaged as above. This sequential passaging was repeated until evolving populations reached approximately 300 generations. During the course of the experiment samples of the evolving populations were taken every 50 generations, frozen at -80°C and stored for future use (see Section 2.1.5.1).

Figure 4.1 Flow chart illustrating the adaptive evolution strategy used to generate 'low ethanol' yeast variants of *S. cerevisiae* BY4742 and AWRI1628.

B1, B2, B3 and A1, A2, A3, represent 'starter populations' isolated from parental strains *S. cerevisiae* BY4742 and *S. cerevisiae* AWRI 1628 respectively. FB1, FB2, FB3 and FA1, FA2, FA3 represent founding populations derived from respective starter populations. EB1, EB2, EB3 and EA1, EA2, EA3 represent the evolving populations in YPD-10S.

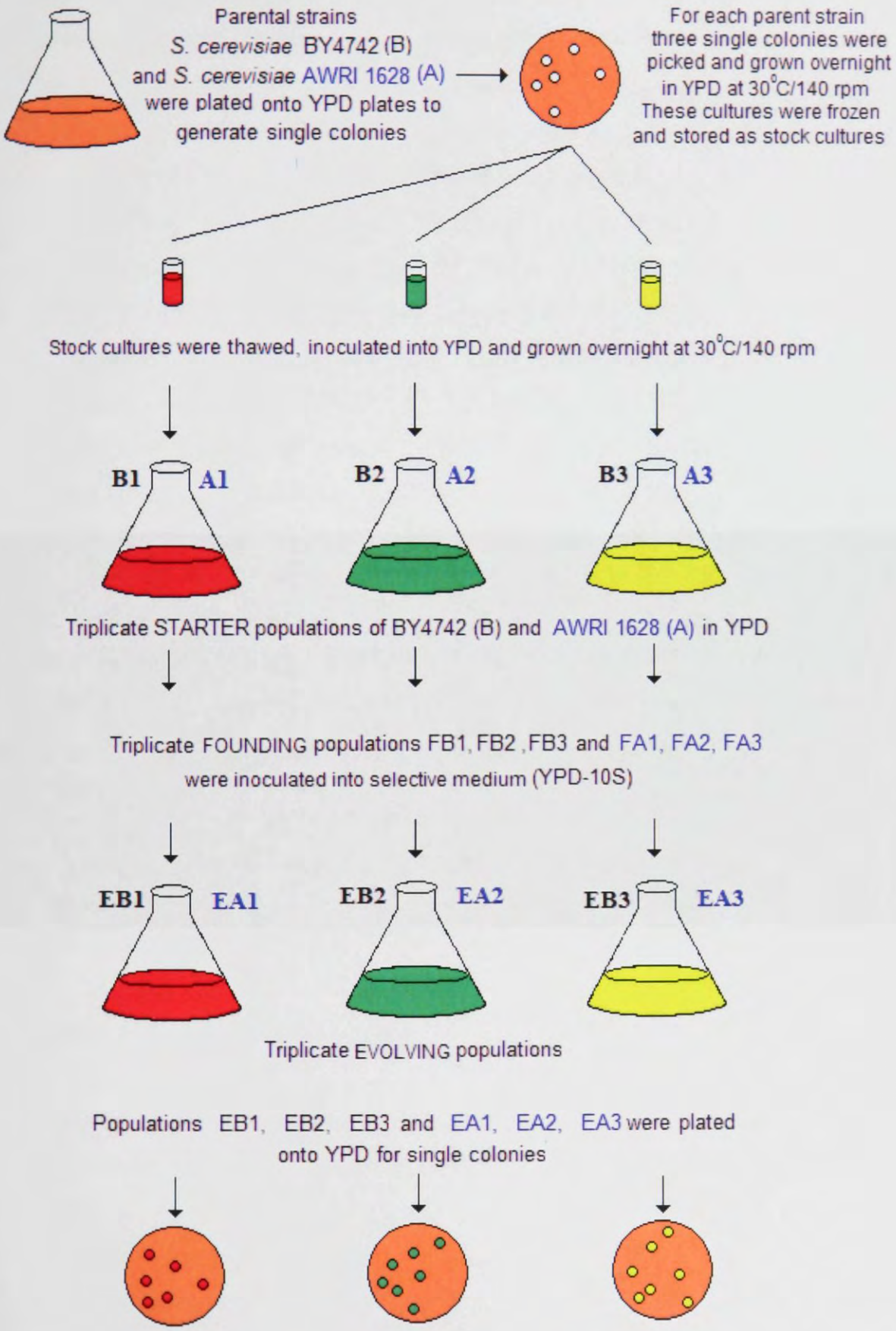
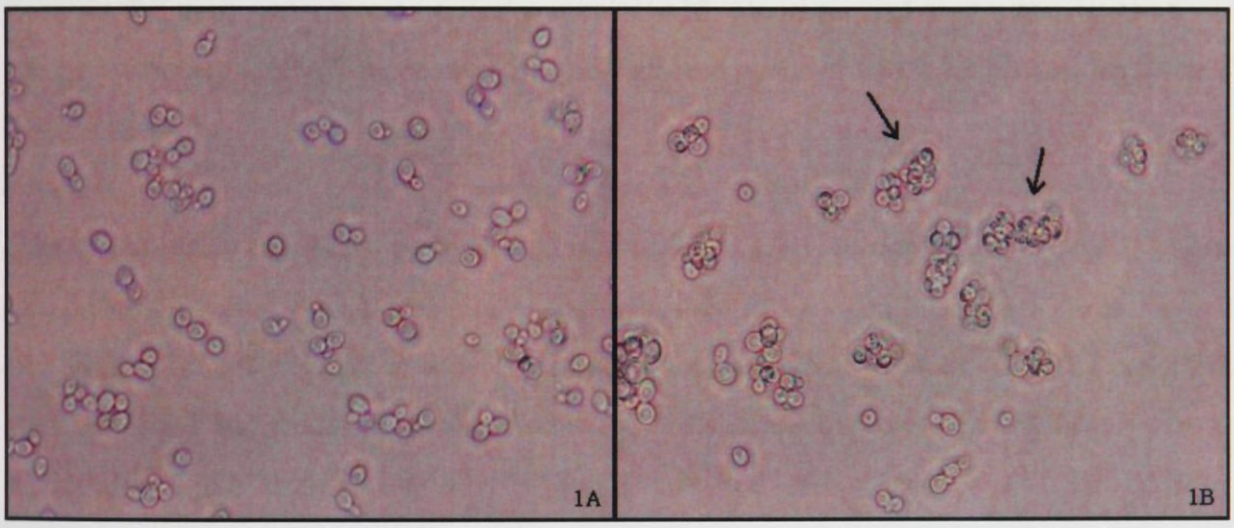

Parental strains
S. cerevisiae BY4742 (B)
and S. cerevisiae AWRI 1628 (A)
were plated onto YPD plates to
generate single colonies
For each parent strain
three single colonies were
picked and grown overnight
in YPD at 30°C/140 rpm
These cultures were frozen
and stored as stock cultures
Stock cultures were thawed, inoculated into YPD and grown overnight at 30°C/140 rpm
B1
A1
B2
A2
B3
A3
Triplicate STARTER populations of BY4742 (B) and AWRI 1628 (A) in YPD
Triplicate FOUNDING populations FB1, FB2 ,FB3 and FA1, FA2, FA3
were inoculated into selective medium (YPD-10S)
EB1
EA1
EB2
EA2
EB3
EA3
Triplicate EVOLVING populations
Populations EB1, EB2, EB3 and EA1, EA2, EA3 were plated
onto YPD for single colonies

Figure 4.2 Microscopic examination of *S. cerevisiae* BY4742 and AWRI 1628

Photographs 1A and 1B show *S. cerevisiae* BY4742 and AWRI1628 respectively, at 40X magnification; cells were taken from stationary phase cultures grown in YPD. Arrows in 1B indicate large clumps of cells, a feature that was common to AWRI1628 in a range conditions.


1A
1B

The six 'starter populations' and the two parental strains were assessed for glycerol, ethanol and acetic acid production following growth in YPD-10; YPD-10, which has 10% glucose, was used (rather than standard YPD which has 2% glucose) to ensure there was sufficient carbon available to generate levels of ethanol, glycerol and acetic acid that could be determined accurately. Frozen samples of the starter populations were inoculated into YPD and incubated overnight at 30°C/140 rpm (see Section 2.1.5.2). The overnight cultures were then inoculated into fresh YPD-10 to an OD_{600} of 0.1 and incubated at 30°C/140 rpm (see Section 2.1.5.4) until fermentation was complete, (i.e. no detectable sugar remained) as confirmed by CliniTest™ analysis (see Section 2.2.5). Samples of the medium were analyzed by HPLC for glycerol, ethanol and acetic acid, as described in Section 2.2.4. B1, B2 and B3 produced almost identical levels of glycerol, ethanol and acetic acid levels to the parent strain, BY4742. Similarly, starter populations A2 and A3 were no different to their parent strain, AWRI1628. However, A1 was different from the parent producing 0.52 g/l more ethanol, 0.44 g/l less glycerol and 0.19 g/l less acetic acid (see Figure 4.3).

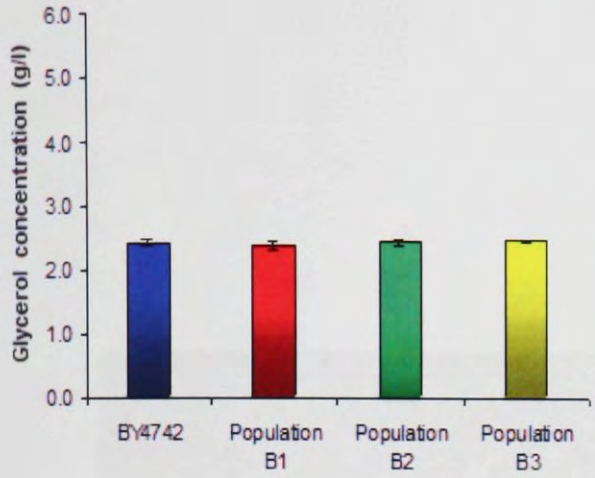
Transposon PCR was used, as described in Section 2.3.7.1, to confirm the identity of the 'starter populations'. DNA from each starter population and both original parent strains was used as template with specific Ty1 transposon primers. Products of PCR reactions were resolved by electrophoresis (Section 2.3.8). The characteristic banding patterns distinguished the two parent strains and confirmed the parentage of each starting populations (Figure 4.4); B1, B2 and B3 had the same transposon fingerprint as BY4742, and A1, A2 and A3 had the same transposon fingerprint as AWRI1628. Transposon banding patterns were also checked for evolving populations at 25 generation intervals during the course of adaptive evolution, (up to 300 generations), Ty1 PCR DNA fingerprints were used to confirm the identity of starter populations B1, B2, B3, and A1, A2, A3, as well as evolving populations (EB1, EB2, EB3) at 300 generations, and (EA1, EA2, EA3) at 100 generations, relative to the parental strains they were derived from. Ty1 PCR products were separated and visualized as described in Section 2.3.8 to confirm the identity of the evolving populations. Ty1 transposon patterns of evolved populations remained unchanged throughout the experiment (see Figure 4.4).

Figure 4.3 Glycerol, ethanol and acetic acid production for parental strains of *S. cerevisiae* BY4742 and AWRI1628 and 'starter' populations B1, B2, B3 and A1, A2, A3

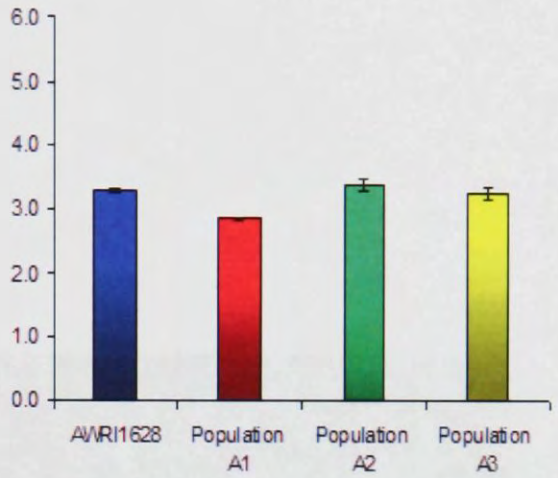
Adaptive evolution experiments were initiated by inoculation from starter populations of *S. cerevisiae* BY4742 (B1, B2 and B3) and AWRI1628 (A1, A2 and A3). The starter populations were tested for glycerol, ethanol and acetic acid production in YPD-10, shaking at 140 rpm/30°C. Fermentations were carried out in triplicate. Values are means ± SD.

Figures 4.3i, 4.3ii and 4.3iii show glycerol, ethanol and acetic acid yields respectively, for starting populations derived from *S. cerevisiae* BY4742. Figures 4.3iv, 4.3v and 4.3vi show glycerol, ethanol and acetic acid levels respectively, for starting populations derived from *S. cerevisiae* AWRI 1628. Blue bars represent original parental strains of both yeasts.

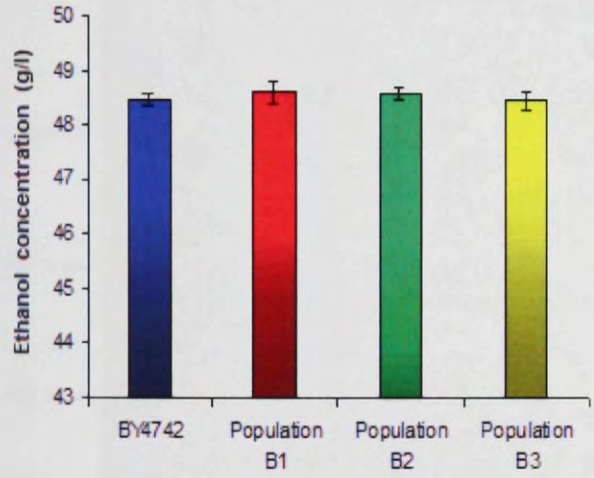
4.3i



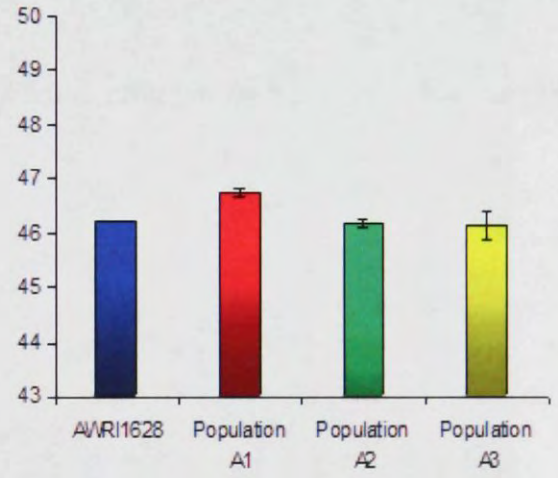
4.3iv



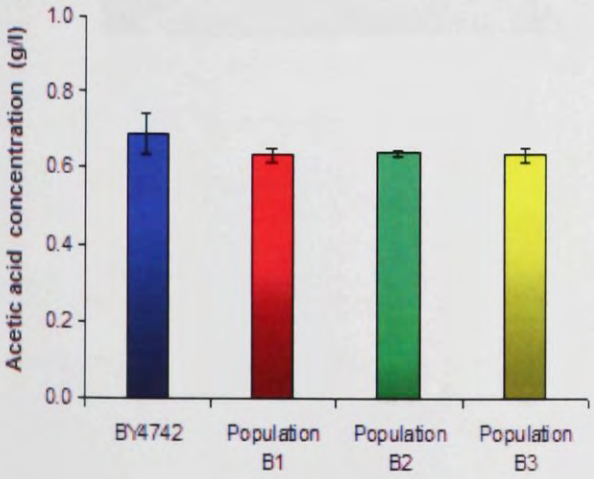
4.3ii



4.3v



4.3iii



4.3vi

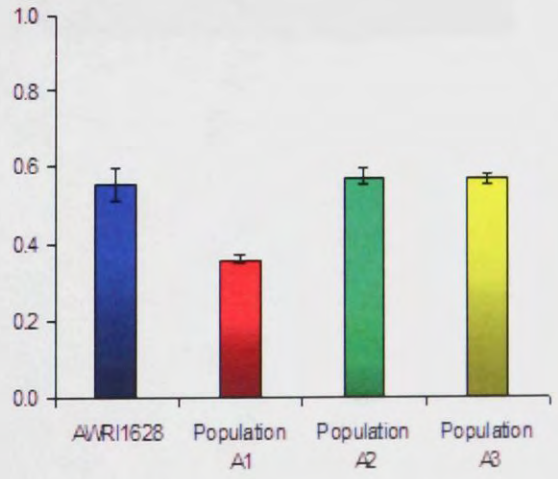
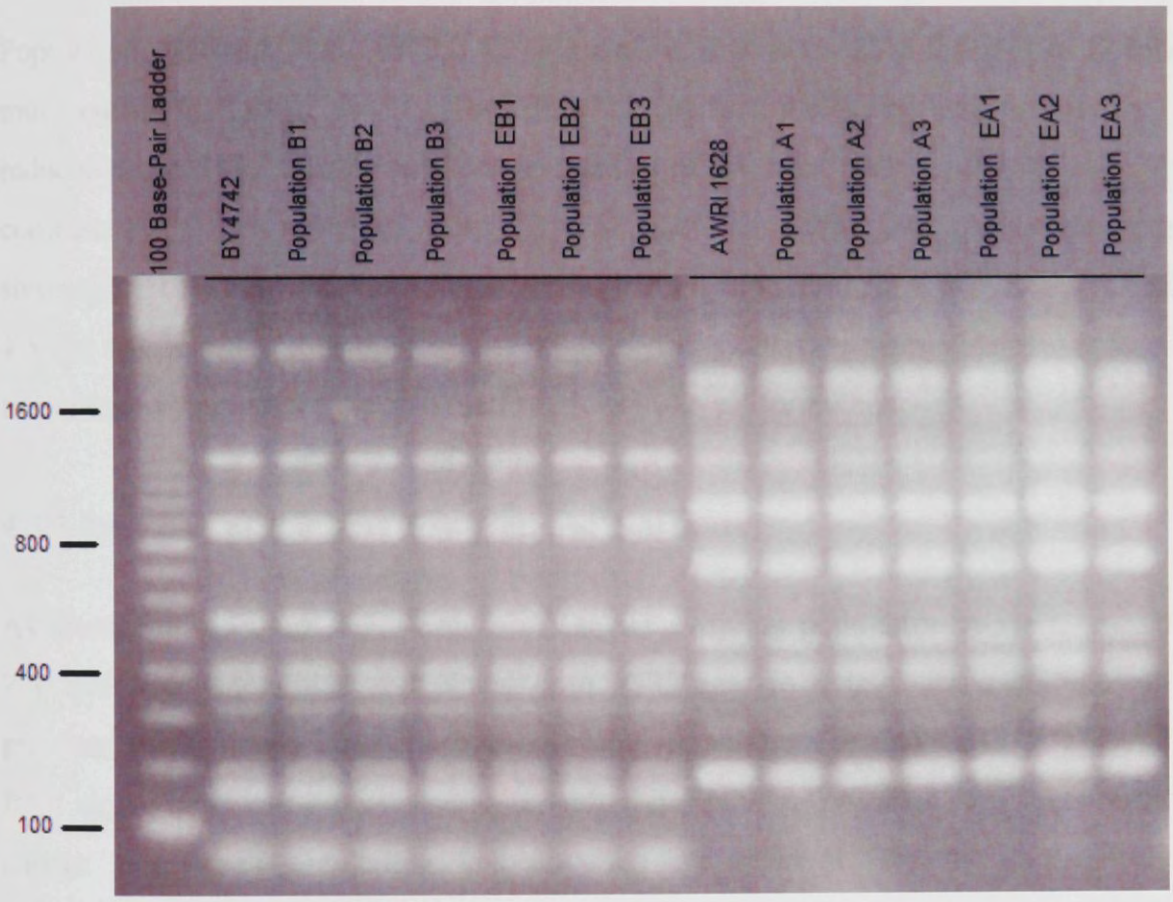


Figure 4.4 Ty1 transposon patterns for *S. cerevisiae* BY4742 and AWRI1628


100 Base-Pair Ladder
BY4742
Population B1
Population B2
Population B3
Population EB1
Population EB2
Population EB3
AWRI 1628
Population A1
Population A2
Population A3
Population EA1
Population EA2
Population EA3
1600
800
400
100

4.3.2 Assessment of phenotypes in evolving populations

Progression of adaptive evolution was followed by assessing samples of the evolving populations at 50, 100... 300 generations. Frozen samples of the populations were used to prepare inocula as described in Section 2.1.5.2. These inocula were then used to inoculate triplicate fermentations, which were incubated at (30°C/140 rpm), until glucose was fully utilized (see Section 2.1.5.4), at which time samples of medium were analyzed by HPLC for glycerol, ethanol and acetic acid production (see Section 2.2.4).

Populations derived from AWRI1628 responded with noticeable changes in phenotype much earlier than those derived from BY4742, producing elevated levels of glycerol and reduced ethanol yields after only 50 generations of selection (see Figures 4.5 and 4.6). In contrast, populations derived from BY4742 acquired measurable phenotypic changes somewhere between 100 and 150 generations. As can be seen from the graphs in Figures 4.5 and 4.6, in most cases the final metabolic redirection away from ethanol synthesis was associated with elevated glycerol and acetic acid levels for both strains.

4.3.3 Isolation of the best 'low-ethanol' variants

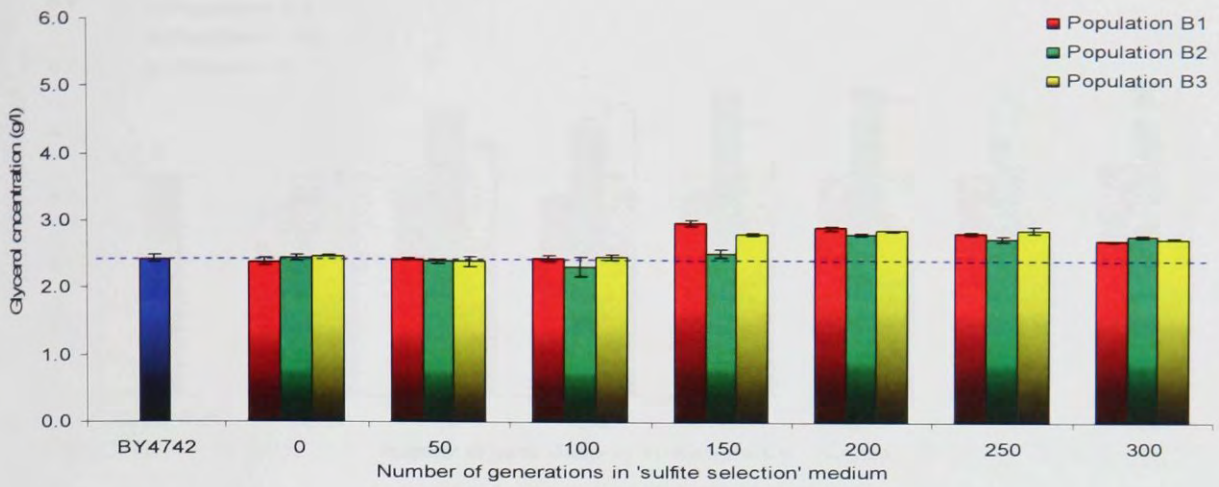
As illustrated at Figure 4.7, samples of the six evolving populations were taken at 100, 200 and 300 generations and plated onto YPD agar to obtain single colonies; as for previous work, single colonies from evolving populations of AWRI1628 were seeded from single cells using a micromanipulator. Five random colonies were then randomly chosen from each population and assessed for glycerol, ethanol and acetic acid production. Incubations were conducted in 15 ml of YPD-10 in 50 ml 'Falcon' tubes, at 30°C/140 rpm, until ferments were dry. Samples from dry fermentations were analyzed using HPLC (Section 2.2.4).

Only glycerol assays gave reproducible results in these screens and therefore data for ethanol and acetic acid concentration assays were not used. Difficulties with ethanol and acetic acid determinations were probably due to the volatility of these compounds, which

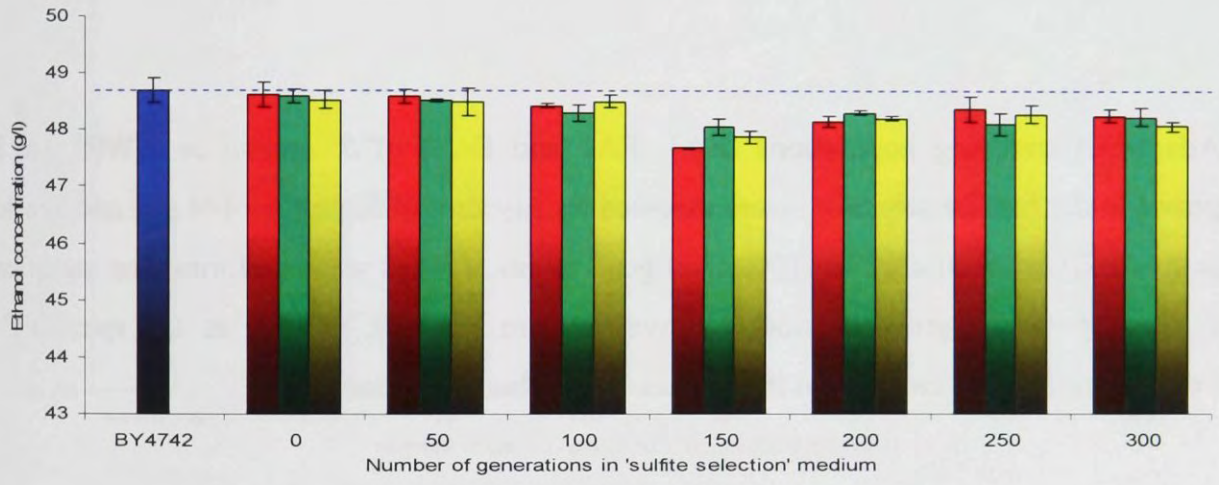
Figure 4.5 Glycerol, ethanol and acetic acid levels for populations EB1, EB2 and EB3 evolving under sulfite stress

Adaptively evolving populations (EB1, EB2 and EB3) of *S. cerevisiae* BY4742, grown under 'sulfite selection' were assessed for glycerol (4.5i), ethanol (4.5ii) and acetic acid (4.5iii) production at 50, 100... 300 generations in SSM. Assays were performed on samples of the populations grown in non-selective medium (YPD-10), shaking at 140 rpm/30°C. Fermentations were carried out in triplicate and values are means ± SD.

4.5i



4.5ii



4.5iii

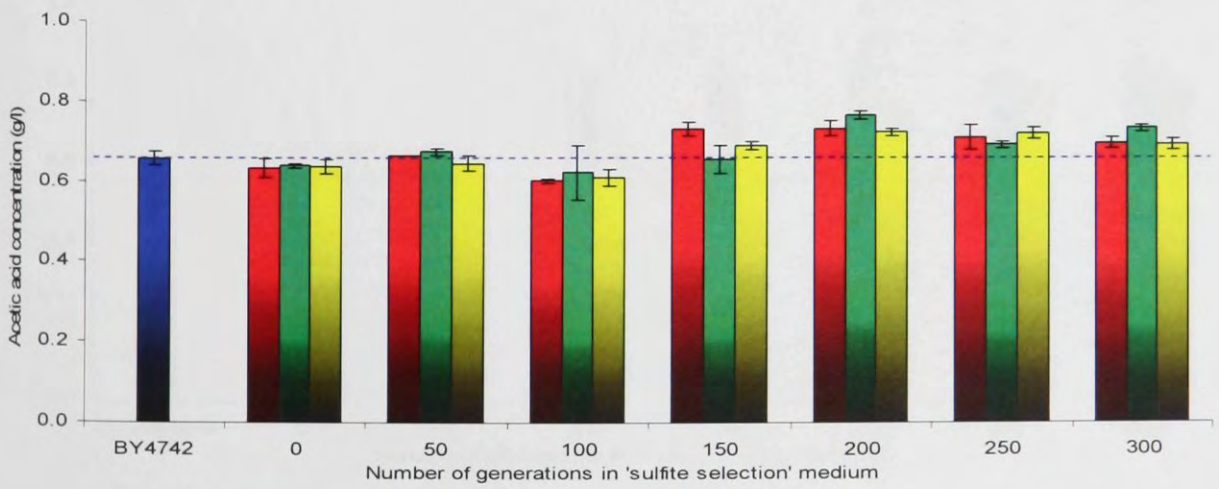
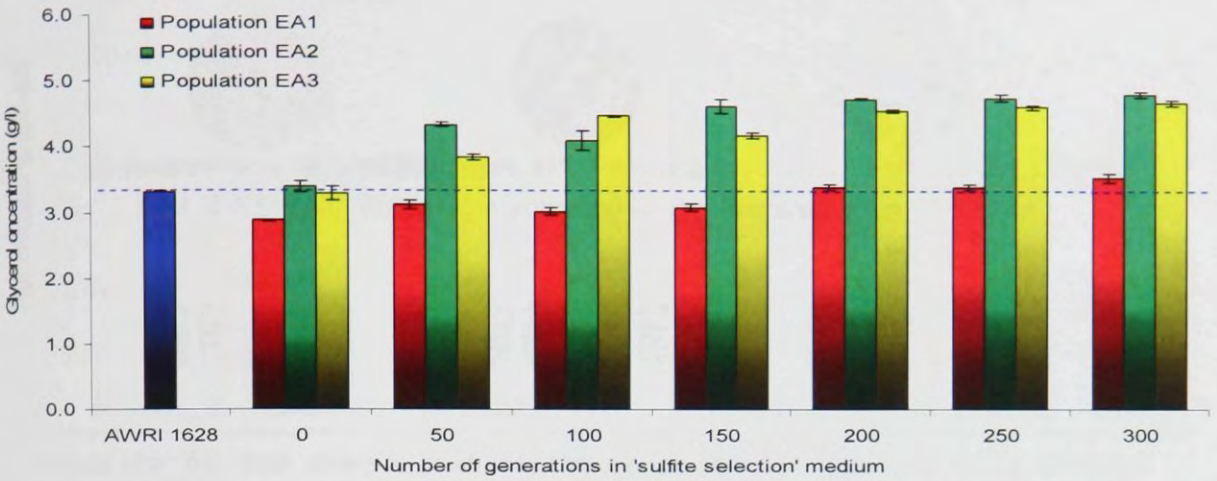


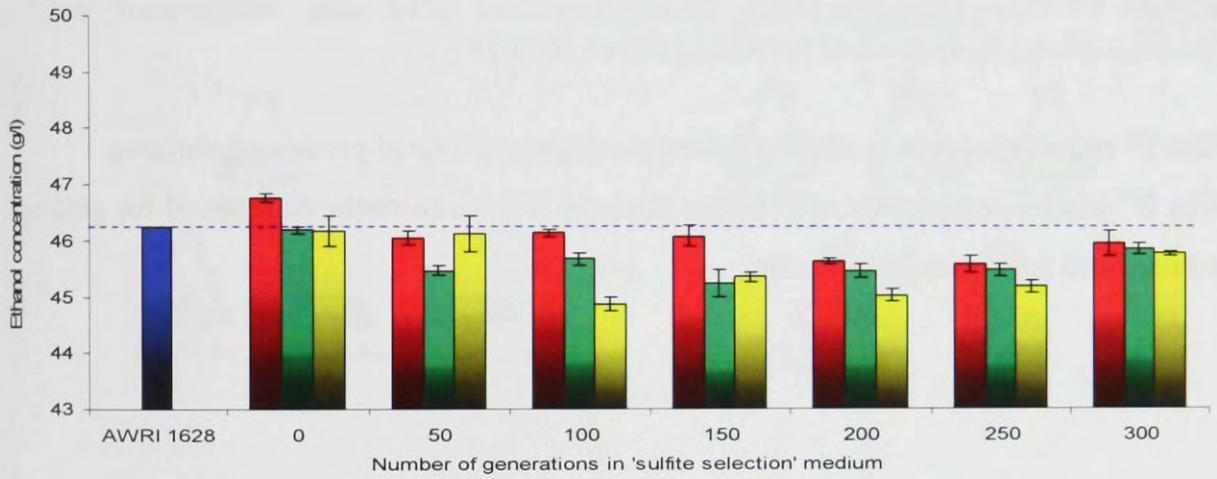
Figure 4.6 Glycerol, ethanol and acetic acid levels for populations EA1, EA2 and EA3 evolving under sulfite stress

Adaptively evolving populations (EA1, EA2 and EA3) of *S. cerevisiae* AWRI 1628 grown under 'sulfite selection' were assessed for glycerol (4.6i), ethanol (4.6ii) and acetic acid (4.6iii) production at 50, 100... 300 generations. Assays were performed on samples of the populations grown in non-selective medium YPD-10, shaking at 140 rpm/30°C. Fermentations were carried out in triplicate and values are means ± SD.

4.6i



4.6ii



4.6iii

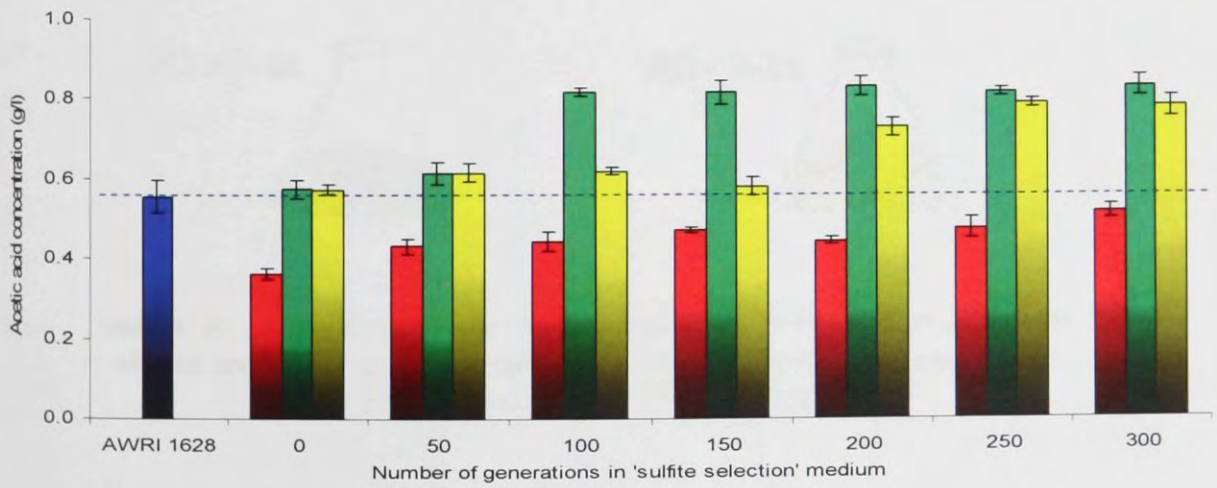
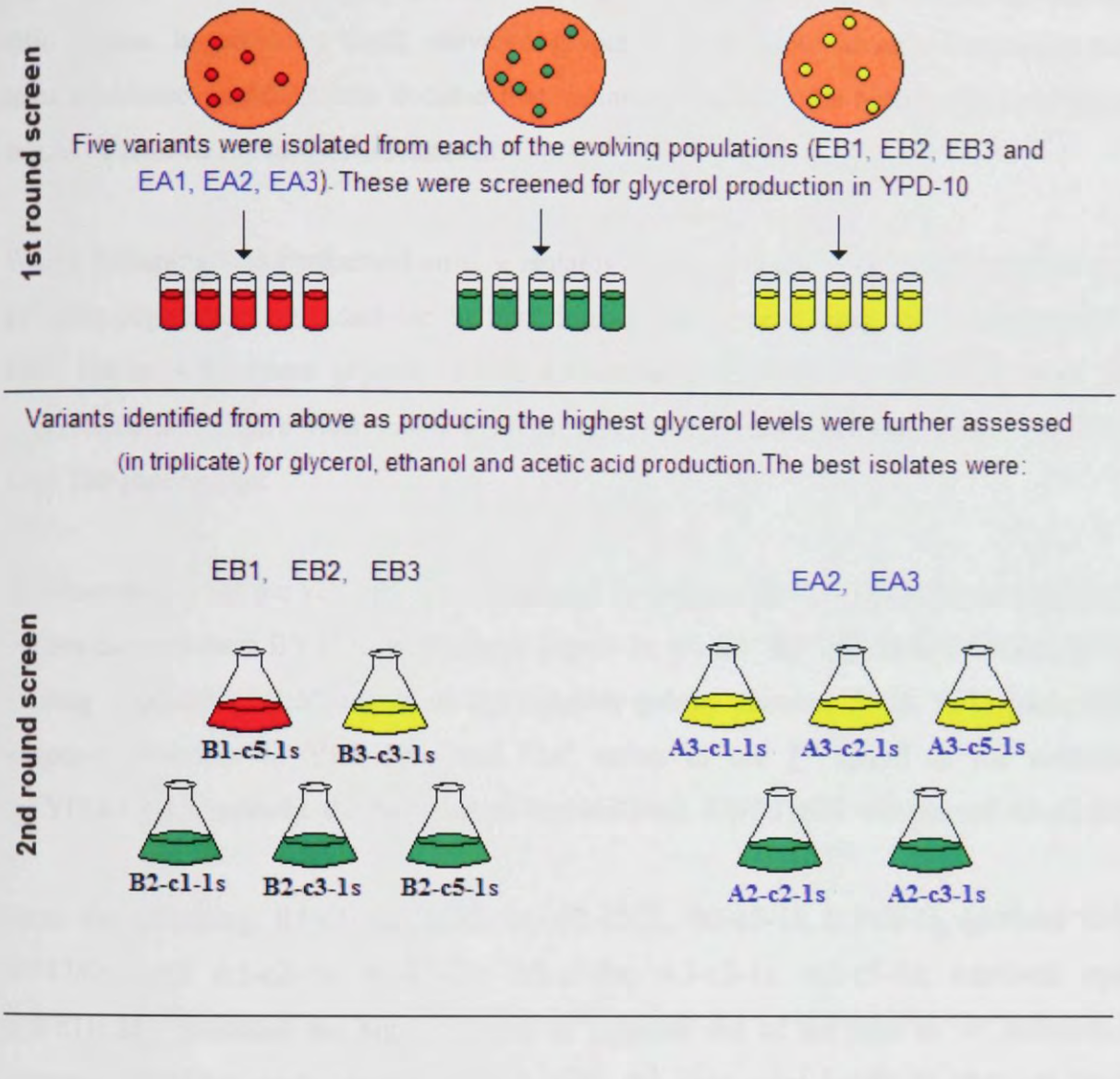


Figure 4.7 Flow chart illustrating the strategy used to identify 'low-ethanol' variants from evolving populations of BY4742 and AWRI1628

The **1st round screen** was used to isolate the highest glycerol-producing variants.

The **2nd round screen** was used to test the best isolates from the first round for glycerol, ethanol and acetic acid production.



Variant B2-c3-1s displayed the greatest redirection of metabolism away from ethanol production for mutants derived from BY4742. A3-c2-1s was the lowest ethanol-producing mutant derived from AWRI1628.

would have been especially problematic in these experiments because fermentations were conducted in small volumes of medium leading to a large [gas-liquid interface]:[volume] ratio. Since, in previous work, elevated glycerol levels were always associated with reduced ethanol yields, it was decided that variants producing the highest glycerol yields would be chosen for further assessment.

Whilst screening was performed on five isolates at 100, 200 and 300 generations for each evolving population, only data for the best 'high-glycerol' producing variants is presented here. Figure 4.8i shows glycerol levels for variants derived from BY4742 after 300 generations and Figure 4.8ii shows glycerol levels for variants derived from AWRI1628 after 100 generations.

A nomenclature for the variants was introduced as follows: the highest glycerol producing variant derived from BY4742 was named **B2-c3-1s**, where "**B2**" refers to the name of the starting population, "**c3**" refers to the random **c**olony number **3** on YPD plate after adaptive evolution in YPD-10S, and "**1s**" refers to the **1**st round of the selection (in YPD-10**S**). Similarly, the best variant derived from AWRI1628 was named **A3-c2-1s**.

From the screening, B1-c5-1a, B2-c1-1s, B2-c3-1s, B2-c5-1s, B3-c3-1s, (derived from BY4742), and A2-c2-1s, A2-c3-1s, A3-c1-1s, A3-c2-1s, A3-c5-1s, (derived from AWRI1628), produced the highest levels of glycerol out of the pool of 90 individuals screened (45 from each parental strain), with the maximum levels of glycerol being 3.9 g/l for B2-c3-1s and almost 5 g/l for A3-c2-1s[1] compared to BY4742 and AWRI1628, which produced 2.8 g/l and 2.9 g/l of glycerol respectively. The above variants were then subjected to further tests to determine glycerol, ethanol and acetic acid production.

4.3.4 Evaluation of phenotypes of high-glycerol producing variants

Isolates producing the highest glycerol levels, chosen from screenings performed at 100, 200 and 300 generations in YPD-10S, were tested in triplicate fermentations for glycerol,

[1] Although the 'best' variant from Figure 4.8 appears to be A2-c3-1s, this strain was later shown to have high petite frequency (see Section 4.3.6) and therefore was not used in subsequent work.

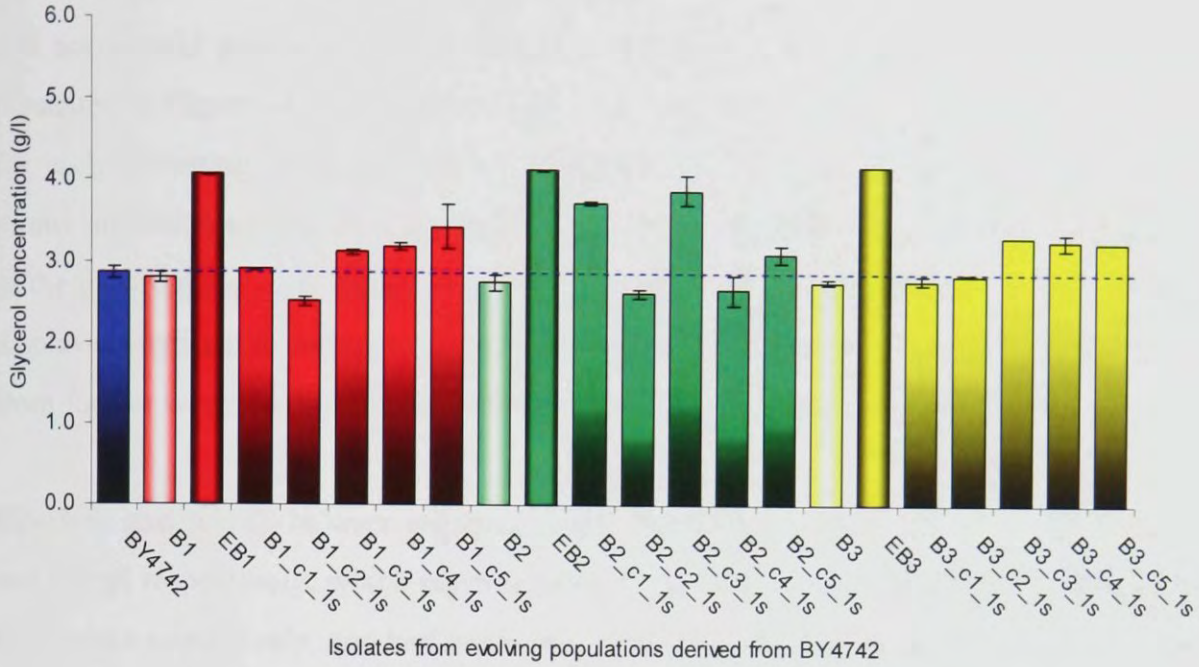
Figure 4.8 Individual isolates assessed for glycerol production after adaptive evolution under 'sulfite stress'

Evolving populations of *S. cerevisiae* BY4742 and *S. cerevisiae* AWRI1628 were sampled after 300 and 100 generations respectively and assessed for glycerol production. Assays were performed as described in Section 2.1.5.4. Fermentations were carried out in triplicate and values are means ± SD.

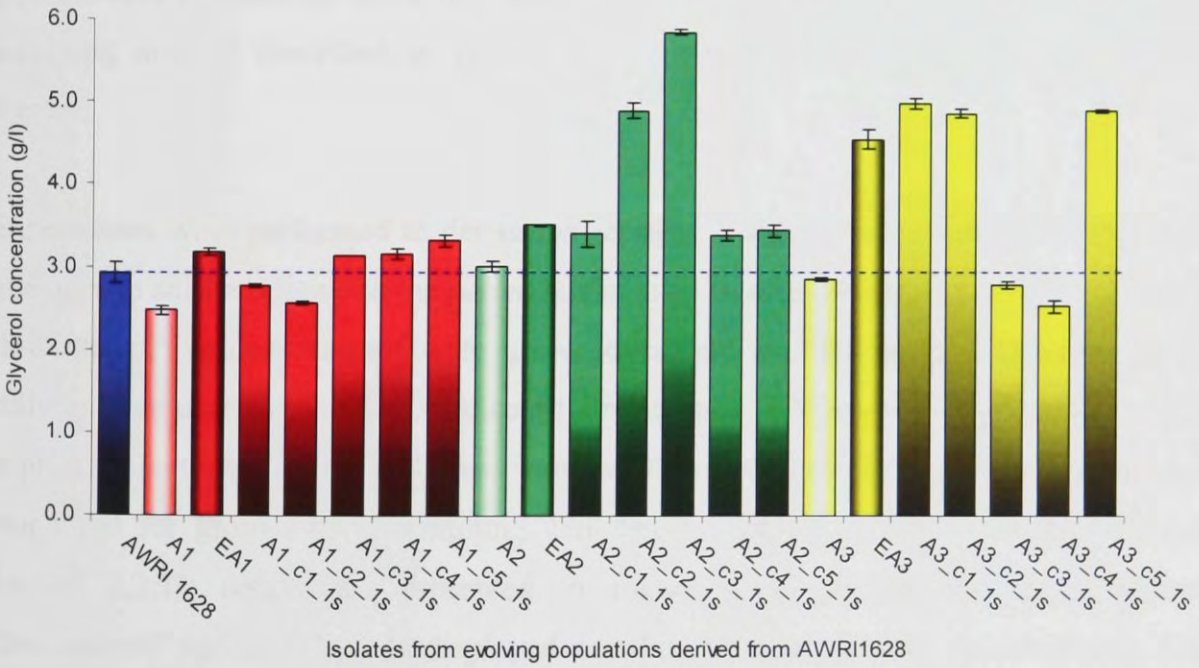
4.8i Glycerol levels for: *S. cerevisiae* BY4742 starter populations B1, B2 and B3; evolving populations EB1, EB2 and EB3 after 300 generations of 'sulfite selection'; and clonal variants from each population isolated from single colonies on YPD plates.

4.8ii Glycerol levels for: *S. cerevisiae* AWRI1628 starter populations; A1, A2 and A3; evolving populations EA1, EA2 and EA3 after 100 generations of 'sulfite selection'; and clonal variants from each population isolated as single colonies on YPD plates.

4.8i


Glycerol concentration (g/l)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
BY4742
B1
EB1
B1_c1_1s
B1_c2_1s
B1_c3_1s
B1_c4_1s
B1_c5_1s
B2
EB2
B2_c1_1s
B2_c2_1s
B2_c3_1s
B2_c4_1s
B2_c5_1s
B3
EB3
B3_c1_1s
B3_c2_1s
B3_c3_1s
B3_c4_1s
B3_c5_1s
Isolates from evolving populations derived from BY4742

4.8ii


Glycerol concentration (g/l)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
AWRI 1628
A1
EA1
A1_c1_1s
A1_c2_1s
A1_c3_1s
A1_c4_1s
A1_c5_1s
A2
EA2
A2_c1_1s
A2_c2_1s
A2_c3_1s
A2_c4_1s
A2_c5_1s
A3
EA3
A3_c1_1s
A3_c2_1s
A3_c3_1s
A3_c4_1s
A3_c5_1s
Isolates from evolving populations derived from AWRI1628

ethanol and acetic acid production. Inoculum cultures were prepared from frozen stocks of 'high-glycerol' producing variants as described in Section 2.1.5.2. These overnight cultures were used to inoculate triplicate fermentations, which were conducted as described in Section 2.1.5.4. Samples of spent media were analyzed for glycerol, ethanol and acetic acid production using HPLC as described in Section 2.2.4, and results are presented in Figures 4.9i, ii, iii and 4.10i, ii, iii. Of the total pool of 30 variants tested, (5 for each screening point per strain), B1-c5-1s and A2-c3-1s appeared to be the most promising isolates, producing the highest glycerol levels and the lowest ethanol yields out of the group of tested isolates (see Figures 4.9 and 4.10). However, both of these isolates displayed high petite frequencies (see Section 4.3.6, Figure 4.13), which excluded them from further work. In addition, B1-c5-1s produced increased acetic acid levels.

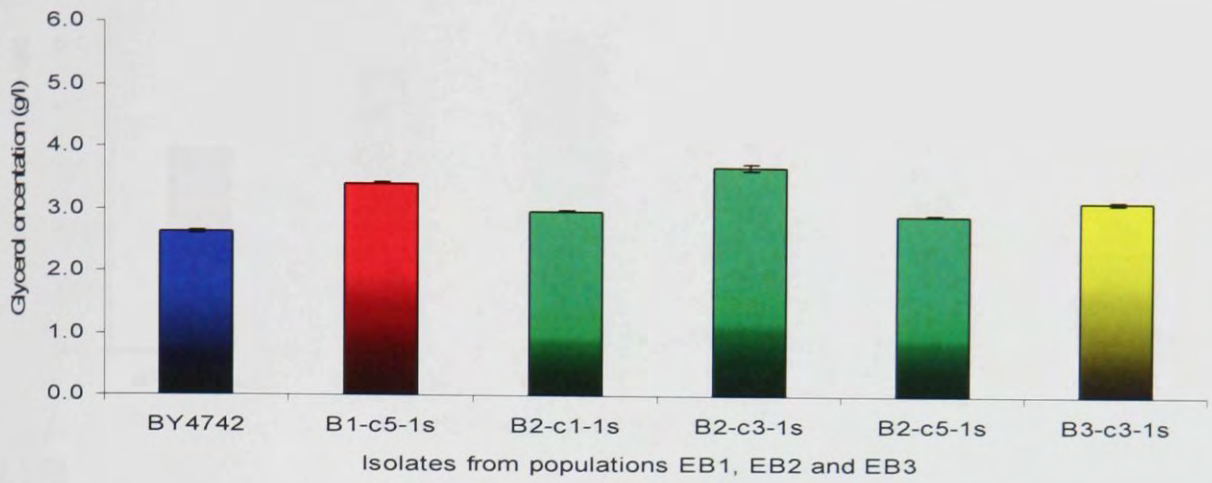
B2-c3-1s and A3-c2-1s were regarded 'best', producing high levels of glycerol: 3.7 g/l and 5.0 g/l respectively, which represented 1.07 g/l and 1.73 g/l increases in yields of this by-product respectively, and had moderate petite frequencies. Ethanol levels for these two isolates decreased by 1.2 g/l for B2-c3-s1 and 1.9 g/l for A3-c2-1s when compared to their parent strains. There was no significant elevation in acetic acid production. The glycerol levels measured using this approach were very close to those identified using screening method described in section 4.3.3, supporting the reliability of screening method.

Experiments were performed to determine whether 'low-ethanol' variants had increased tolerance to sulfite relative to the parent strains they were evolved from. Parent stains and 'low-ethanol' variants thereof were grown overnight in YPD at 30°C/140 rpm. These cultures were used to inoculate triplicate fermentations in YPD-10S (30g/l sodium sulfite at pH 8) to an OD_{600} of 0.1, and these were incubated at 30°C/140 rpm. The cultures were monitored for growth by determining cell density, OD_{600}, at 12 hours intervals (see Section 2.2.1); results are presented in Figure 4.11. During exponential growth 'low-ethanol' variant B2-c3-1s displayed doubling time reduced by approximately 69% relative to its parental strain. Variant A3-c2-1s also grew more rapidly than its parental strain, displaying an approximate 25% reduction in doubling time during exponential growth.

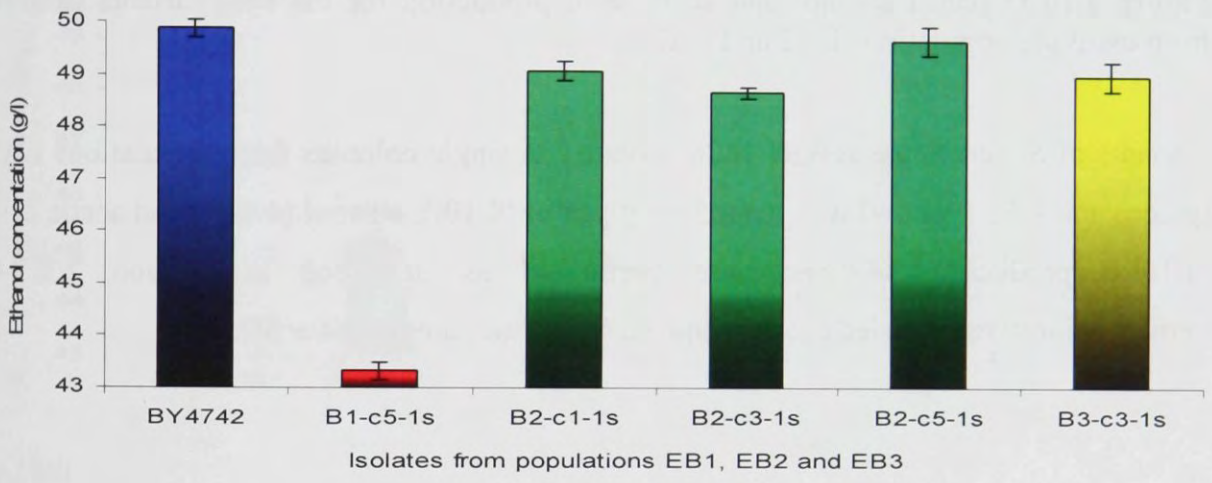
Figure 4.9 Glycerol ethanol and acetic acid production for the best variants isolated from evolving populations EB1, EB2 and EB3

Variants of *S. cerevisiae* BY4742, isolated as single colonies from populations EB1 (red), EB2 (green) and EB3 (red) were tested for glycerol (4.9i), ethanol (4.9ii) and acetic acid (4.9iii) production. Assays were performed as described in Section2.1.5.4. Fermentations were carried out in triplicate and values are means ± SD.

4.9i



4.9ii



4.9iii

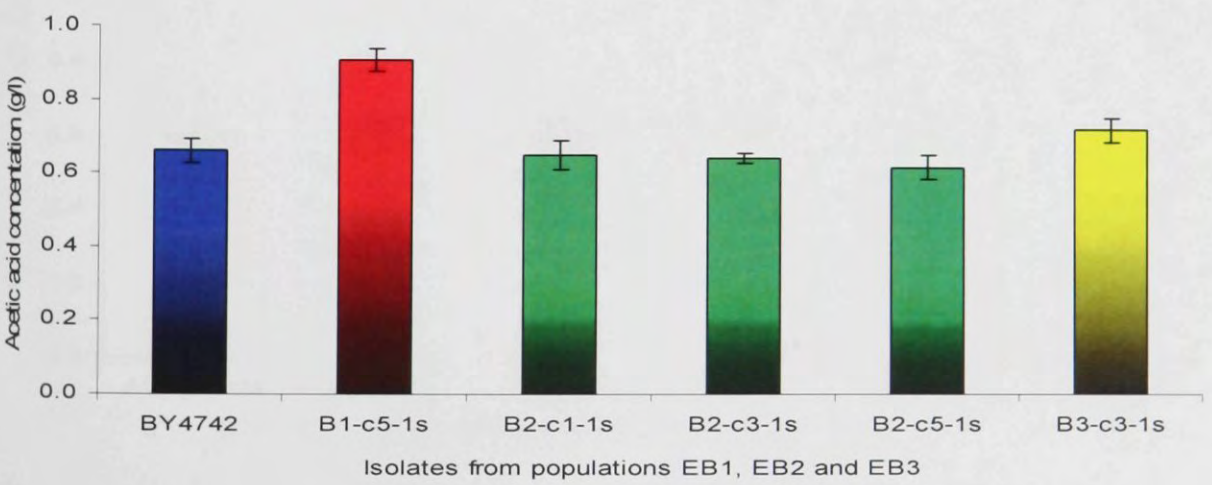
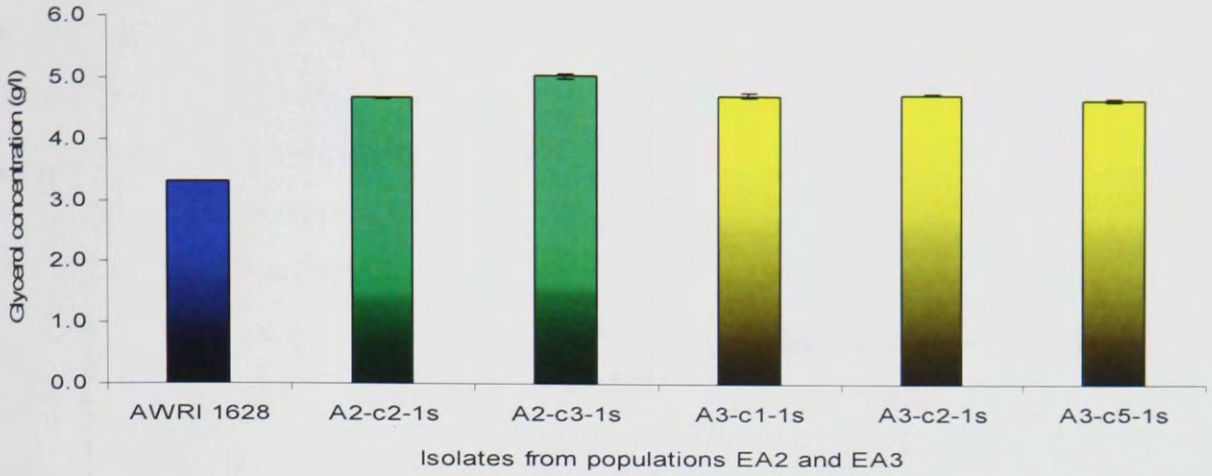


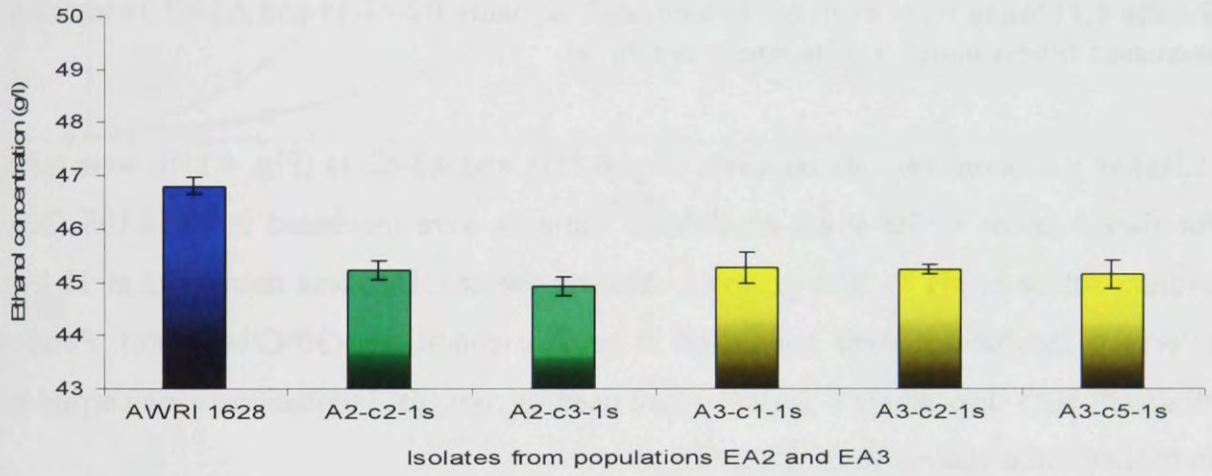
Figure 4.10 Glycerol ethanol and acetic acid production for the best variants isolated from evolving populations EA2 and EA3

Variants of *S. cerevisiae* AWRI 1628, isolated as single colonies from populations EA2 (green) and EA3 (yellow) were tested for glycerol (4.10i), ethanol (4.10ii) and acetic acid (4.10iii) production. Assays were performed as described in Section 2.1.5.4. Fermentations were carried out in triplicate and values are means ± SD.

4.10i



4.10ii



4.10iii

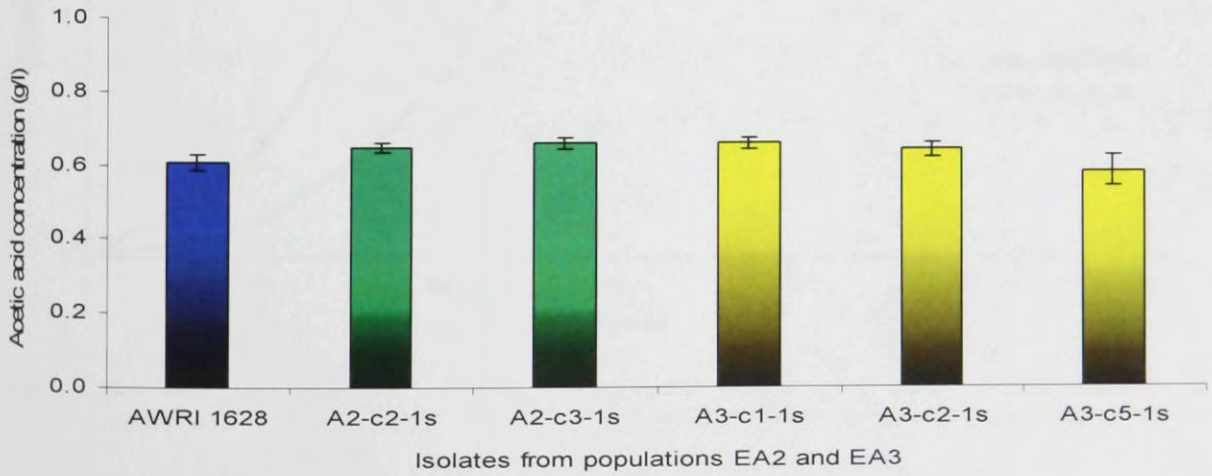
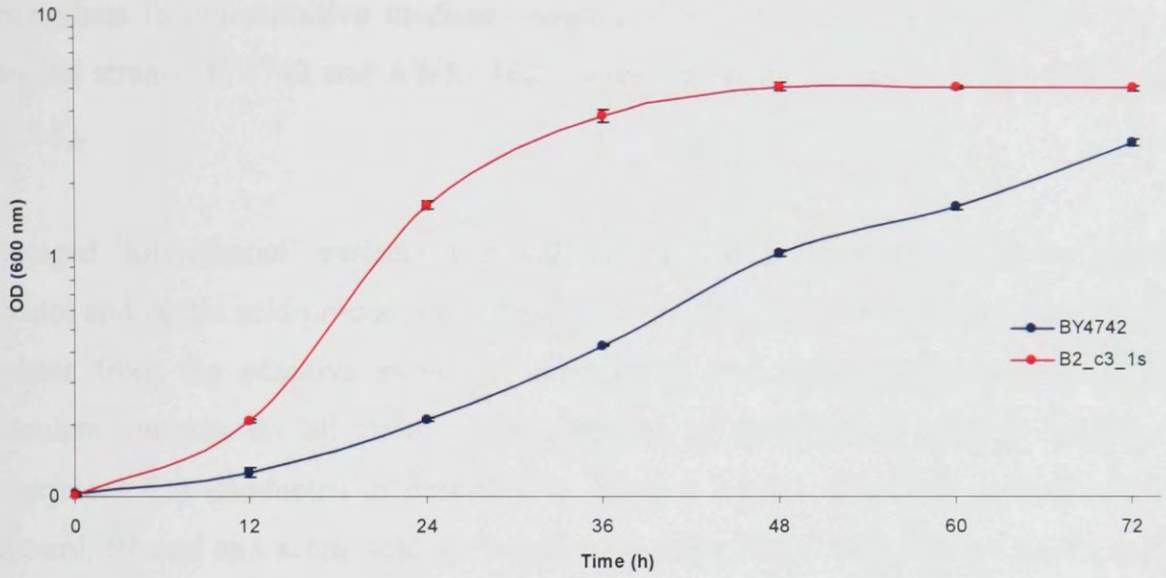


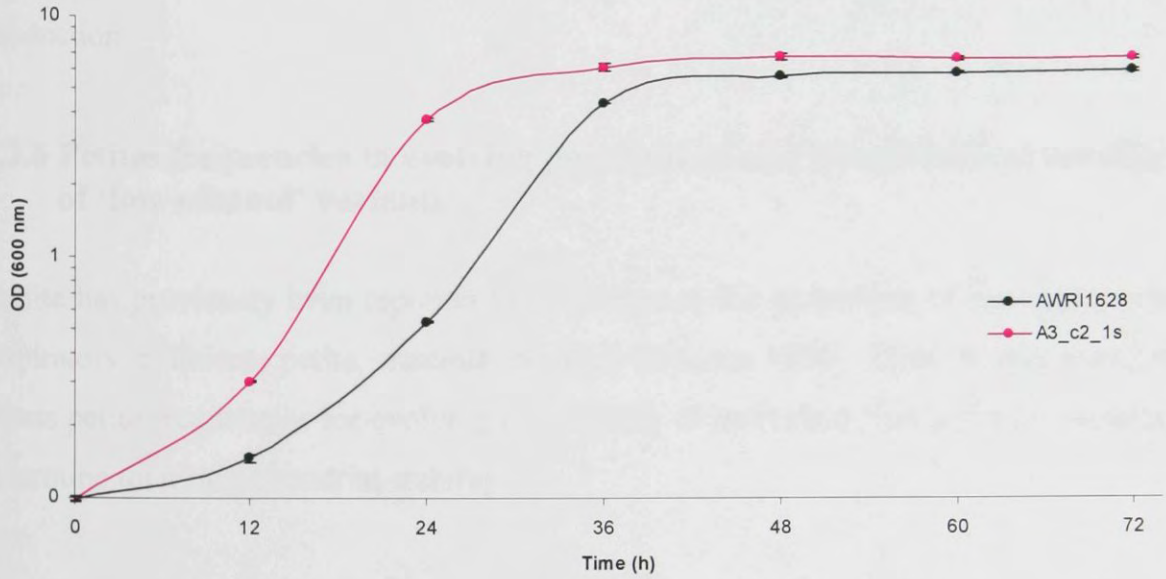
Figure 4.11 Adaptively evolved 'low-ethanol' variants B2-c3-1s and A3-c2-1s tested for increased fitness under 'sulfite stress' condition

Adaptively evolved variants B2-c3-1s (Fig. 4.11i), and A3-c2-1s (Fig. 4.11ii) were tested for fitness under sulfite stress conditions. Variants were incubated in YPD-10S (30g/l sodium sulfite at pH 8, 10% glucose). Absorbance at OD_{600} was monitored at 12 hour intervals. Incubations were conducted in aerobic conditions (30°C/140 rpm). Parental strains of both 'low-ethanol' variants were used as controls. Incubations were carried out in triplicate and values are means ± SD.

4.11i


10
1
0
OD (600 nm)
0
12
24
36
48
60
72
Time (h)
BY4742
B2_c3_1s

4.11ii


10
1
0
OD (600 nm)
0
12
24
36
48
60
72
Time (h)
AWRI1628
A3_c2_1s

4.3.5 Assessment of stability of the 'low-ethanol' mutants

To test the stability of 'low-ethanol' variants, the best variants and the original parent strains were cultured and sequentially transferred in non-selective medium for approximately 50 generations. The experiment was conducted in serial batch cultures, (YPD at 30°C/140 rpm), passaging approximately every 5 generations. After 50 generations in non-selective medium samples of variants B2-c3-1s and A3-c2-1s, and parental strains BY4742 and AWRI 1628, were frozen and stored at -80°C (see Section 2.1.5.1).

Passaged 'low-ethanol' variants and both parental strains were assessed for glycerol, ethanol and acetic acid production compared to the original, non-passaged 'low-ethanol' isolates from the adaptive evolution experiment, and non-passaged parental strains. Inoculum cultures for all strains were prepared as described in Section 2.1.5.2, and experiment was conducted as described in Section 2.1.5.4. The final concentrations of glycerol, ethanol and acetic acid were measured using HPLC (see Section 2.2.4), and are shown in Figure 4.12. Isolates and parental strains cultured in non-selective medium for 50 generations the 'ethanol-, glycerol-, and acetic acid-production' phenotypes were stable; there were no shifts in phenotype with respect to glycerol, ethanol and acetic acid production.

4.3.6 Petites frequencies in evolving populations and mitochondrial stability of 'low-ethanol' variants

Sulfite has previously been reported to contribute to the generation of elevated levels of respiratory deficient, petite, mutants in yeast (Schimz 1980). Thus, it was decided to assess petite frequencies for evolving populations of individual 'low-ethanol' variants, to determine their mitochondrial stability.

The 'Tetrazolium Overlay Technique', essentially as described by Ogur et al. (1957), was used for this purpose (see Section 2.2.7). The three evolving populations of each parental strain, and five of the best 'low-ethanol' variants chosen from these evolving populations

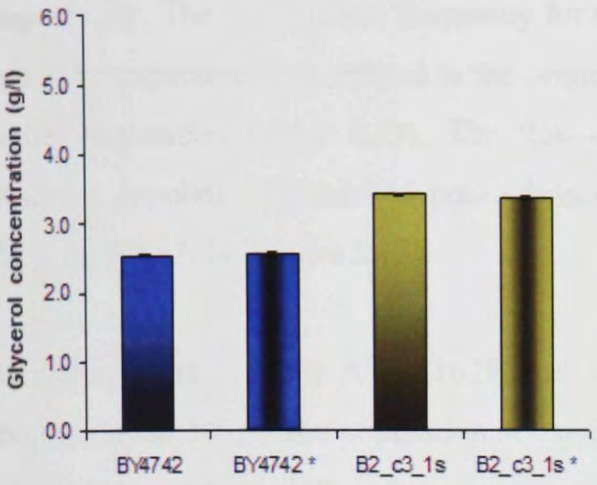
Figure 4.12 Stability test for adaptively evolved 'low-ethanol' variants of BY4742 and AWRI1628; B2-c3-1s and A3-c2-1s respectively

Parental strains and adaptively evolved variants of *S. cerevisiae* BY4742 and AWRI 1628 were grown in non-selective medium (YPD-10) for approximately 50 generations in order to assess stability. Strains were assessed for glycerol, ethanol and acetic acid production. Assays were performed as described in Section 2.1.5.4. Fermentations were carried out in triplicate and values are means ± SD.

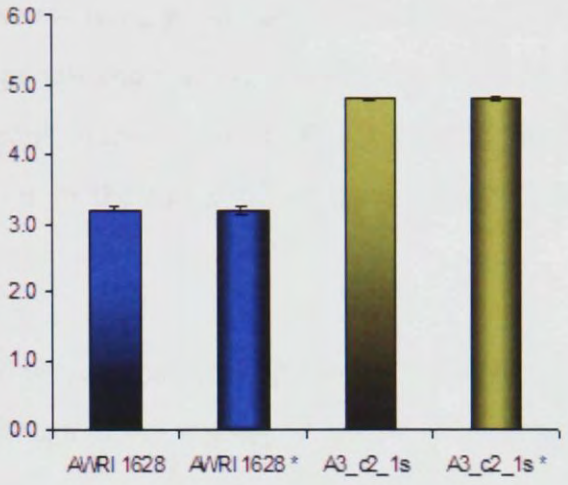
* indicates strains that have undergone 50 generations in non-selective medium.

Figures (4.12i, 4.12ii and 4.12iii) indicate glycerol, ethanol and acetic acid levels respectively for parental strain BY4742 and 'low-ethanol' variant B2-c3-1s. Figures 4.12iv, 4.12v and 4.12vi show glycerol, ethanol and acetic acid yields respectively for parental strain AWRI1628 and 'low-ethanol' variant A3-c2-1s.

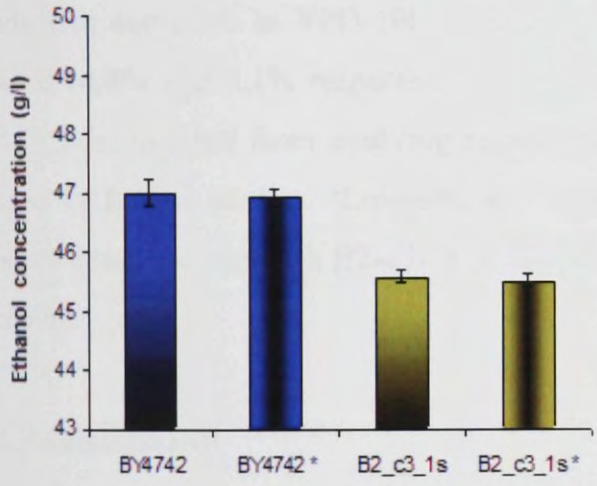
4.12i



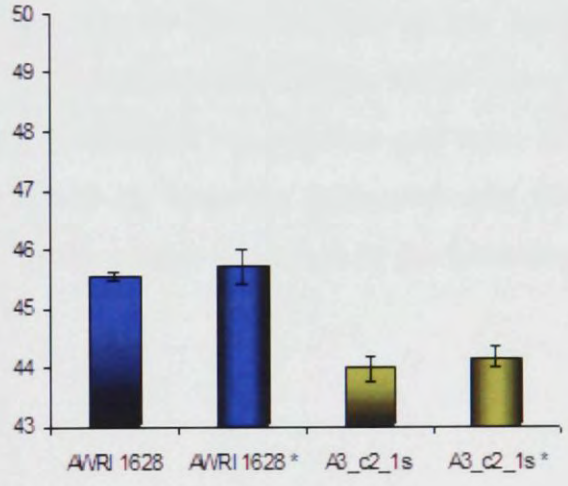
4.12iv



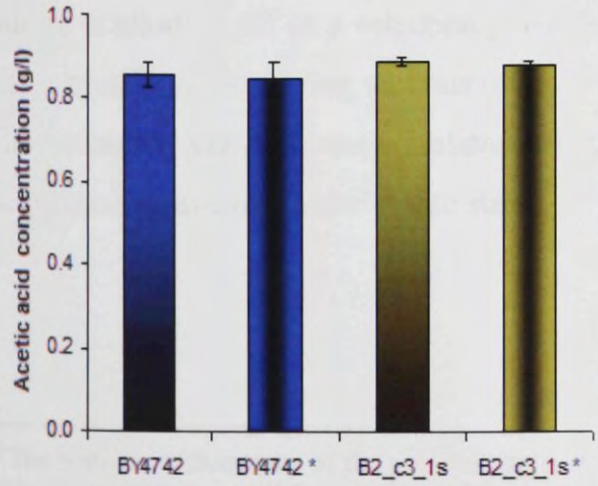
4.12ii



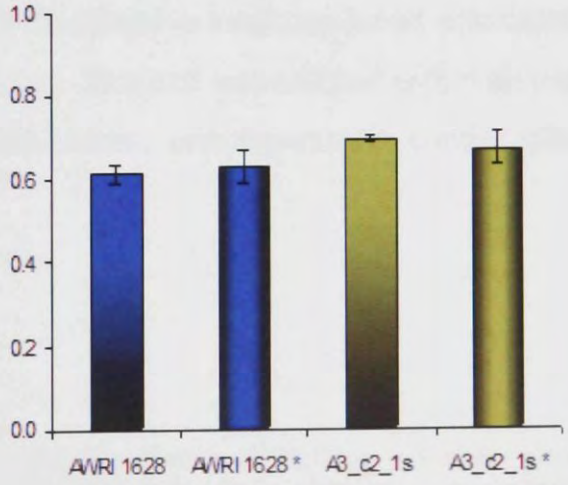
4.12v



4.12iii



4.12vi



(see Section 4.3.3), were assessed for petite frequencies. At 300 generations populations EB1, EB2 and EB3 increased their petite frequencies by 353%, 184% and 456% respectively. The final petites frequency for these evolving populations were 23%, 12.8% and 35% respectively, compared to the original parent and starting populations which had petite frequencies below 6.5%. The 'low-ethanol' variants of BY4742 selected from evolving populations exhibited petite frequencies in the range of 44% for B2-c5-1s to 7.5% for B2-c3-1s (Figure 5.13).

Surprisingly, the parent AWRI1628 and starting populations A2 and A3 had petite frequencies of 20%[2]. The population A1, unlike A2 and A3, displayed only 1.8%, petites, which increased to 11% after 100 generations in YPD-10S. Perhaps surprisingly, populations EA2 and EA3 displayed reduced petite frequencies at 100th generation of adaptive evolution in YPD-10S. The petite frequencies for EA2 and EA3 at that point were 14.8% and 9.1% respectively (Figure 4.13). 'Low-ethanol' variants A2-c3-1a and A3-c5-1s, isolated from evolving populations of AWRI1628 were petites and were not used in further studies. 'Low-ethanol' variant A3-c2-1s, however, generated only 8% petite colonies, and with B2-c3-1s, was chosen for the studies described in the following chapter.

4.3 Discussion

The primary aim of work presented in this chapter was to assess the effectiveness of sulfite at alkaline pH as a selection pressure for the adaptive evolution-based generation of 'low-ethanol' producing variants of *S. cerevisiae*. This aim was realized in that several 'low-ethanol' variants were isolated from both wine-, and laboratory strains, after sequential cultivation under sulfite stress.

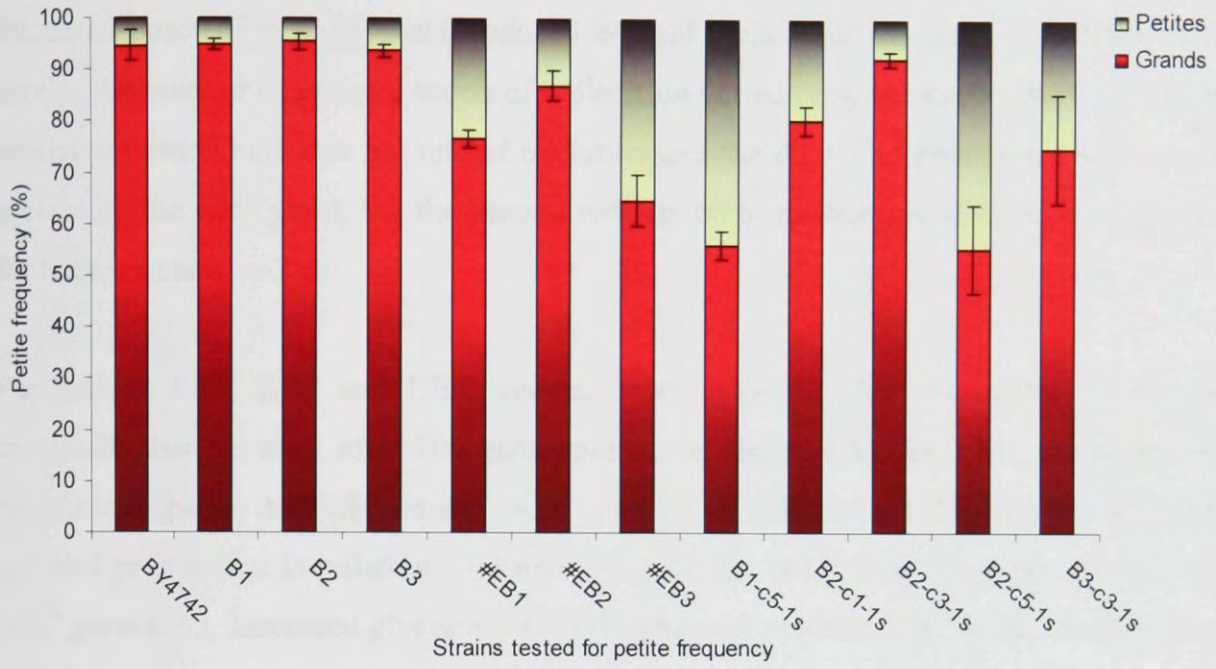
[2] The high petite frequency of the parental strain, AWRI1628, was discovered relatively late in this work, when isolates were tested for mitochondrial genome stability. With hindsight it might have been better to start with more stable parental strain. However, this observation does not negate the findings of this chapter.

Figure 4.13 Petite frequencies were determined (as described in 4.3.6) for adaptively evolved populations and 'low-ethanol' mutants of *S. cerevisaiae* BY4742 and AWRI1628. Values represent means of triplicates ± SD.

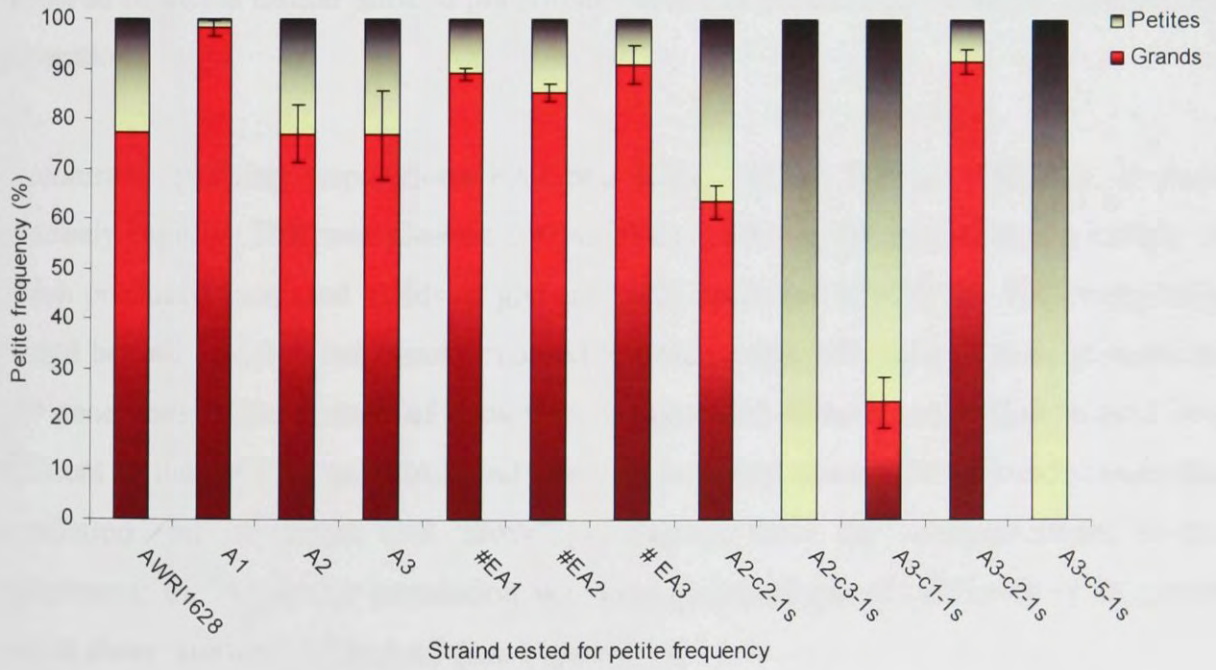
13i Petites frequency for parental strain of *S. cerevisiae* BY4742, starter populations B1, B2 and B3, evolving populations after 300 generations of 'sulfite selection' #EB1, #EB2 and #EB3, as well as variants selected from those populations.

13ii Petites frequency for parental strain of *S. cerevisiae* AWRI1628, starter populations A1, A2 and A3, evolving populations after 100 generations of 'sulfite selection' #EA1, #EA2 and #EA3, as well as variants selected from those populations.

4.13i


Petites
Grands
Petite frequency (%)
100
90
80
70
60
50
40
30
20
10
0
BY4742
B1
B2
B3
#EB1
#EB2
#EB3
B1-c5-1s
B2-c1-1s
B2-c3-1s
B2-c5-1s
B3-c3-1s
Strains tested for petite frequency

4.13ii


Petites
Grands
Petite frequency (%)
100
90
80
70
60
50
40
30
20
10
0
AWRI1628
A1
A2
A3
#EA1
#EA2
EA3
A2-c2-1s
A2-c3-1s
A3-c1-1s
A3-c2-1s
A3-c5-1s
Straind tested for petite frequency

The two strains of *S. cerevisiae* used for this work were chosen to test how differently two unrelated, divergent strains (laboratory, BY4742, and wine, AWRI1628) would evolve. Six experimental populations (three from each strain) were established, and in all cases, adaptive evolution under sulfite stress clearly drove metabolic changes towards increased production of glycerol, reduced ethanol yields and an increase in acetic acid levels. However, the rates and levels of redirection varied between strains. It is clear from results presented here that the rate of evolution and the degree of phenotypic shifts were greater for the wine yeast, but the general redirection of phenotypic changes were similar for both strains.

Populations EB1, EB2 and EB3, derived from BY4742, did not evolve significant metabolic changes until after 100 generations (see Figure 4.5). By 150 generations the populations began to differentiate, with samples of EB1 and EB3 showing elevated glycerol production. Population EB2 evolved a similar redirection of metabolism by the 200th generation. Increased glycerol was, in most cases, associated with decreased ethanol and elevated acetate production. At 300 generations, samples of all three EB populations displayed similar phenotypes, and these appeared to have reached equilibrium; i.e. there appeared to be no further shift in phenotypes of these populations between 200 and 300 generations.

In contrast, evolving populations EA2 and EA3, derived from AWRI1628, evolved relatively rapidly. This was clearest for population EA2 at 50 generations, a sample of which produced increased yields of glycerol with decreases in ethanol. EA3 marginally lagged behind this, but had clearly evolved to produce less ethanol and more glycerol by 100 generations. The pattern of evolution of decreased ethanol production in EA1 was different to that of EA2 and EA3, and this was probably due to differences between this population and its parent and 'sister' populations from the commencement of the experiment; the A1 starter population was clearly phenotypically different to its parent and its sister 'starters' A2 and A3 (see Figure 4.3).

As discussed in Section 1.3.3.3, glycerol and ethanol production are closely coupled to the generation of acetate. This is because production of these three metabolic

intermediates is associated with redox balance, specifically NAD^+/NADH; glycerol and ethanol production both lead to the oxidation of NADH, and acetate production leads to reduction of NAD^+. Generally, in *S. cerevisiae* an overproduction of glycerol typically leads to increased acetate production as a means of compensating for the overproduction of NAD^+ (Remize et al. 1997, Michnick et al. 1996). However, as discussed in Section 1.3.5.3, sulfite sequesters acetaldehyde, which is a major substrate for acetic acid production, therefore this selection agent was expected to minimize the risk of increased acetic acid yields; i.e. it was expected, that sulfite stress, whilst promoting glycerol production, would not lead large increases in acetic acid production. Results from Section 3.3.3 suggest that this is possible, at least for BY4742.

Unfortunately, in the experiments described in this chapter, acetic acid yields generally increased with increasing glycerol and decreasing ethanol production for all six evolving populations (see Figure 4.5, 4.6). However, the increase in acetic acid yields for population EA3 were not as tightly linked to increased glycerol production as was the case for the other strains at the early stages of evolution; there was only a marginal increase in acetic acid production relative to the other populations up to 150 generations in sulfite (see Figure 4.6). This observation suggests that during evolution in sulfite some phenotypes may evolve that would not tightly couple glycerol overproduction with increased acetate yields. This is supported by the observation that 'low-ethanol' variant A3-c2-1s isolated from population EA3 at 100 generations of evolution produced elevated glycerol, lowered ethanol and unchanged acetic acid yields (see Figure 4.10).

Evolving populations EB1, EB2, EB3, EA2 and EA3 appeared to reach 'equilibrium' by 300 generations. However, the degree of metabolic redirection differed between BY4742- and AWRI1628-derived populations. The question arises: why did different strains evolve to produce different amounts of glycerol, ethanol and acetate? Bearing in mind that the two parental strains used in this study were quite different, (AWRI1628 is a wine-derived strain and BY4742 is a laboratory strain), it is likely that the different genetic backgrounds of these strains impose different constrains on what is possible.

Triplicate founding populations were derived from starter cultures inoculated from single colonies derived from the same 'parent' stock. Therefore one would predict that founding populations should display very similar, if not identical, phenotypes to their parents and 'sibling' populations. This was the case for all 'sibling' founding populations derived from BY4742, (EB1, EB2, EB3), and two of the populations from AWRI1628, (EA2, EA3). Thus, except for EA1[3], evolving 'sibling' populations should presumably have started the evolution under sulfite stress with very similar genetic material for evolution to work on, and therefore might have been predicted to evolve similarly over time in identical conditions. However, the evolutionary paths differed between 'sibling' populations, and this is apparent in the metabolic differences for populations EB1, EB2 and EB3 at 150 generations, and for populations EA2 and EA3 at 50 - 150 generations (see Figures 4.5 and 4.6). This leads to the question: what might have contributed to different metabolic patterns that the evolving populations displayed at early and intermediate stages of evolution?

Sulfite has at least two known toxic effects on yeast cells: it is mutagenic (Schimz 1980), and it inhibits the activity of several enzymes including glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase (Hinze and Holzer 1985 and 1986, Maier et al 1985). It also binds acetaldehyde, which critically influences glycolytic and fermentative carbon flux, impairing energy and biomass production. To counter the deleterious effects of sulfite *S. cerevisiae* has sulfite transporters in its plasma membrane (encoded by *SSU1*) that secrete sulfite from the cell, thus lowering its cellular concentration (Xu et al. 1994, Park and Bakalinsky 2000). Therefore, in an evolutionary context, there are several potential targets for adaptations that might influence the fitness of yeast cells under sulfite stress. One may reasonably speculate that, for instance, mutations leading to increased activity of sulfite transporters, may yield variants that would be fitter to cope with sulfite stress conditions, or, redirection of metabolic carbon fluxes that compensate for NADH accumulation when ethanol production is reduced, may

[3]EA1 is not compared to its parent and 'sibling' populations in the above because, whilst retaining the same genetic fingerprint, it was clearly phenotypically different from the outset, presumably due to a mutation(s) that did not impact on the DNA fingerprint

lead to similar improvement of fitness. Thus, different evolving populations may carry individuals that have gained different mutation(s) with similar consequences in fitness, but resulting in different metabolic profiles for the populations at different points during the course of evolution. Interestingly, all evolving 'sibling' populations, apart from EA1, reached very similar 'end points' at 300 generations.

Another possible explanation for the observed differences between evolving 'sibling' populations is sampling error. Samples of evolving populations were taken at 50 generation intervals and stored frozen (see Section 2.1.5.1). Small samples of these, (1 bead from cryo-vials), were then inoculated into fresh YPD medium and grown overnight in YPD (see Section 2.1.5.2). If the evolving populations had a high level of genetic variation at the early/mid stages of evolution, it is possible that samples used for the initiation of these experiment might not have been representative; i.e. they might not have carried an adequate amount of cells to reflect a total genetic diversity of the evolving populations. To test this, additional experimental work would be required that includes multiple replicates of each population.

One criticism that might be leveled at the strategy used for the work described in this chapter is that each of the evolving 'sibling' populations was seeded from a separate single colony. Clearly this would have minimized genetic variation as each population was effectively clonal at the commencement of the experiment. This might seem an odd approach considering that natural selection (i.e. adaptive evolution) requires genetic variation for the generation of novel adaptations; the greater amount of genetic variation available, the more rapidly evolution will proceed (see, for example, Chambers at al. 2007). However, the work described in this chapter aimed to assess the way that populations evolve long-term under sulfite stress, and, in this context, one of the important questions to be addressed was: will this strategy always deliver the same outcome for a particular genotype. The approach used here lowered variability across replicate populations, allowing this question to be addressed.

In summary, 'low-ethanol' variants were isolated and displayed a clear redirection of carbon flux towards elevated glycerol production at the expense of ethanol. The changes

in ethanol production, however, were small. From a medium containing 10% (w/v) glucose, 'low-ethanol' variants, B2-c3-1s and A3-c2-1s, produced 0.15% (v/v) and 0.19% (v/v) less ethanol than their respective parental strains. A decrease in ethanol yield of this magnitude would not be considered adequate by the wine industry, and therefore further improvements of the 'low-ethanol' phenotype are required; the aim of the work described in the next chapter was to generate variants that display greater reductions in ethanol yield during fermentation.

4.4 Conclusions

- It was demonstrated that selection of *S. cerevisiae* yeast strains under sulfite stress drives adaptation of the evolving populations towards enhanced glycerol production, which, in the majority of cases, was associated with lowered ethanol production.
- Populations evolved under sulfite stress exhibited enhanced acetic acid production associated with increased glycerol and lowered ethanol yields. However, some individual isolates with elevated glycerol and lowered ethanol yields, had unchanged acetic acid production.
- The rate of evolution taken to generate the 'low-ethanol' phenotype was strain dependent.
- 'Low-ethanol' variants were stable; variants grown for 50 generations in non-selective medium retained their 'low-ethanol' phenotype.
- Variants B2-c3-1s and A3-c2-1s were assessed as suitable for future work. These variants showed enhanced glycerol production connected with lowered ethanol and not significantly changed acetic acid yields. These variants also had low frequencies of petite formation.

CHAPTER 5

FURTHER ADAPTIVE EVOLUTION OF 'LOW-ETHANOL' VARIANTS

5.1 Aim

The aim of the work described in this chapter was to further improve the 'low-ethanol' phenotypes of previously isolated variants B2-c3-1s and A3-c2-1s; that is, to generate variants of B2-c3-1s and A3-c2-1s that display even greater reductions in ethanol yield compared to original parent strains they were derived from. Two different sets of conditions were trialed to achieve this goal:

- increasing the selection pressure (i.e. the sulfite concentration) relative to that used for work described in Chapter 4
- imposing a different, glycerol-inducing, selection pressure to that which was used for work described in Chapter 4.

5.2 Introduction

Natural selection leads to the evolution of novel adaptations in response to new selection pressures. Once adapted, however, and if no additional changes occur in the environment, natural selection is essentially a conservative force, stabilizing and maintaining 'well suited' genotypes. This is very important in the context of applying adaptive evolution strategies in the laboratory to generate microorganisms with desirable traits: if a desired phenotype has reached equilibrium, it is unlikely that it will shift further, unless the selection pressure is increased or an alternative selective agent, favoring the same trait, is used.

It was shown in Chapter 3 that, for both AWRI1628 and BY4742, the greater the sulfite concentrations used in the medium, the greater the redirection of metabolism away from ethanol production. It is also the case that increasing sulfite concentration is associated with increasing toxicity. Thus, introducing higher sulfite concentration for the second

round of adaptive evolution of the best isolates from Chapter 4 should lead to the evolution of variants displaying a greater redirection of metabolism away from ethanol production.

An alternative selection pressure to drive increased glycerol production and, thereby, potentially reduce ethanol yields is osmotic stress. It is well known that osmotic stress induces yeast cells to produce increased levels of glycerol, which acts as a compatible solute (Nevoigt and Stahl 1997, Petrovska et al. 1999). The effect of metabolic redirection of carbon towards glycerol production in response to osmotic stress should lead to decreases in ethanol production, and such an effect has been previously reported (Petrovska at al. 1999). Thus, introduction of osmotic stress as a selection pressure in adaptive evolution experiments may lead to the generation of 'low-ethanol' variants. For work described in this chapter, potassium chloride (KCl) was chosen as an osmotic stressing agent because of its relatively low toxicity on yeast (Murguia et al. 1996, Mulet et al. 1999).

5.3 Results

5.3.1 Determination of the sulfite concentrations for use in the second round of adaptive evolution of 'low-ethanol' yeast variants

In choosing a sulfite concentration that would further push the evolution of 'low-ethanol' phenotypes that were generated previously, it was decided to aim for a concentration that inhibited growth to a similar extent as was achieved using 30 g/l on the parental strain (as described in previous chapter). A preliminary screening experiment was conducted in which 'low-ethanol' variants B2-c3-1s and A3-c2-1s were exposed to a range of sulfite concentrations, with the aim of finding a concentration that, after 48 hours growth, delivered a similar culture density to that of the original parental strains grown in 30 g/l sulfite. From these experiments it was found that sulfite concentrations of approximately 38 g/l and 40 g/l provide suitable growth inhibition for B2-c3-1s and A3-c2-1s respectively (data not shown). This finding was confirmed in microplate experiments conducted in triplicate as described in Section 2.1.5.6. Data from the confirmation experiments underlying the clear inhibitory effect of increased sulfite concentrations on

both B2-c3-1s and A3-c2-1s is shown in Figure 5.1. It is interesting to note that the growth profile of BY4742 in 30 g/l of sulfite differed from the four growth profiles of B2-c3-1s exposed to a range sulfite concentrations (30, 36, 38 and 40 g/l). In fact, in several independent experiments BY4742 had a similar growth profile in the presence of 30 g/l of sulfite (see Figures 3.3i and 4.11i). It was therefore not possible to identify a concentration of sulfite that inhibited growth of B2-c3-1s to the same extant as BY4742 exposed to 30 g/l of sulfite. Nevertheless, sulfite concentrations of 38 g/l and 40 g/l inhibited growth of B2-c3-1s and A3-c2-1s respectively so that after approximately 48 hours they reached similar culture densities to their parental strains exposed to 30 g/l of sulfite. The above concentrations were used for the purposes of the second round of adaptive evolution.

5.3.2 Determination of potassium chloride (KCl) concentration for use in the adaptive evolution of 'low-ethanol' variants

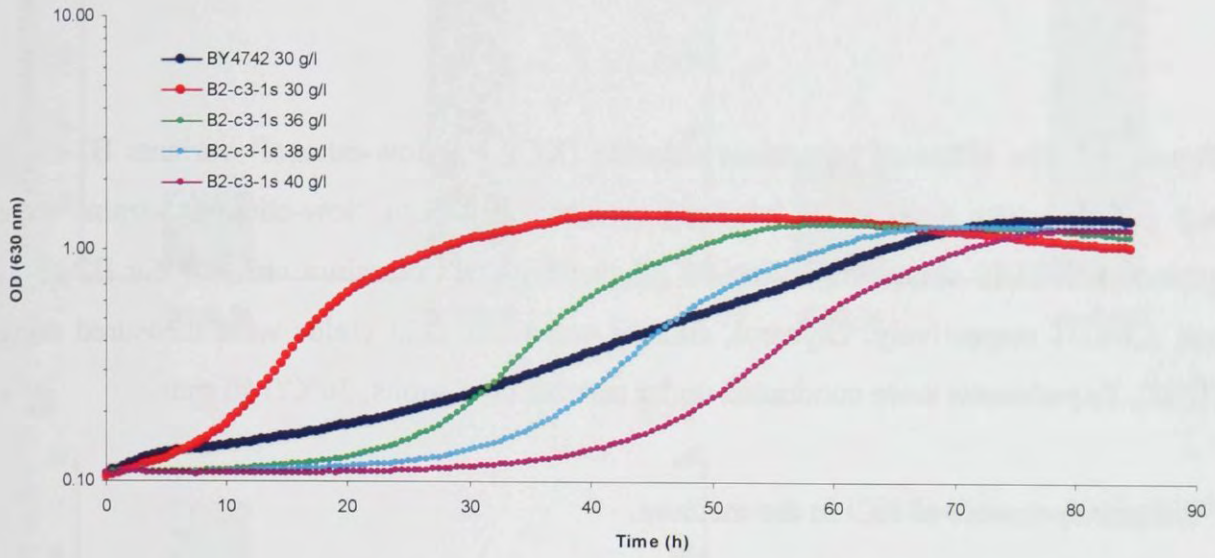
Along with adaptive evolution under increased sulfite concentrations, B2-c3-1s and A3-c2-1s were also exposed to selection under osmotic stress, using KCl as the stressing agent. A range of KCl concentrations was trialed to find a level that would increase glycerol and decrease ethanol production, but not completely prevent growth. This was performed in YPD-10, pH 4.5, supplemented with KCl. From preliminary screening experiments the KCl concentrations that were most suitable for adaptive evolution experiments were approximately 90 g/l and 95 g/l for B2-c3-1s and A3-c2-1s respectively; these concentrations led to substantial and similar decreases in ethanol production, and reduced growth rate (data not shown). To confirm this, triplicate B2-c3-1s and A3-c2-1s fermentations were conducted in YPD-10K medium at pH 4.5, supplemented with 90 g/l and 95 g/l KCl for B2-c3-1s and A3-c2-1s respectively. Assays for fermentation products were performed as described in Section 4.3.1.

Figure 5.2 and Table 5.1 show glycerol, ethanol and acetic acid yields for the B2-c3-1s and A3-c2-1s, measured using HPLC as described in (Section 2.2.4). All fermentations had gone to completion; there was no remaining glucose as determined by HPLC

Figure 5.1 Growth profiles of B2-c3-1s and A3-c2-1s and their parent strains at range of sulfite concentrations

Growth of 'low-ethanol' variants B2-c3-1s and A3-c2-1s were assessed in a range of sulfite concentrations in microplate experiments. Variants were grown in YPD-10S supplemented with 30, 36, 38 or 40 g/l of sulfite. All incubations were conducted in triplicate, 30°C (no shaking). Parental strains, BY4742 and AWRI1628, were incubated in YPD-10S supplemented with 30 g/l of sulfite as used for these strains in the first round of the adaptive evolution (see Chapter 4)

5.1i


10.00
1.00
0.10
OD (630 nm)
BY4742 30 g/l
B2-c3-1s 30 g/l
B2-c3-1s 36 g/l
B2-c3-1s 38 g/l
B2-c3-1s 40 g/l
0
10
20
30
40
50
60
70
80
90
Time (h)

5.1ii

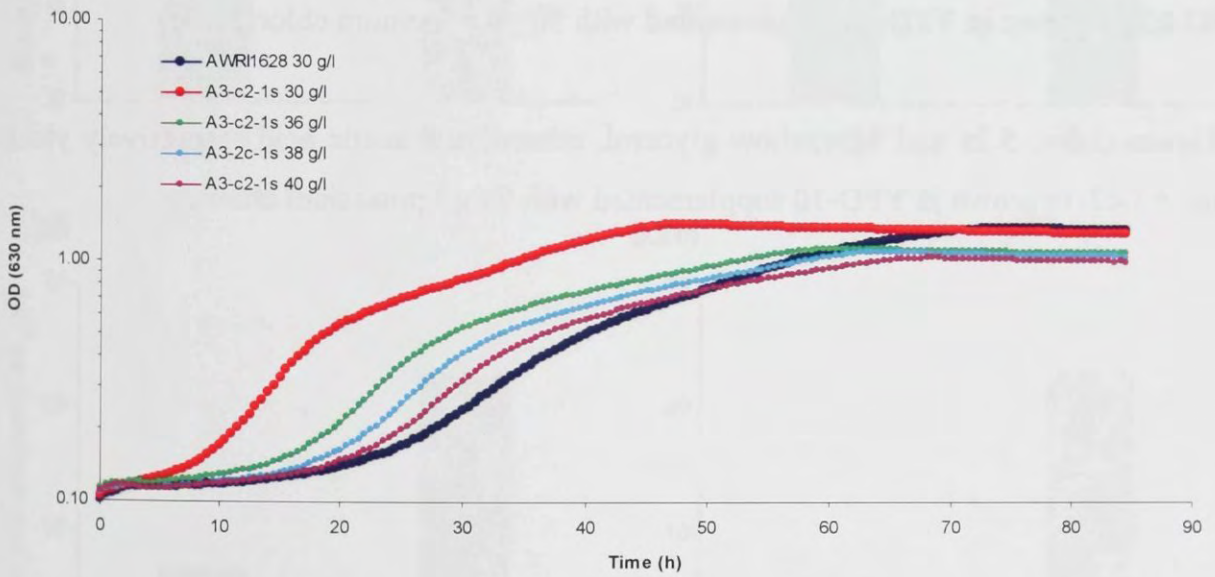

10.00
1.00
0.10
OD (630 nm)
AWRI1628 30 g/l
A3-c2-1s 30 g/l
A3-c2-1s 36 g/l
A3-2c-1s 38 g/l
A3-c2-1s 40 g/l
0
10
20
30
40
50
60
70
80
90
Time (h)

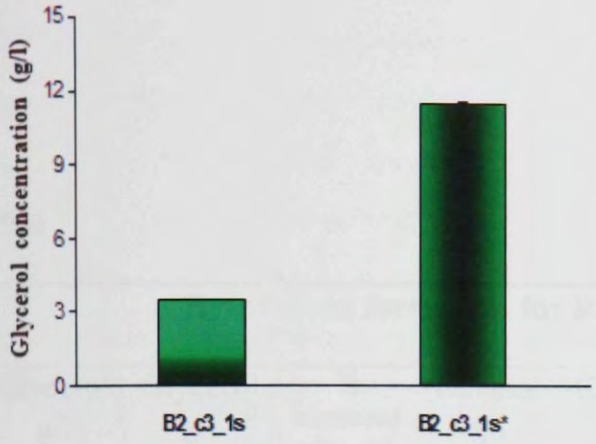
Figure 5.2 The effect of potassium chloride (KCl) on 'low-ethanol' variants B2-c3-1s and A3-c2-1 was assessed in triplicate incubations. Both 'low-ethanol' strains were grown in YPD-10 containing either 90 g/l or 95 g/l of potassium chloride for B2-c3-1s and A3-c2-1 respectively. Glycerol, ethanol and acetic acid yields were measured using HPLC. Experiments were conducted under aerobic conditions, 30°C/140 rpm.

* indicates presence of KCl in the medium.

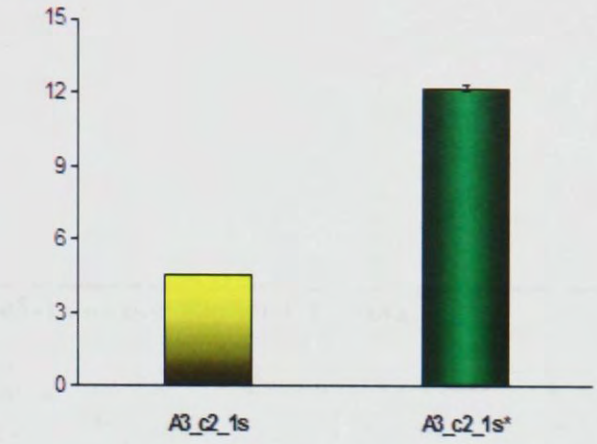
Figures 5.2i, 5.2ii and 5.2iii show glycerol, ethanol and acetic acid yields respectively for B2-c3-1s grown in YPD-10 supplemented with 90 g/l potassium chloride.

Figures 5.2iv, 5.2v and 5.2vi show glycerol, ethanol and acetic acid respectively yields for A3-c2-1s grown in YPD-10 supplemented with 95 g/l potassium chloride.

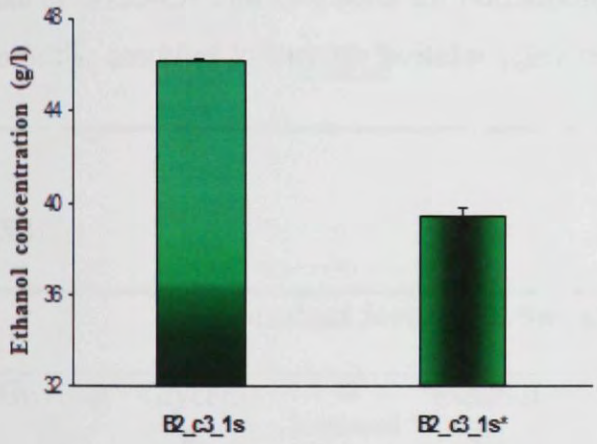
5.2i



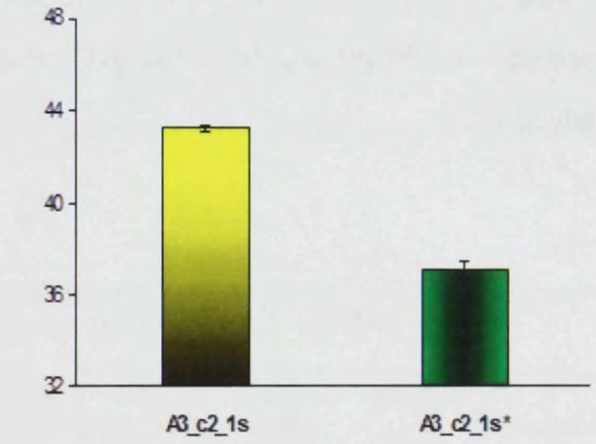
5.2iv



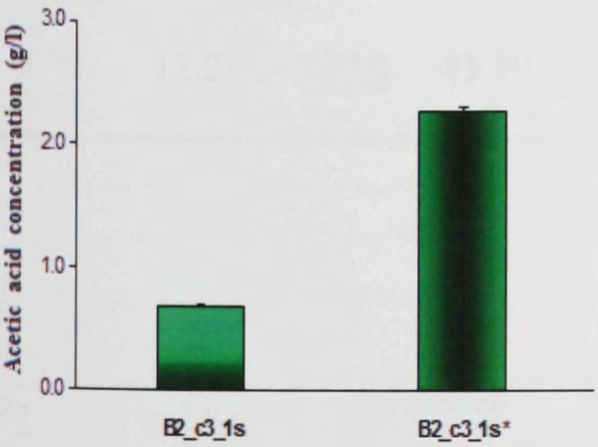
5.2ii



5.2v



5.2iii



5.2vi

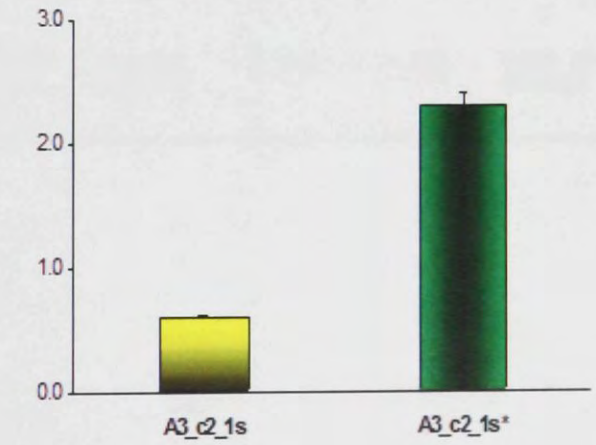


Table 5.1 Glycerol, ethanol and acetic acid production for B2-c3-1s and A3-c2-1s in the presence of 90 g/l and 95 g/l of KCl respectively, relative to control cultures with no added KCl.

5.1i

By-product formation for B2-c3-1s under 90g/l KCl stress

Glycerol g/l Unstressed control	Glycerol g/l KCl stress	% increased glycerol KCl stress	Ethanol g/l Unstressed control	Ethanol g/l KCl stress	% decreased ethanol KCl stress	Acetic acid g/l Unstressed control	Acetic acid g/l KCl stress	% increased acetic acid KCl stress
3.43	11.43	**233.2**	46.14	39.40	**14.6**	0.69	2.26	**227.5**

5.1ii

By-product formation for A3-c2-1s under 95g/l KCl stress

Glycerol g/l Unstressed control	Glycerol g/l KCl stress	% increased glycerol KCl stress	Ethanol g/l Unstressed control	Ethanol g/l KCl stress	% decreased ethanol KCl stress	Acetic acid g/l Unstressed control	Acetic acid g/l KCl stress	% increased acetic acid KCl stress
4.57	12.23	**167.6**	43.19	37.12	**14.05**	0.59	2.30	**289.8**

analysis. For both variants tested under osmotic stress conditions glycerol production greatly increased while ethanol yields were down relative to control (non-osmotically stressed) cultures. B2-c3-1s grown in YPD-10K supplemented with 90 g/l of potassium chloride produced 6.7 g/l more glycerol and 8 g/l less ethanol compared to incubation in YPD-10 only. A3-c2-1s produced 6.1 g/l more glycerol and 7.65 g/l less ethanol, when grown in YPD-10K supplemented with 95 g/l KCl. Both variants produced elevated levels of acetic acid under osmotic stress; 1.57 g/l and 1.71 g/l increases were measured for B2-c3-1s and A3-c2-1s respectively (see Table 5.1).

The toxicity of the above potassium chloride concentrations was also determined in microplate experiments for both 'low-ethanol' variants as described in Section 2.1.5.6. As shown in Figure 5.3 growth of these variants was significantly inhibited under the above salt concentrations. 'Low-ethanol' variants B2-c3-1s and A3-c2-1s grown under KCl stress reached an OD_{600} of 0.2 and 0.26 respectively after 12 hours of incubation. In comparison, the growth of these variants growth in non-stress conditions reached an OD_{600} of 1 and 1.05 after the same incubation time.

5.3.3 Second round of adaptive evolution

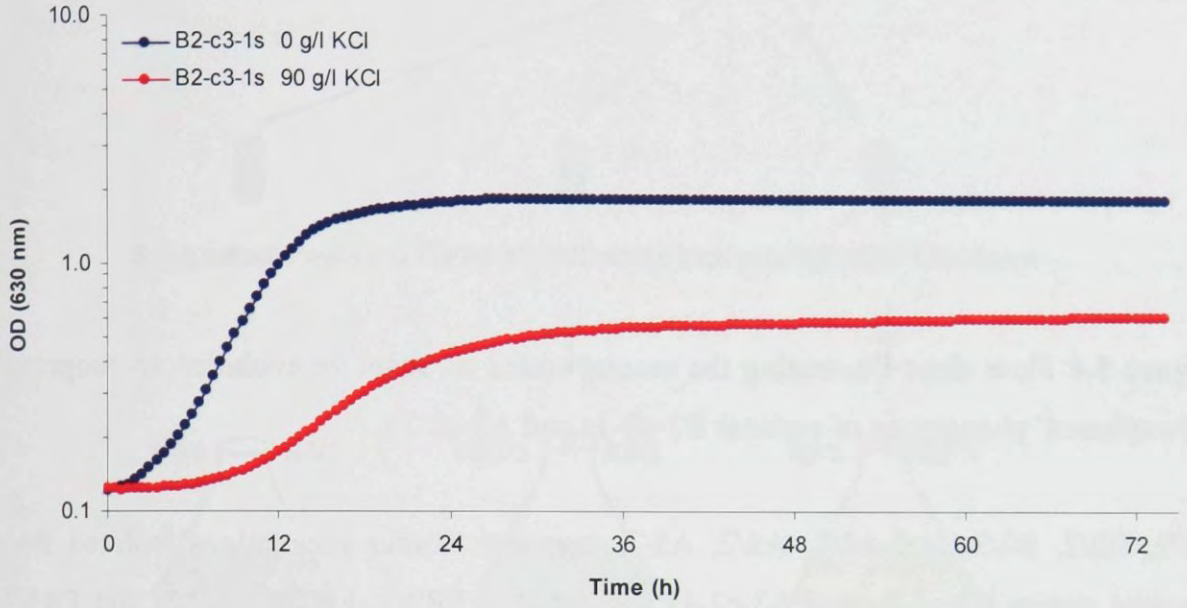
Three random colonies of 'low-ethanol' variants B2-c3-1s and A3-c2-1s were used to generate 'starter populations' for the second round of adaptive evolution (see Figure 5.4). Single colonies were obtained as described in Section 4.3.1. Stock cultures were prepared from these colonies and kept at -80°C as described in Section 2.1.5.1. Single colonies of A3-c2-1s were obtained using a micromanipulator (Singer MSM System 300). For the initiation of adaptive evolution cultures triplicate 'starter populations' were inoculated into YPD from the above frozen stocks, and grown overnight at 30°C/140 rpm. The starter populations were named **B2**/1, **B2**/2, **B2**/3, and **A3**/1, **A3**/2, **A3**/3, seeded from **B2**-c3-1s and **A3**-c2-1s respectively.

As shown in Figure 5.4, triplicate founding populations FB2/1, FB2/2, FB2-3, and FA3/1, FA3/2, FA3/3 were inoculated from 'starter populations' into YPD-10S containing 38 g/l

Figure 5.3 Growth profiles of B2-c3-1s and A3-c2-1s under osmotic stress

Growth of 'low-ethanol' variants B2-c3-1s and A3-c2-1s were assessed in presence of KCl in microplate experiments. Variants were grown in YPD-10K supplemented with 90 g/l and 95 g/l of KCl respectively compared to growth in non-supplemented medium. All incubations were conducted in triplicate at 30°C (no shaking).

5.3i


10.0
1.0
0.1
OD (630 nm)
B2-c3-1s 0 g/l KCl
B2-c3-1s 90 g/l KCl
0
12
24
36
48
60
72
Time (h)

5.3ii

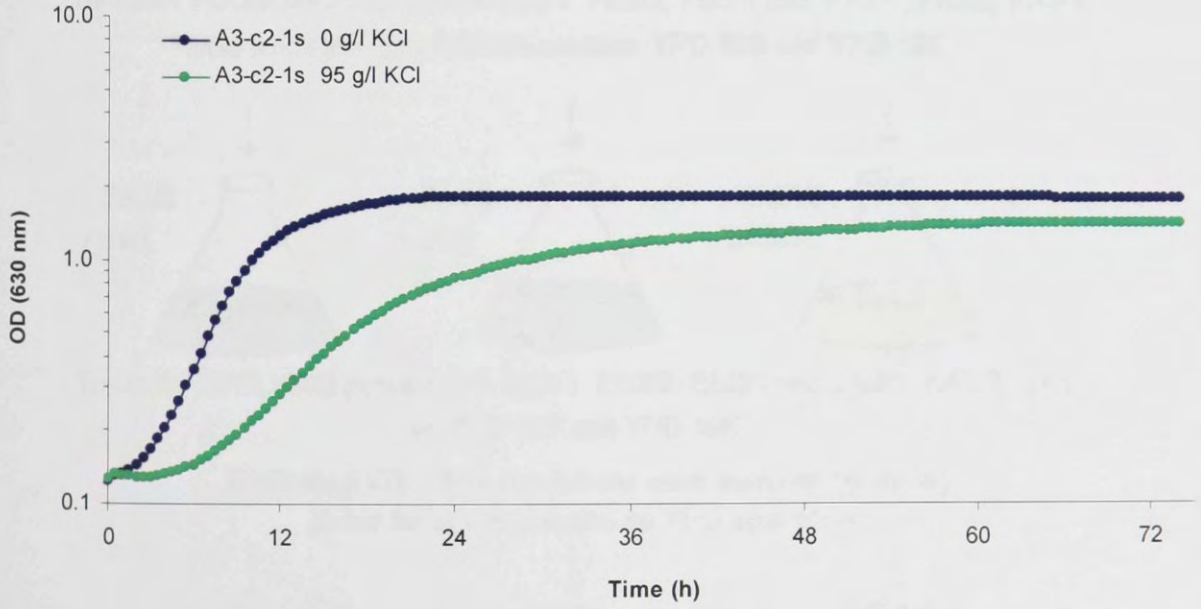
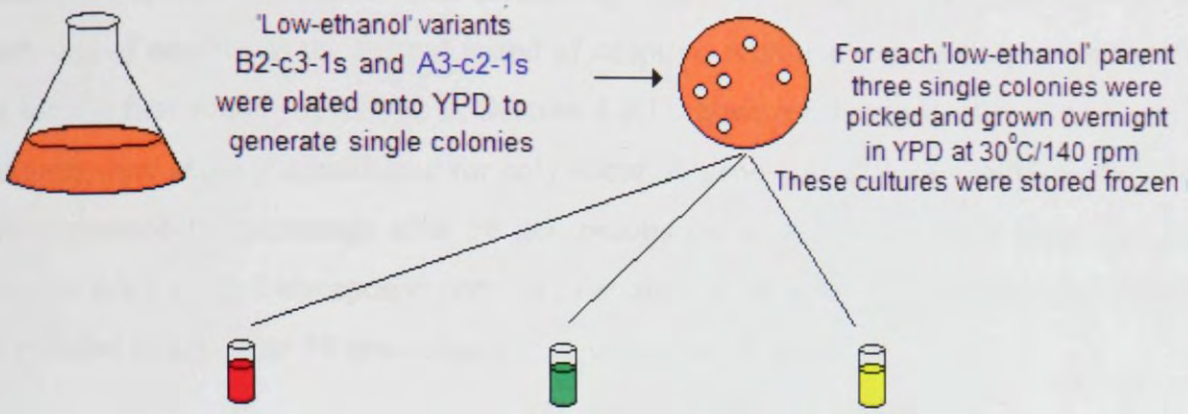
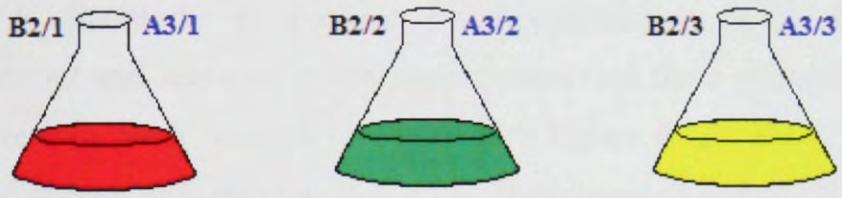

10.0
1.0
0.1
OD (630 nm)
A3-c2-1s 0 g/l KCl
A3-c2-1s 95 g/l KCl
0
12
24
36
48
60
72
Time (h)

Figure 5.4 Flow chart illustrating the second round of adaptive evolution to 'improve' 'low-ethanol' phenotypes of variants B2-c3-1s and A3-c2-1s.

B2/1, B2/2, B2/3, and A3/1, A3/2, A3/3 represents starter populations isolated from parental strains B2-c3-1s and A3-c2-1s respectively. FB2/1, FB2/2, FB2/3, and FA3/1, FA3/2, FA3/3 represent founding populations derived from respective starting populations. EB2/1, EB2/2, EB2/3, and EA3/1, EA3/2, EA3/3 represent respective evolving populations after 50 generations in selective medium YPD-10S or YPD-10K.

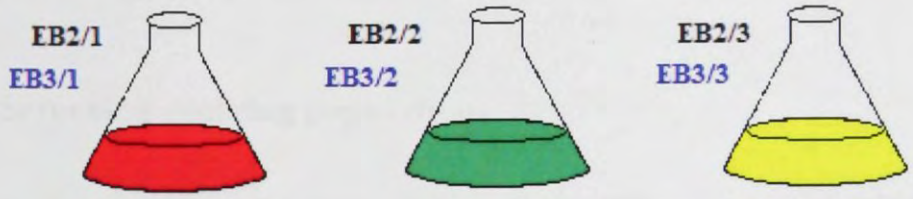


Stock cultures were inoculated into YPD and grown overnight at 30°C/140 rpm



Triplicate STARTER populations of B2-c3-1s (B2) and A3-c2-1s (A3) in YPD

Triplicate FOUNDING populations FB2/1, FB2/2, FB2/3 and FA3/1, FA3/2, FA3/3
were inoculated into selective medium YPD-10S and YPD-10K



Triplicate EVOLVING populations EB2/1, EB2/2, EB2/3 and EA3/1, EA3/2, EA3/3
in YPD-10S and YPD-10K

Triplicate EVOLVING populations were sampled by plating
plated for single colonies on YPG agar plates

and 40 g/l of sodium sulfite respectively. The same starting populations were used to seed founding populations in YPD-10K containing 90 g/l and 95 g/l of KCl respectively. For both sets of conditions the second round of adaptive evolution was conducted essentially as for the first round (described in Section 4.3.1), however, because of limited time the cultures were serially subcultured for only about 50 generations. All evolving populations were assessed for parentage after 50 generations using transposon PCR technique (see Section 2.3.7.1). Ty1 transposon patterns of evolved populations were unchanged relative to parental strains after 50 generations of selection (see Figure 5.5).

Samples of 'starter populations' were assessed for fermentation products as described in Section 4.3.1. Glycerol, ethanol and acetate levels produced by 'starter populations' and parental 'low-ethanol' variants B2-c3-1s and A3-c2-1s were measured using HPLC (see Section 2.2.4). The results revealed that all starter populations produced similar levels of glycerol, ethanol and acetic acid, (data not shown) and these were close to the values found in previous work with these strains (see Figure 4.12). Samples of the 'starter populations' of both 'low-ethanol' variants were prepared as described in Section 2.1.5.1 stored at -80°C for future use.

Petite frequencies were determined for 'starter populations' as described in Section 2.2.7, and were found to be 7.5, 7.61 and 7.45 for B2/1, B2/2 and B2/3 respectively, and 8.1, 8.12 and 7.88 for A3/1, A3/2 and A3/3 respectively ,which is almost identical to parental strains from which they were seeded.

5.3.4 Screening evolving populations

Evolving populations were screened after 50 generations, essentially as for the first round of adaptive evolution, (see Section 4.3.3). However, it was found in the previous chapter that growth in sulfite-containing medium can induce petite mutations (see Section 4.3.6), and therefore YPG plates (on which petites are unable to grow) were used to isolate single colonies from evolving populations. As for previous experiments involving strains derived from AWRI1628 (which tend to form clumps), colonies were seeded from single cells isolated using a using micromanipulator.

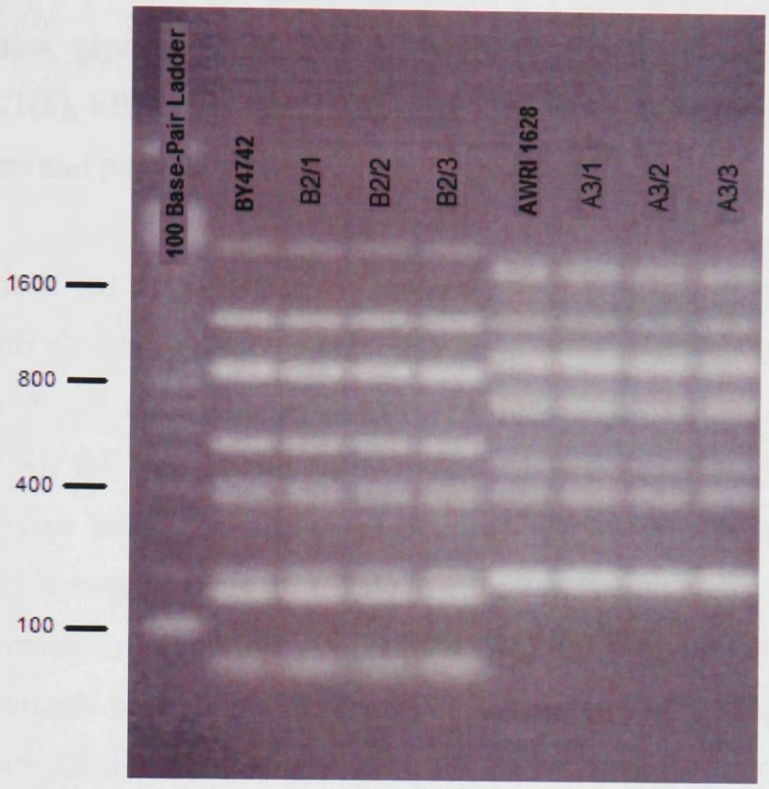
Figure 5.5 Ty1 transposon PCR fingerprints for the 'starter populations' and evolving populations at 50 generations in YPD-10S and YPD-10K.

Figure 5.5i Ty1 transposon fingerprints for original parental strains BY4742 and AWRI1628, and 'starter populations' derived from B2-c3-1s (B2/1, B2/2 and B2/3) and A3-c2-1s (A3/1, A3/2 and A3/3)

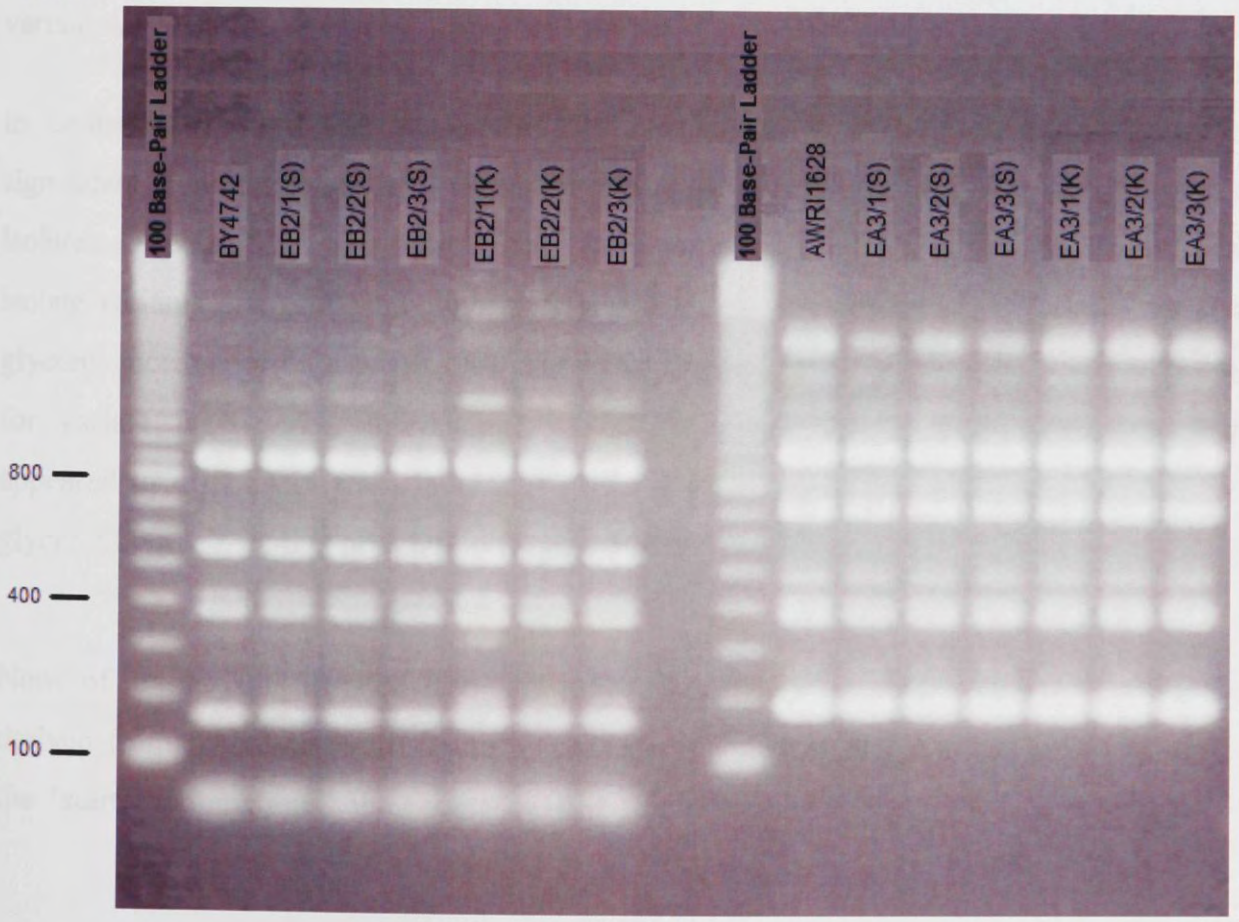
Figure 5.5ii Ty1 transposon fingerprints for original parental strains BY4742 and AWRI1628 and evolving populations EB2/1, EB2/2, EB2/3, and EA3/1, EA3/2, EA3/3 in YPD-10S (S) and YPD-10K (K). The transposon fingerprints of evolving populations were assessed after 50 generations of adaptive evolution.

NOTE: The transposon fingerprint for evolving population EB2/1(K) (see Figure 5.5ii), were slightly different from the parental strain BY4742. This result was checked in a separate experiment and found to be an artifact; the fingerprint was identical to the parental strain BY4742 (data not shown). Such artifacts are not unusual when using this PCR-based technique and must be confirmed or rejected in respect experiments.

5.5i


100 Base-Pair Ladder
BY4742
B2/1
B2/2
B2/3
AWRI 1628
A3/1
A3/2
A3/3
1600
800
400
100

5.5ii


100 Base-Pair Ladder
BY4742
EB2/1(S)
EB2/2(S)
EB2/3(S)
EB2/1(K)
EB2/2(K)
EB2/3(K)
100 Base-Pair Ladder
AWRI1628
EA3/1(S)
EA3/2(S)
EA3/3(S)
EA3/1(K)
EA3/2(K)
EA3/3(K)
800
400
100

Five random colonies were chosen from each of the six evolving populations, and assessed for glycerol production in non-selective, YPD-10, medium (Figure 5.6). Figures 5.7i and 5.8i show glycerol levels for individual variants selected from evolving populations EB2/1(S), EB2/2(S), EB2/3(S), and EA3/1(S), EA3/2(S), EA3/3(S), the starting populations and parental strains BY4742 and AWRI1628.

Evolving populations EB2/1(S), EB2/2(S) and EB2/3(S) clearly underwent changes in metabolism towards elevated glycerol production after 50 generations of increased sulfite selection (Figure 5.7i). Four 'high-glycerol' producing isolates, two chosen from population EB2/1(S); B2/1-c2-2s and B2/1-c4-2s, and two from EB2/3(S); B2/3-c2-2s and B2/3-c3-2s, were taken for further assessment. These isolates had increases in glycerol production in range from 1 g/l (27%) to 1.9 g/l (52%) compared to their 'starter' populations. Screening of evolving populations EA3/1(S), EA3/2(S) and EA3/3(S) revealed a few variants with minor increases in glycerol levels compared to 'starter populations' (Figure 5.8i). These variants were A3/1-c3-2s, A3/2-c2-2s, A3/2-c3-2s and A3/3-c2-2s; the maximum increase in glycerol production of 0.24 g/l was noted for variant A3/1-c3-2s.

In contrast to adaptive evolution in sulfite, selection in YPD-10K did not lead to significant increases in glycerol yields for most evolving populations or randomly chosen isolates (Figures 5.7ii and 5.8ii). Only from population EB2/1(K) it was possible to isolate variants that produced elevated glycerol levels, and within this group the highest glycerol increase of 0.28 g/l (8.1%), compared to the 'starter' population, was measured for variant B2/1-c1-2k. Interestingly, B2/3-c1-2k, derived from a population that appeared to drift away from increased glycerol yields, produced 0.27 g/l (7.4%) more glycerol than its 'starter' population (Figure 5.8ii).

None of the variants isolated from the three osmotically stressed evolving populations derived from A3-c2-1s, were found to produce elevated levels of glycerol compared to the 'starter populations' they were seeded from (Figure 5.7ii). More surprisingly, a

Figure 5.6 Flow chart illustrating the strategy used for isolation of 'low-ethanol' variants from evolving populations B2-c3-1s and A3-c2-1s.

The **1st round screen** was used to isolate the highest glycerol-producing variants.

The **2nd round screen** was used to test the best isolates from the above. The isolates were assessed in triplicate for glycerol, ethanol and acetic acid production as described in Section 2.1.5.4.

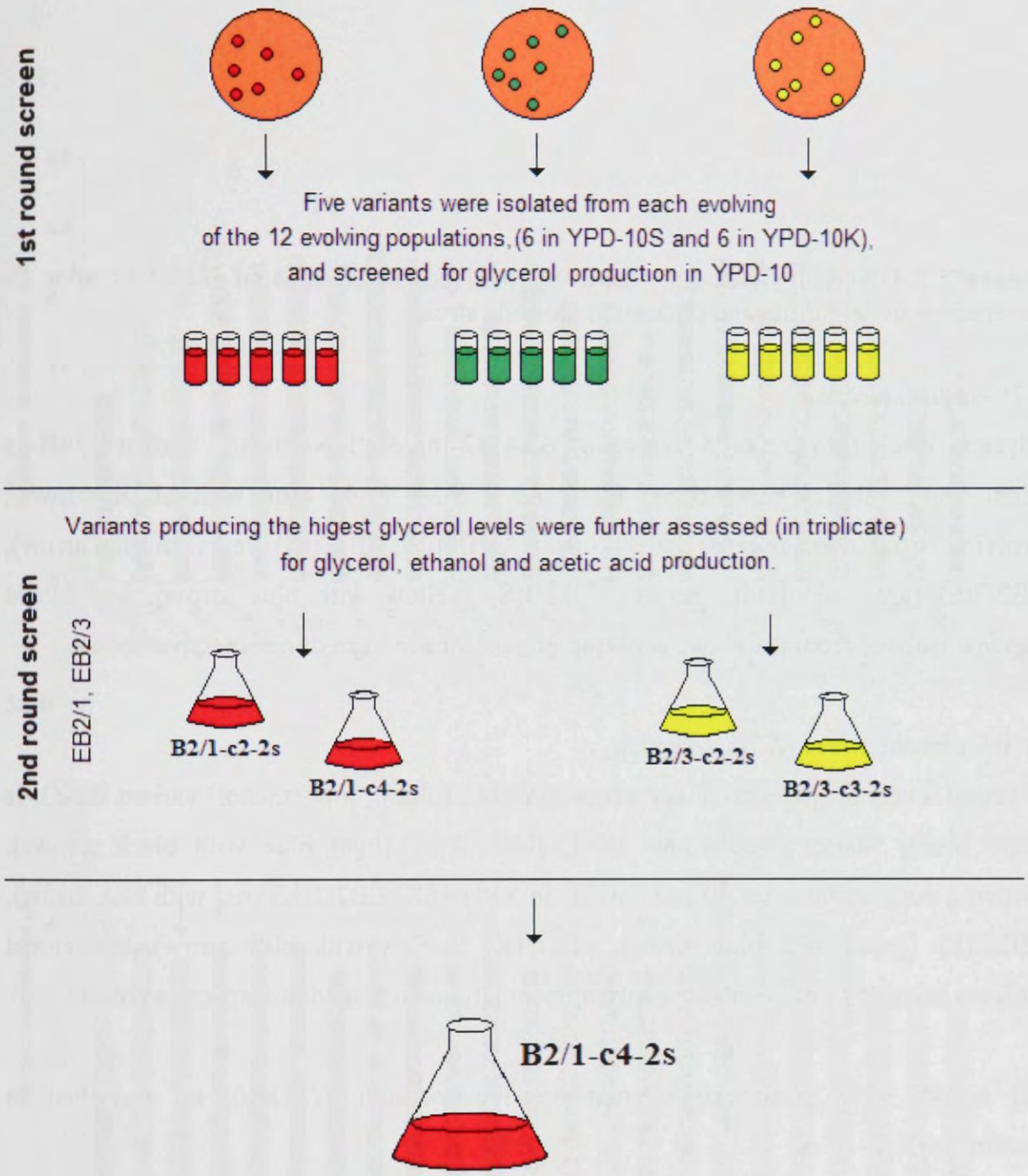

1st round screen
Five variants were isolated from each evolving
of the 12 evolving populations, (6 in YPD-10S and 6 in YPD-10K),
and screened for glycerol production in YPD-10
2nd round screen
Variants producing the higest glycerol levels were further assessed (in triplicate)
for glycerol, ethanol and acetic acid production.
EB2/1, EB2/3
B2/1-c2-2s
B2/1-c4-2s
B2/3-c2-2s
B2/3-c3-2s
B2/1-c4-2s
Variant B2/1-c4-2s displayed the greatest redirection of metabolism away from
ethanol production

Figure 5.7 Glycerol yields for adaptively evolved populations of B2-c3-1s after 50 generations under sulfite and potassium chloride stress.

5.7i –*sulfite selection*

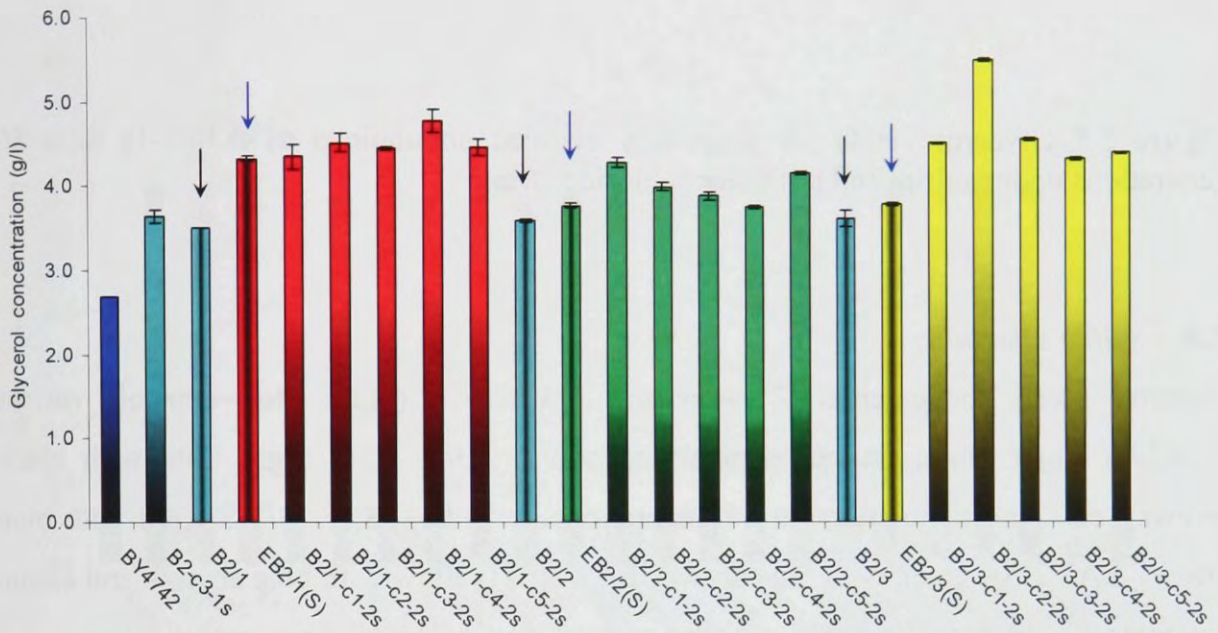
Glycerol levels for parental *S. cerevisiae* BY4742 (blue), 'low-ethanol' variant B2-c3-1s (light blue), 'starter populations' B2/1, B2/2, B2/3 (light blue with black arrows), evolving populations after 50 generations in YPD-10S; EB2/1(S) (red with blue arrow), EB2/2(S) (green with blue arrow), EB2/3(S) (yellow with blue arrow), and clonal variants isolated from the above evolving populations indicated in respective colors.

5.7ii – *osmotic stress (KCl) selection*

Glycerol levels for parental *S. cerevisiae* BY4742 (blue), 'low-ethanol' variant B2-c3-1s (light blue), 'starter populations' B2/1, B2/2, B2/3 (light blue with black arrows), evolving populations after 50 generations in YPD-10K; EB2/1(K) (red with blue arrow), EB2/2(K) (green with blue arrow), EB2/3(K) (yellow with blue arrow), and clonal variants isolated from the above evolving populations indicated in respective colors.

All assays were performed in non-selective medium, YPD-10, as described in Section 5.3.5

5.7i


Glycerol concentration (g/l)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
BY4742
B2-c3-1s
B2/1
EB2/1(S)
B2/1-c1-2s
B2/1-c2-2s
B2/1-c3-2s
B2/1-c4-2s
B2/1-c5-2s
B2/2
EB2/2(S)
B2/2-c1-2s
B2/2-c2-2s
B2/2-c3-2s
B2/2-c4-2s
B2/2-c5-2s
B2/3
EB2/3(S)
B2/3-c1-2s
B2/3-c2-2s
B2/3-c3-2s
B2/3-c4-2s
B2/3-c5-2s

5.7ii

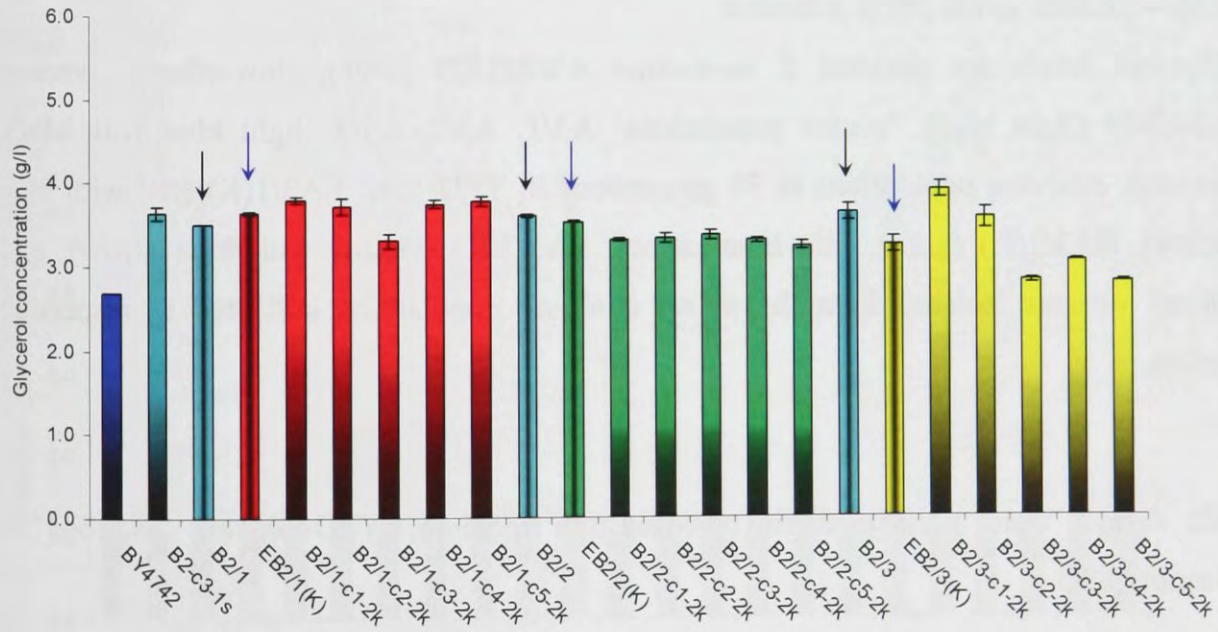

Glycerol concentration (g/l)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
BY4742
B2-c3-1s
B2/1
EB2/1(K)
B2/1-c1-2k
B2/1-c2-2k
B2/1-c3-2k
B2/1-c4-2k
B2/1-c5-2k
B2/2
EB2/2(K)
B2/2-c1-2k
B2/2-c2-2k
B2/2-c3-2k
B2/2-c4-2k
B2/2-c5-2k
B2/3
EB2/3(K)
B2/3-c1-2k
B2/3-c2-2k
B2/3-c3-2k
B2/3-c4-2k
B2/3-c5-2k

Figure 5.8 Glycerol yields for adaptively evolved populations of A3-c2-1s after 50 generations under sulfite and potassium chloride stress

5.8i – *sulfite selection*

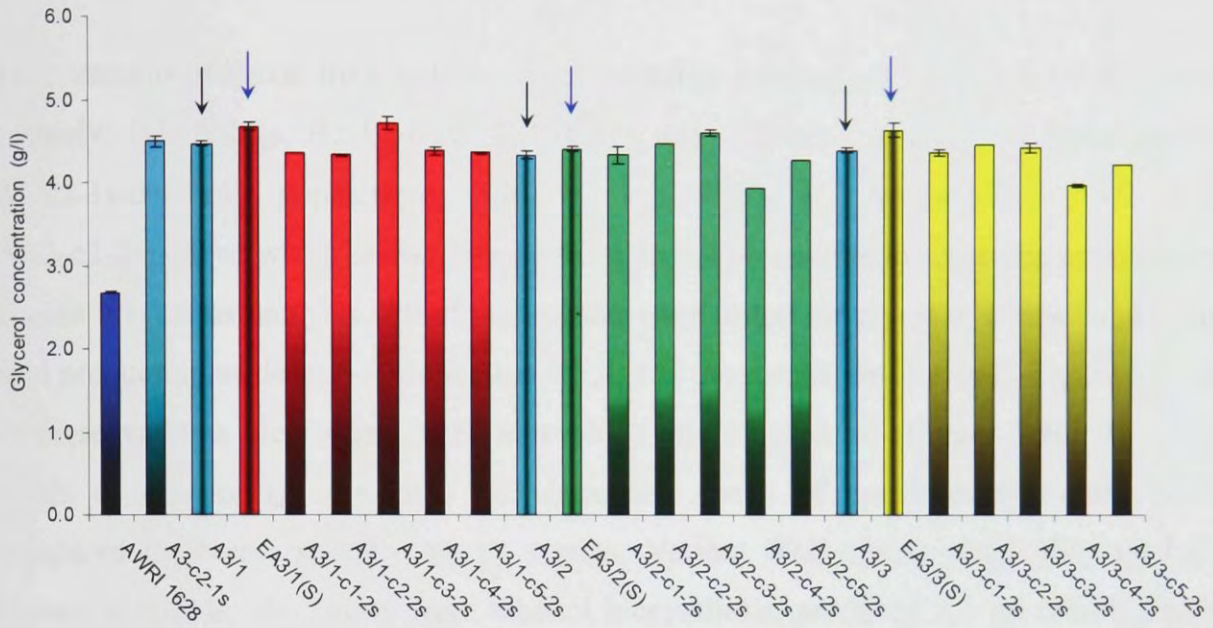
Glycerol levels for parental *S. cerevisiae* AWRI1628 (blue), 'low-ethanol' variant A3-c2-1s (light blue), 'starter populations' A3/1, A3/2, A3/3 (light blue with black arrows), evolving populations at 50 generations in YPD-10S; EA3/1(S) (red with blue arrow), EA3/2(S) (green with blue arrow), EA3/3(S) (yellow with blue arrow), and clonal variants isolated from the above evolving populations indicated in respective colors.

5.8ii – *osmotic stress (KCl) selection*

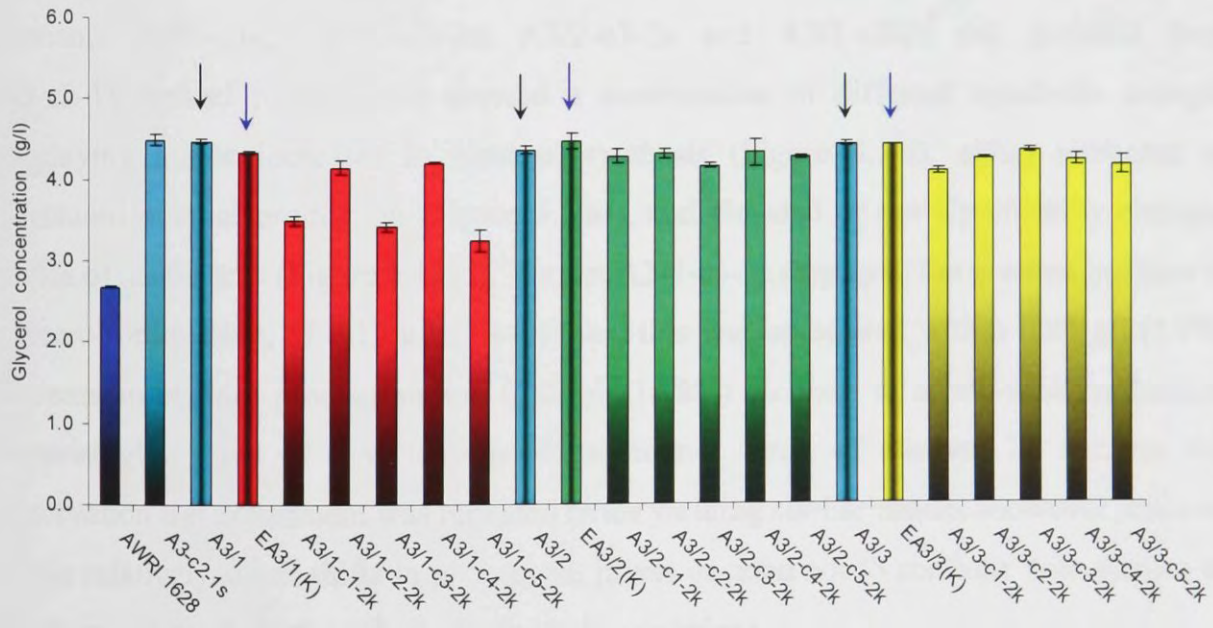
Glycerol levels for parental *S. cerevisiae* AWRI1628 (blue), 'low-ethanol' variant A3-c2-1s (light blue), 'starter populations' A3/1, A3/2, A3/3 (light blue with black arrows), evolving populations at 50 generations in YPD-10K; EA3/1(K) (red with blue arrow), EA3/2(K) (green with blue arrow), EA3/3(K) (yellow with blue arrow), and clonal variants isolated from the above evolving populations indicated in respective colors.

All assays were performed in non-selective medium, YPD-10, as described in Section 5.3.5

5.8i


Glycerol concentration (g/l)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
AWRI 1628
A3-c2-1s
A3/1
EA3/1(S)
A3/1-c1-2s
A3/1-c2-2s
A3/1-c3-2s
A3/1-c4-2s
A3/1-c5-2s
A3/2
EA3/2(S)
A3/2-c1-2s
A3/2-c2-2s
A3/2-c3-2s
A3/2-c4-2s
A3/2-c5-2s
A3/3
EA3/3(S)
A3/3-c1-2s
A3/3-c2-2s
A3/3-c3-2s
A3/3-c4-2s
A3/3-c5-2s

5.8ii


Glycerol concentration (g/l)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
AWRI 1628
A3-c2-1s
A3/1
EA3/1(K)
A3/1-c1-2k
A3/1-c2-2k
A3/1-c3-2k
A3/1-c4-2k
A3/1-c5-2k
A3/2
EA3/2(K)
A3/2-c1-2k
A3/2-c2-2k
A3/2-c3-2k
A3/2-c4-2k
A3/2-c5-2k
A3/3
EA3/3(K)
A3/3-c1-2k
A3/3-c2-2k
A3/3-c3-2k
A3/3-c4-2k
A3/3-c5-2k

significant group of isolates, as well as some founding populations, partially lost the original phenotype producing lower glycerol levels. None of these variants were used in further studies.

5.3.5 Phenotypic assessment of variants

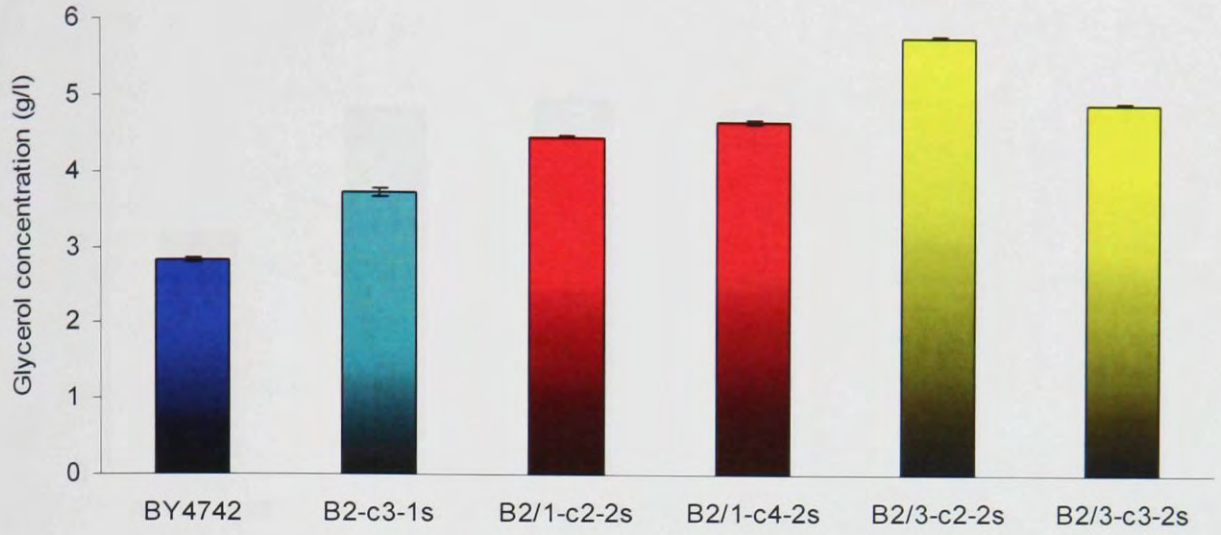
Four variants isolated from sulfite-driven evolving populations seeded from B2-c3-1s, (namely: B2/1-c2-2s, B2/1-c4-2s, B2/3-c2-2s and B2/3-c3-2s), and four isolates from A3-c2-1s-derived populations, (namely: A3/1-c3-2s, A3/2-c2-2s, A3/2-c3-2s, A3/3-c2-2s), all of which showed improved glycerol production in screening assays, were chosen for further analysis. All of the variants were tested for glycerol, ethanol and acetic acid production as described in Section 4.3.4, and the results are shown in Figure 5.9. All of these variants clearly produced elevated levels of glycerol (Figure 5.9i), decreased levels of ethanol (Figure 5.9ii) and increased levels of acetic acid (Figure 5.9iii) compared to their respective parent strains. Variant B2/3-c2-2s, which displayed the highest metabolic shift away from ethanol biosynthesis, produced 2.1 g/l (56.5%) more glycerol, 2.6 g/l (5.4%) less ethanol and 0.36 g/l (47.8%) more acetic acid levels, compared to its parental strain, B2-c3-1s.

Variants A3/1-c3-2s, A3/2-c2-2s, A3/2-c3-2s and A3/3-c2-2s (all isolated from A3-c2-1s-derived populations) showed a combination of different metabolic changes displaying minor increases in glycerol synthesis (Figure 5.10i), either increased or decreased ethanol production (Figure 5.10ii), and elevated or not significantly changed yields of acetic acid (Figure 5.10iii). Variant A3/2-c3-2s displayed the greatest increase in glycerol production, of 0.15 g/l (3.14%), but this was associated with a 0.85 g/l (1.9%) increase in ethanol production and 0.12 g/l (16.8%) increase in acetic acid production. Surprisingly, 3 out of 4 variants produced higher levels of ethanol. To confirm this observation the experiment was repeated twice yielding similar results. However, because of the relatively small shifts in phenotype, it was decided not to continue with studies on the four isolates from the A3-c2-1s-derived populations.

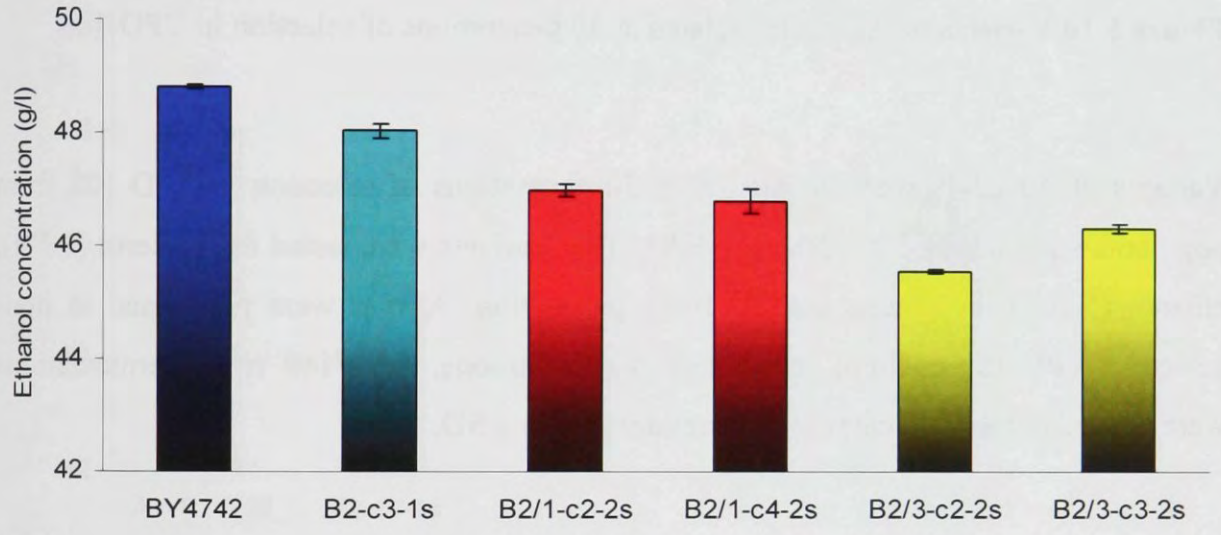
Figure 5.9 Variants of B2-c3-1s isolated at 50 generations of selection in YPD-10S

Variants of B2-c3-1s were isolated after 50 generations of selection in YPD-10S from populations EB2/1(S), EB2/2(S) and EB2/3(S). Variants were tested for glycerol (5.9i), ethanol (5.9ii) and acetic acid (5.9iii) production. Assays were conducted in non-selective YPD-10 medium, semi-anaerobic conditions, 30°C/140 rpm. Fermentations were carried out in triplicates and values are means ± SD.

5.9i



5.9ii



5.9iii

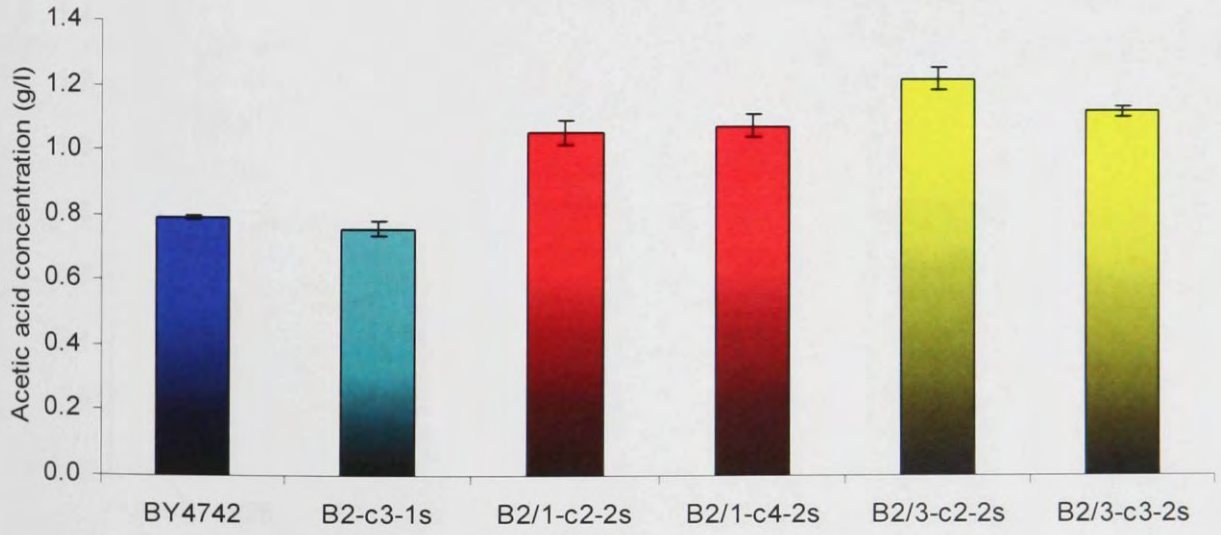
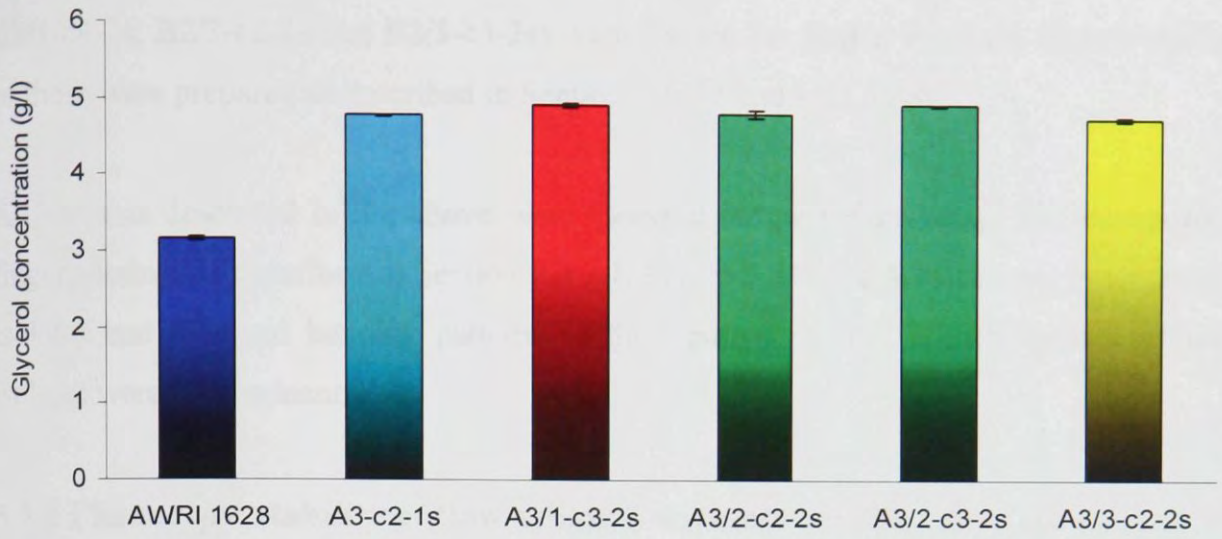


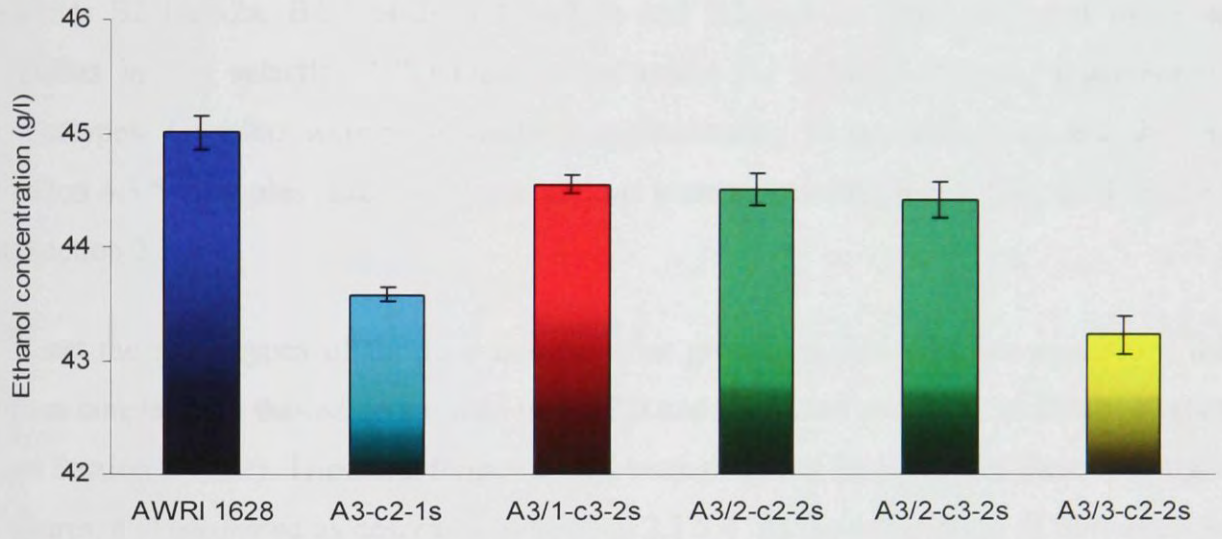
Figure 5.10 Variants of A3-c2-1s isolated at 50 generations of selection in YPD-10S

Variants of A3-c2-1s were isolated after 50 generations of selection in YPD-10S from populations EA3/1(S), EA3/2(S) and EA3/3(S). Variants were tested for glycerol (5.10i), ethanol (5.10ii) and acetic acid (5.10iii) production. Assays were performed in non-selective YPD-10 medium, semi-anaerobic conditions, 30°C/140 rpm. Fermentations were carried out in triplicates and values are means ± SD.

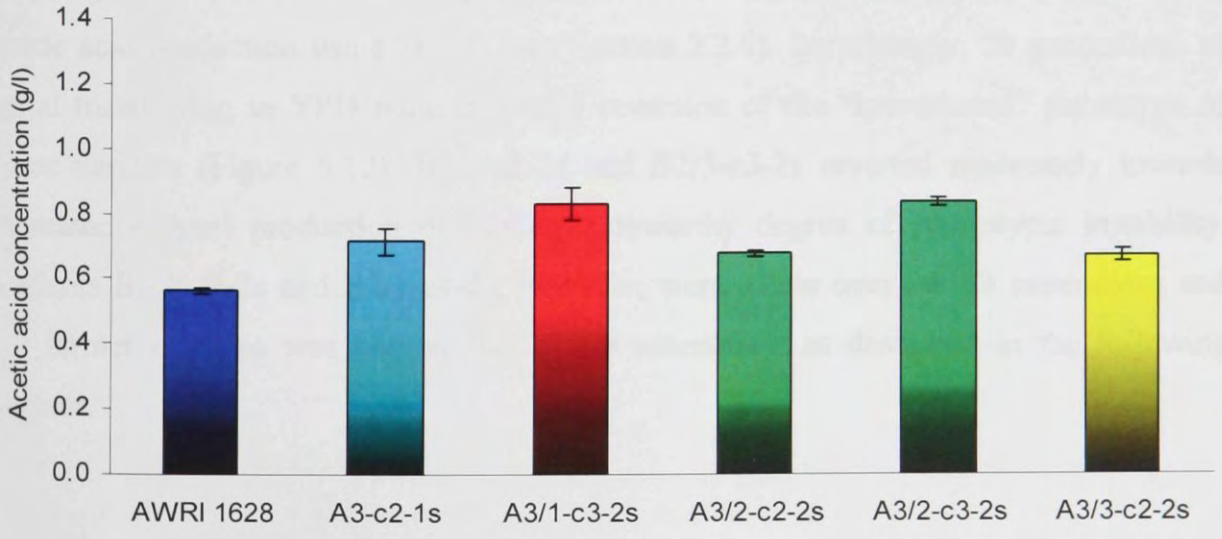
5.10i



5.10ii



5.10iii



Four variants derived from evolving populations of B2-c3-1s (namely: B2/1-c2-2s, B2/1-c4-2s, B2/3-c2-2s and B2/3-c3-2s) were chosen for further analysis. Frozen stocks of these were prepared as described in Section 2.1.5.1 and kept at -80°C.

All variants described in the above were assessed for parentage using Ty1 transposon fingerprinting, as described in Section 2.3.7.1. Figure 5.11i and 5.11ii show, in all cases, isolates had identical banding patterns to their parent strains. Therefore none of the isolates were contaminants.

5.3.6 Phenotypic stability of 'low-ethanol' variants

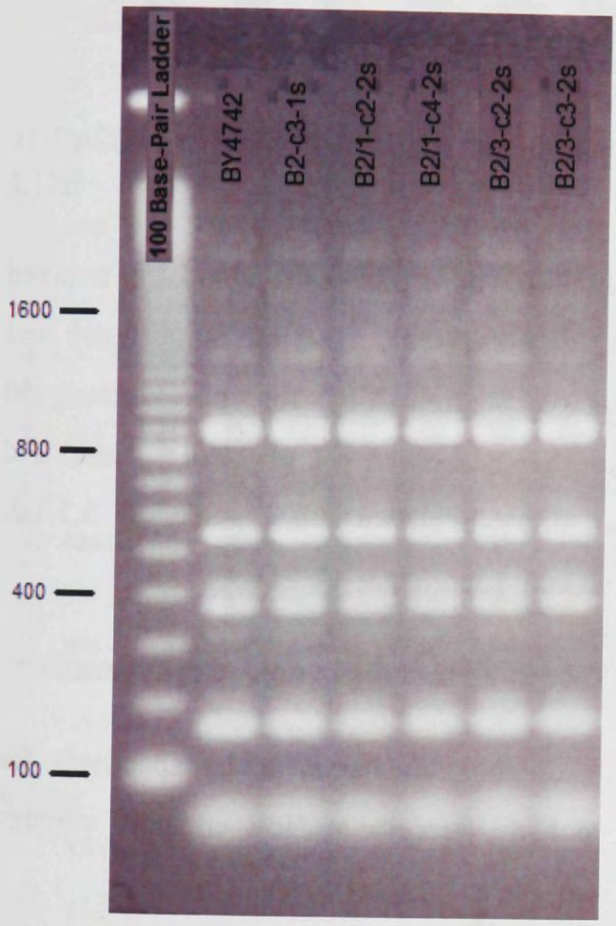
Variants B2/1-c2-2s, B2/1-c4-2s, B2/3-c2-2s and B2/3-c3-2s were subjected to serial transfers in non selective YPD medium, to assess the stability of their 'low-ethanol' phenotypes. Transfers were performed for approximately 50 generations as described in Section 4.3.5. Samples taken at 50 generations were stored frozen at -80°C, as described in Section 2.1.5.1.

To test the phenotypes of the four isolates after growth in non-selective conditions the frozen samples were thawed, inoculated into YPD and incubated overnight at 30°C/140 rpm (see Section 2.1.5.2). Triplicate fermentations were prepared from each of these overnight cultures, and incubated as described in Section 2.1.5.4. At the completion of fermentation (when glucose was fully utilized) all cultures were assessed for glycerol, ethanol and acetic acid production using HPLC (see Section 2.2.4). Surprisingly, 50 generations of serial transferring in YPD induced partial reversion of the 'low-ethanol' phenotype of some variants (Figure 5.12). B2/3-c2-2s and B2/3-c3-2s reverted moderately towards increased ethanol production displaying noteworthy degree of phenotypic instability. Variants B2/1-c2-2s and B2/1-c4-2s, however, were stable over the 50 generations and the former of these was chosen for further assessment as described in the following chapter.

Figure 5.11 Ty1 transposon patterns for 'low-ethanol' variants isolated after the second round of adaptive evolution in sulfite

The best variants selected from evolving populations EB2/1(S), EB2/2(S), EB2/3(S), and EA3/1(S), EA3/2(S), EA3/3(S), isolated following 50 generations of adaptive evolution in YPD-10S, were assessed for Ty1 transposon patterns.

5.11i


100 Base-Pair Ladder
BY4742
B2-c3-1s
B2/1-c2-2s
B2/1-c4-2s
B2/3-c2-2s
B2/3-c3-2s
1600
800
400
100

5.11ii

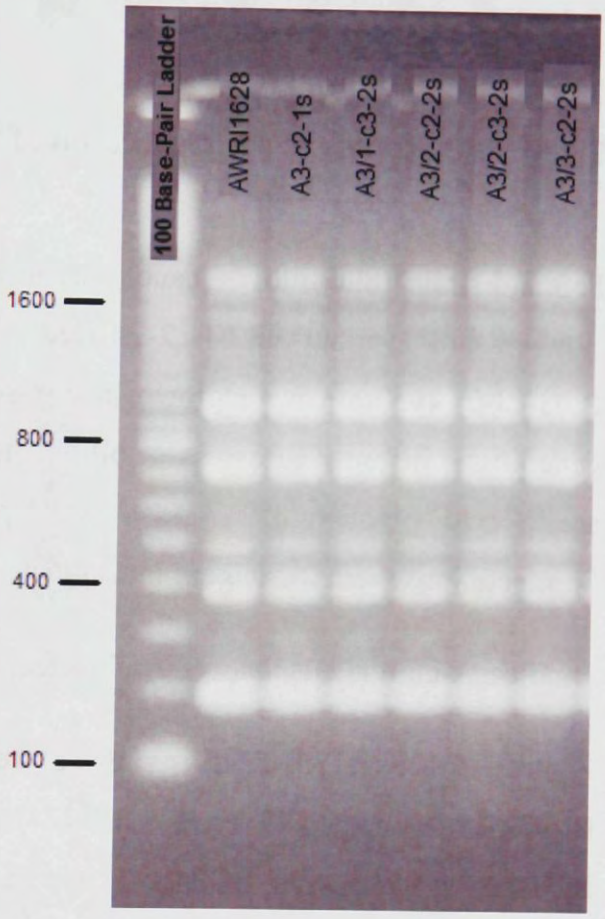

100 Base-Pair Ladder
AWRI1628
A3-c2-1s
A3/1-c3-2s
A3/2-c2-2s
A3/2-c3-2s
A3/3-c2-2s
1600
800
400
100

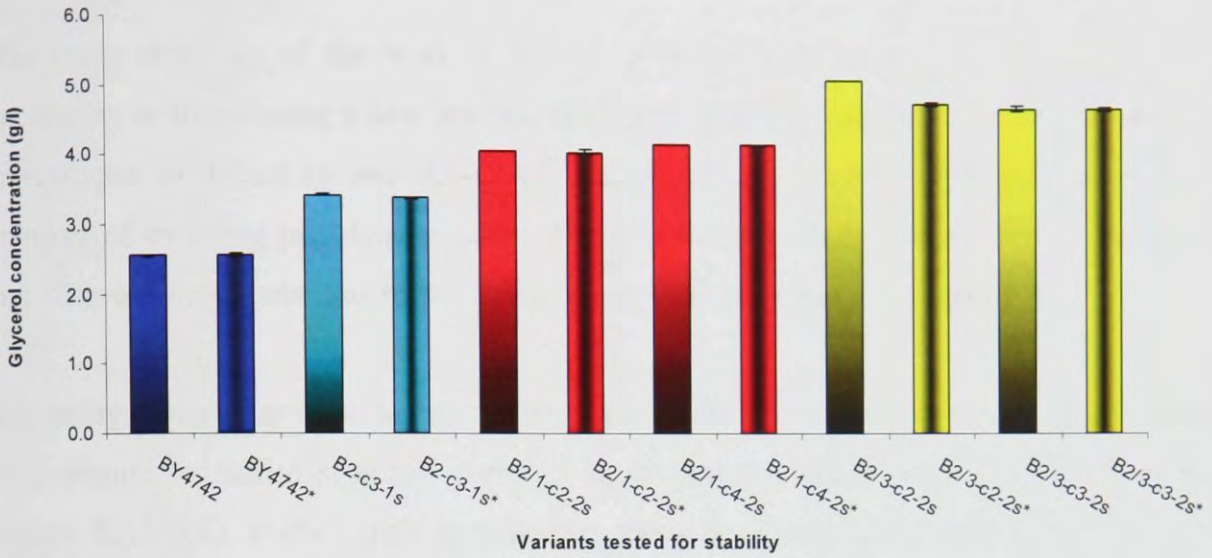
Figure 5.12 Stability of adaptively evolved 'low-ethanol' variants derived from B2-c3-1s

Parental strain (blue), low ethanol variant B2-c3-1s (light blue) and adaptively evolved 'low-ethanol' variants B2/1-c2-2s (red), B2/1-c4-2s (red), B2/3-c2-2s (yellow) and B2/3-c3-2s (yellow) were grown in non-selective medium YPD for approximately 50 generations in order to assess stability. Strains were assayed for glycerol, ethanol and acetic acid production. Assays were performed as described in Section 5.3.3.2. Fermentations were carried out in triplicate and values are means ± SD.

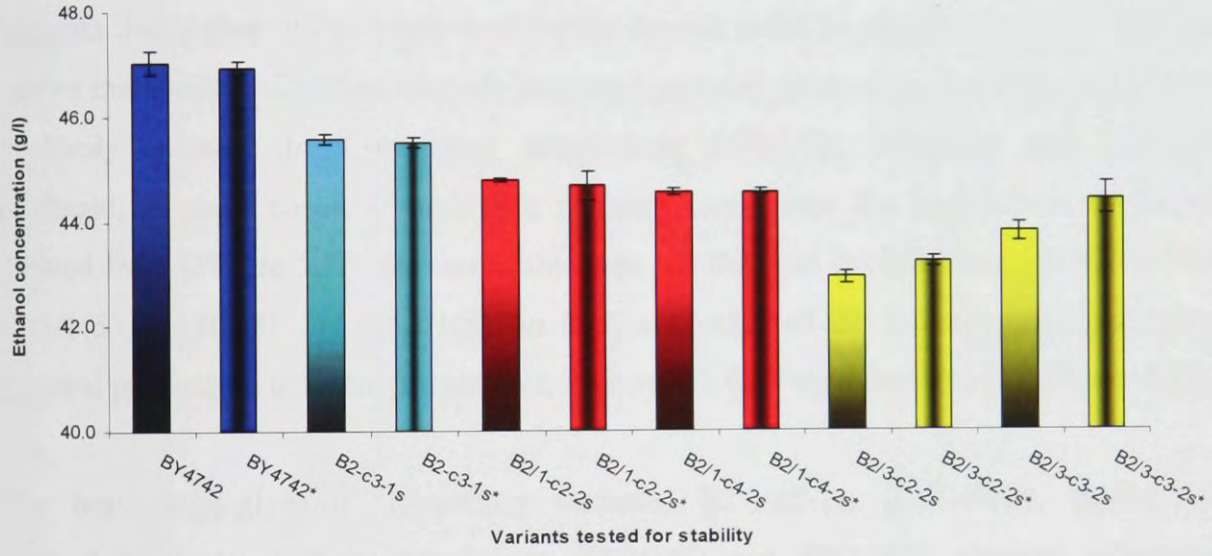
* indicates individuals that were subjected to sequential transfer in non-selective medium

Figures (5.12i, 5.12ii, 5.12iii) show glycerol, ethanol and acetic acid respectively for BY4742, 'low-ethanol' variant B2-c3-1s, and its successive 'low-ethanol' variants B2/1-c2-2s, B2/1-c4-2s, B2/3-c2-2s and B2/3-c3-2s.

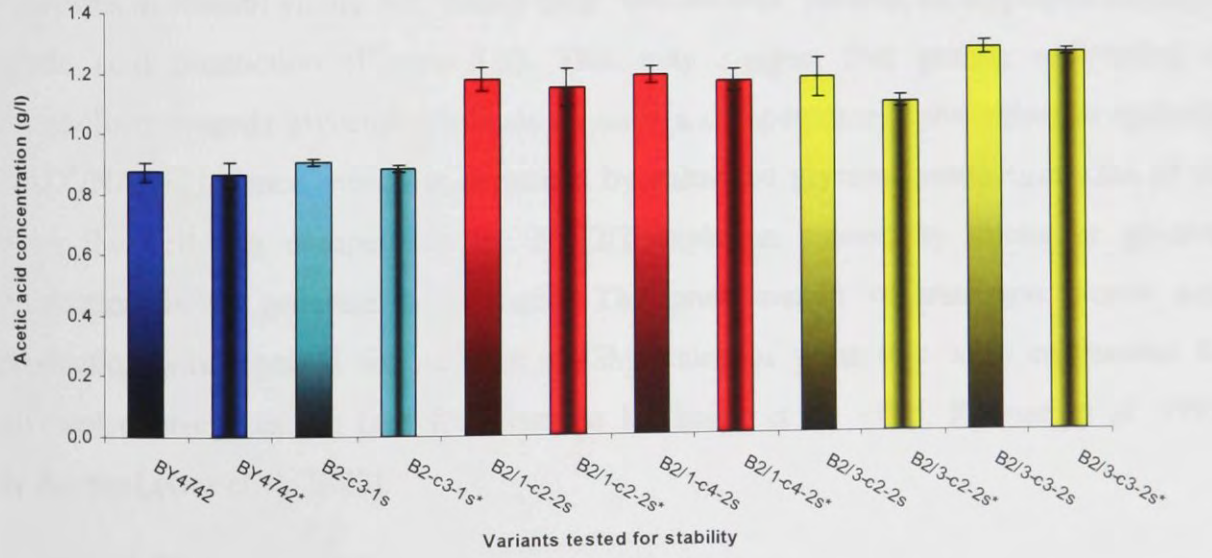
5.12i



5.12ii



5.12iii



5.4 Discussion

The main objective of the work described in this chapter was to investigate whether increasing or introducing a new selection pressure would further improve the low-ethanol phenotypes of B2-c3-1s and A3-c2-1s. This goal was successfully achieved since a number of evolving populations adapted to produce elevated glycerol levels. However, not all evolving populations responded to the selection pressures as expected.

Screening experiments conducted on six populations after approximately 50 generations of exposure to sulfite selective pressure show that the majority of these populations, except EA3/2(S), shifted their metabolism towards enhanced glycerol production; the greatest shift was noted for population samples derived from B2-c3-1s; EB2/1(S). This suggests that higher sulfite levels used for the second round of adaptive evolution induced further metabolic redirection towards increased glycerol production. Individuals that were randomly selected from evolving populations EB2/1(S), EB2/2(S) and EB2/3(S) produced, in most cases, even higher glycerol levels than the populations they were derived from (Figure 5.7i). However, this was not the case for individuals isolated from EA3/1(S), EA3/2(S) and EA3/1(S); in fact, a number of the isolates displayed lower glycerol production than the populations from which they were isolated (see Figure 5.8i).

The best 'high-glycerol' producing variants, B2/1-c2-2s, B2/1-c4-2s, B2/3-c2-2s, B2/3-c3-2s, isolated from populations EB2/1(S) and EB2/3(S), showed substantial decreases in ethanol yields, but, unlike their 'low-ethanol' parents, all displayed increased acetic acid production (Figure 5.9). This may suggest that greater redirection of metabolism towards glycerol synthesis requires a compensatory mechanism to maintain NAD^+/NADH balance, which is perturbed by enhanced glycerol production. One of the ways the cell can compensate for NADH depletion caused by excessive glycerol production is to generate acetic acid. The phenomenon of excessive acetic acid production was reported for number of GM stains of yeast that were engineered for glycerol over-expression (see for example Michnick et al. 1996, Remize et al. 1999, de Barros Lopes et al. 2003).

The question arises: why increased glycerol production did not lead to increased acetic acid yields in evolved, 'low-ethanol' variants from the first round of the selection (see Chapter 4). The answer may lie in the degree to which glycerol production was enhanced; perhaps there is a limit to how far glycerol yields can be driven before redox balance becomes problematic and above this threshold, acetic acid production becomes essential to maintained NADH levels.

Interestingly, unexpected phenotypic changes were observed for individuals derived from evolving populations EA3/1(S), EA3/2(S), EA3/3(S): instead of further improvement of 'low-ethanol' phenotype, 3 out of 4 variants, A3/1-c3-2s, A3/2-c2-2s and A3/2-c3-2s displayed elevated ethanol production (see Figure 5.10). Out of these variants, only A3/1-c3-2s and A3/2-c3-2s had negligible increases in glycerol production, which was coupled with elevated acetic acid production. The reason for a shift away from the desired phenotype is not known, but the result suggests alternative adaptations to sulfite stress may have arisen in the populations, making redirection of metabolism to increased glycerol and decreased ethanol disadvantageous.

The intended effect of introducing osmotic stress was to introduce a glycerol-promoting selection pressure that was different to sulfite; the expectation was that this would lead to a 'build up' of additional 'low-ethanol'-conferring mutations in a genetic background of 'low-ethanol' variants selected in the first round of adaptive evolution in sulfite. Adaptive evolution in YPD-10K, however, did not generate the expected changes. In fact, in most evolving populations there was a reversion parental phenotype. One possible explanation for this is that under entirely different selection pressure, mutations acquired under sulfite selection, conferring 'low-ethanol' phenotype, were disadvantageous. Thus, it would be of interest to conduct adaptive evolution experiments under osmotic stress starting with the original parental strains. In such setting, potential adaptations that have been acquired by 'low-ethanol' variants arisen in sulfite, and pose disadvantageous genetic background for the osmotic stress-driven selection, could have been omitted.

One criticism of the work described in this chapter is that adaptive evolution was performed over a very limited number (i.e. 50) of generations. Nonetheless, for the work

described in Chapter 4, however, this was sufficient to see shifts towards 'low-ethanol' phenotypes, at least for evolving populations derived from AWRI1628; shifts in phenotype of evolving populations derived from BY4742 occurred at some stage between 100 and 150 generations. After 50 generations of the second round of adaptive evolution in sulfite, however, the most 'responsive' strain appeared to be BY4742-derived variant, B2-c3-1s. Why should a BY4742-derived isolate be more 'responsive' to increased sulfite selection pressure than AWRI1628-derived isolate in this second round of adaptive evolution? There is no empirical data to help answer this question, but it is reasonable to argue that B2-c3-1s is genetically different to its original parent, (it clearly has a different phenotype and this difference is inheritable), and therefore its 'new' genetic background may have created the possibility for new adaptations to take place.

Outcomes from the work described in this chapter were undoubtedly compromised by limited time. It would have been desirable to run the adaptive evolution experiments for at least another 50 generation, but this was not possible in the time available. Future experiments in this work should take this into account, and plan for sufficient time to allow at least 100, and preferably 200 generations.

5.5 Conclusions

- Increasing the selection pressure, (i.e. the concentration of sulfite), in adaptive evolutions experiments on 'low-ethanol' variants of laboratory and wine yeast strains, led to isolation of variants with further decrease in ethanol yields.
- Introducing an alternative selection pressure, (i.e. osmotic stress), in adaptive evolutions experiments on 'low-ethanol' variants of laboratory and wine yeast strains, did not lead, in most of cases, to the isolation of variants with improved glycerol production; only a few isolates produced slightly more glycerol than their parental strains .
- The 'best' 'low-ethanol' variant isolated from the work described in this chapter, B2/1-c4-2s, was found to be genetically stable and was chosen for the work described in the following chapters.

CHAPTER 6

PHYSIOLOGICAL CHARACTERIZATION OF THE 'LOW-ETHANOL' VARIANTS

6.1 Objectives

The objective of the work described in this chapter was to better characterize the phenotypes of 'low-ethanol' producing variants B2-c3-1s, B2/1-c4-2s and A3-c2-1s, and their parental strains.

6.2 Introduction

The previous chapters described the isolation of 'low-ethanol' variants B2-c3-1s and B2/1-c4-2s derived from *S. cerevisiae* BY4742, and A3-c2-1s derived from *S. cerevisiae* AWRI1628. Preliminary comparisons of these variants with their parent strains identified statistically significant differences in glycerol, ethanol and acetic acid production, providing some evidence of the 'low-ethanol' producing phenotype. A more comprehensive characterization of the variant phenotypes was however required to test the effectiveness of developing 'low-ethanol' producing wine strains using sulfite and adaptive evolution.

With the above in mind, phenotype characterization in this chapter included quantification of metabolite formation, including glycerol, ethanol and acetic acid, in variant and parent strains as well as their growth and substrate profiles during fermentation; these traits were assessed for incubations conducted under various pH and oxygen concentration conditions. It was anticipated that such results would provide some evidence on how the variants altered their metabolism to adapt to the high sulfite concentrations used in the adaptive evolution experiments described in Chapters 4 and 5.

The response of yeast to sulfite exposure was previously shown to be significantly affected by culture pH (Sections 3.3.2 and 3.3.3). The previous two chapters describe the isolation of variants from adaptive evolution experiments using high sulfite concentrations and a pH of 8. Given that wine fermentations are generally conducted at

an acidic pH, it was important to test the stability of the variant phenotypes when incubated at a pH similar to that of wine fermentations. Such a comparison will indicate whether pH has any influence on the 'low-ethanol' phenotype of the variants.

Wine fermentations typically commence under semi-aerobic conditions but yeast activity uses all available oxygen and the fermentation quickly becomes self-anaerobic. The fermentations described in Chapters 4 and 5, which were used for preliminary assessment of the 'low-ethanol' phenotypes of the isolated variants, were conducted entirely under semi-aerobic conditions, which meant that oxygen was available for growth, especially during the early-mid growth stages. The presence of oxygen meant that there was possible mitochondrial-based TCA cycle activity which could have an impact on the redox balance of the cell. These conditions, taking into account the relatively short incubation time, are dissimilar to standard wine fermentations. Given the importance of redox balance in influencing ethanol, glycerol and acetic acid production in yeast and its role in obtaining the 'low-ethanol' producing variants, it was decided to examine and compare the phenotypes of the parent and mutant strains under aerobic and anaerobic conditions.

6.3 Results

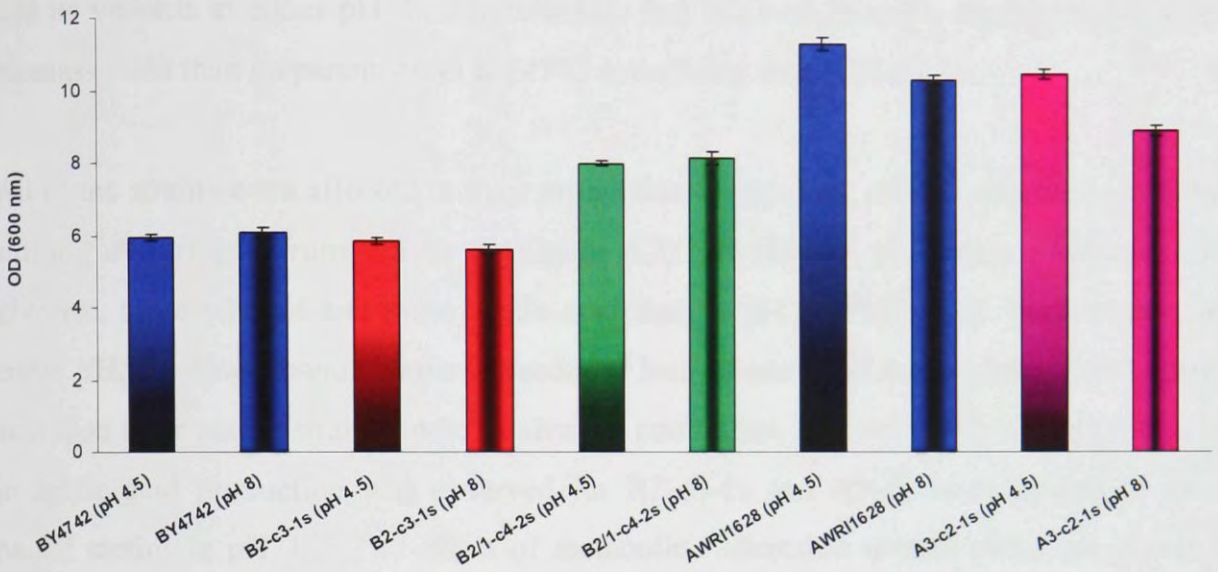
6.3.1 The influence of pH on fermentation profile

The influence of pH on performance of the 'low-ethanol' variants B2-c3-1s, B2/1-c4-2s and A3-c2-1s and their parent stains was investigated by conducting incubations in YPD-10, at pH 4.5 and pH 8 (see Section 2.1.5.4). Incubations were conducted in triplicate and samples were taken at the end point of the fermentation after glucose exhaustion; the samples were analyzed for biomass (Sections 2.2.3) and metabolite production using HPLC (Section 2.2.4.).

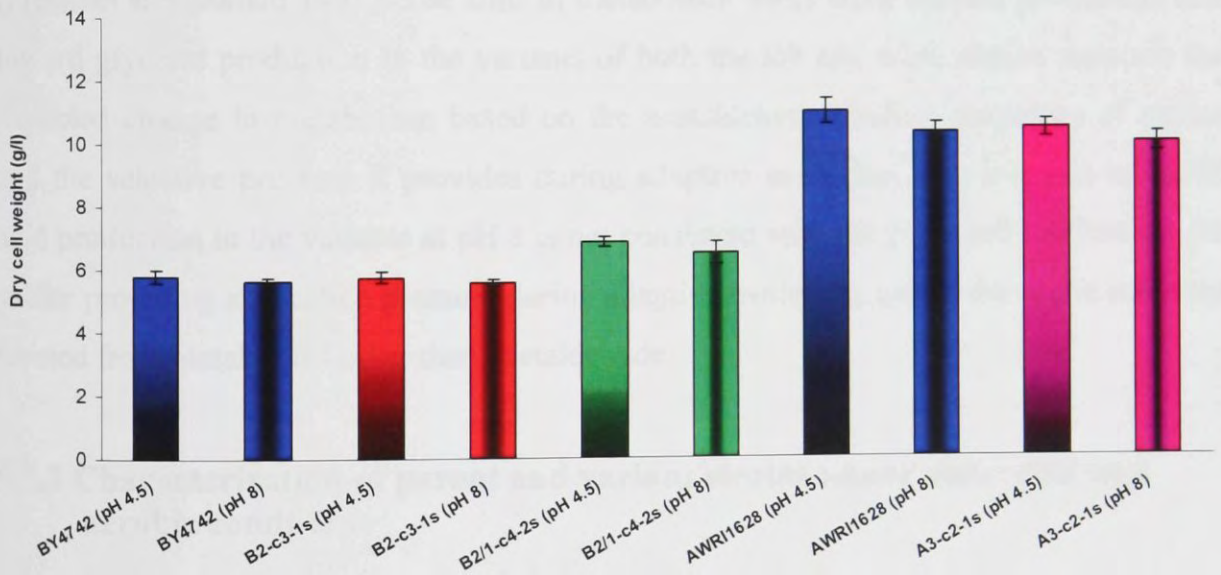
The effect of pH on biomass levels of parent and variant strains at the point of sugar exhaustion are shown in Figure 6.1. The results show that BY4742 and its variants B2-c3-1s and B2/1-c4-2s were not significantly affected in their OD_{600} and dry cell

Figure 6.1 Biomass and absorbance at OD_{600} were measured for *S. cerevisiae* BY4742 (blue) and its 'low-ethanol' variants B2-c3-1s (green) and B2/1-c4-2s (red), and *S. cerevisiae* AWRI1628 (blue) and its 'low-ethanol' variant A3-c2-1s (purple). Incubations were conducted in YPD-10 at pH 4.5 and 8, (30°C/140 rpm), as described in Section 2.1.5.4. Experiments were carried out in triplicate and the error bars represent standard deviation from the mean.

6.1i


OD (600 nm)
0
2
4
6
8
10
12
BY4742 (pH 4.5)
BY4742 (pH 8)
B2-c3-1s (pH 4.5)
B2-c3-1s (pH 8)
B2/1-c4-2s (pH 4.5)
B2/1-c4-2s (pH 8)
AWRI1628 (pH 4.5)
AWRI1628 (pH 8)
A3-c2-1s (pH 4.5)
A3-c2-1s (pH 8)

6.1ii


Dry cell weight (g/l)
0
2
4
6
8
10
12
14
BY4742 (pH 4.5)
BY4742 (pH 8)
B2-c3-1s (pH 4.5)
B2-c3-1s (pH 8)
B2/1-c4-2s (pH 4.5)
B2/1-c4-2s (pH 8)
AWRI1628 (pH 4.5)
AWRI1628 (pH 8)
A3-c2-1s (pH 4.5)
A3-c2-1s (pH 8)

weight by a shift in culture pH from 4.5 to 8. The OD_{600} of AWRI1628 and its variant A3-c2-1s did decrease slightly when the culture pH was increased from 4.5 to 8; this was not reflected in their dry cell weight which showed no statistical difference at either pH. Overall, AWRI1628 and its variant, A3-c2-1s had higher biomass yields than BY4742 and its variants at either pH. It is noteworthy that B2/1-c4-2s had a significantly higher biomass yield than its parent strain BY4742 and sibling strain B2-c3-1s.

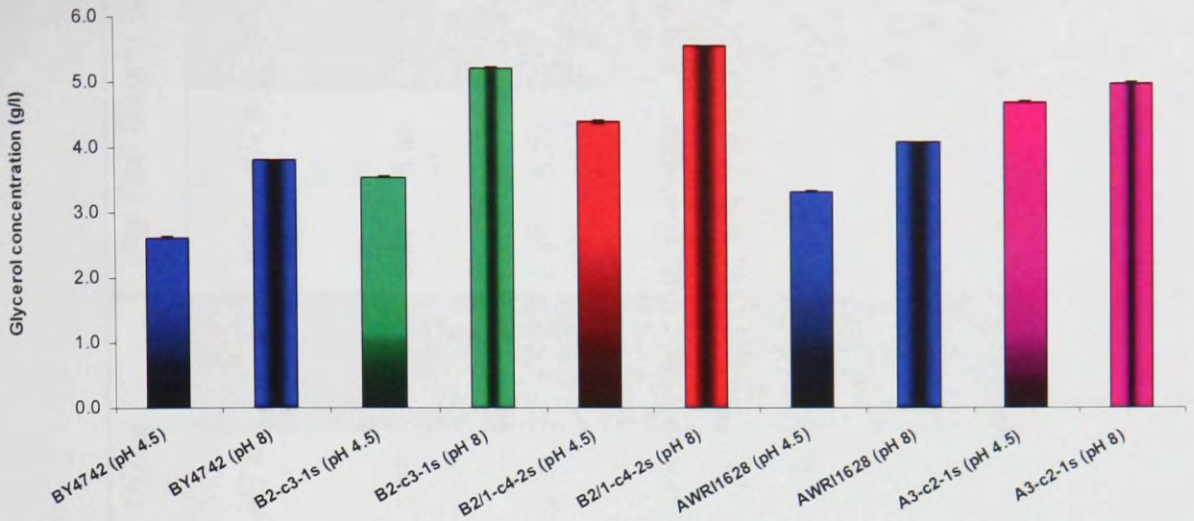
All of the strains were affected in their production of glycerol, ethanol and acetic acid by shifting culture pH from 4.5 to 8 (Figure 6.2). At pH 4.5 all strains produced less glycerol, more ethanol and more acetic acid than at pH 8 (Table 6.1). Furthermore, at either pH, all 'low-ethanol' variants produced less ethanol, and more glycerol and acetic acid than their parent strains under equivalent conditions, however, only a slight increase in acetic acid production was observed for B2-c3-1s and A3-c2-1s compared to their parent strains at pH 4.5. The effect of metabolic redirection toward enhanced glycerol production at alkaline pH has previously been experimentally demonstrated (Freeman and Donald 1957). The shift in metabolism away from ethanol production and toward glycerol production in the variants of both the lab and wine strains supports the expected change in metabolism based on the acetaldehyde-binding properties of sulfite and the selective pressure it provides during adaptive evolution. The increase in acetic acid production in the variants at pH 8 is not consistent with the proposed mechanism for sulfite providing a selection pressure during adaptive evolution, unless the acetic acid was formed from metabolites other than acetaldehyde.

6.3.2 Characterization of parent and variant strains under anaerobic and aerobic conditions

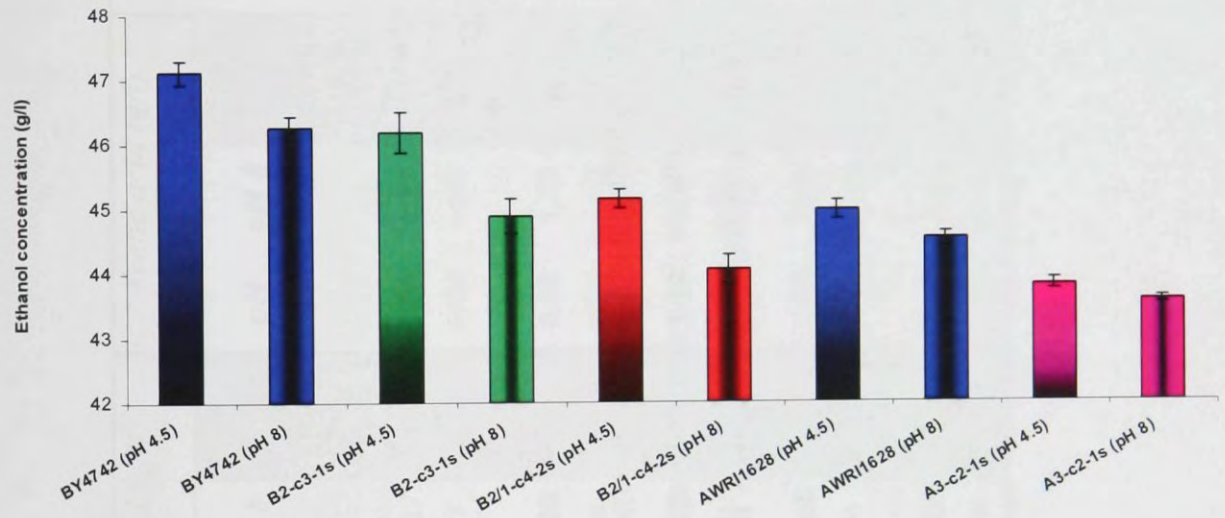
Triplicate aerobic and anaerobic experiments were conducted in YPD-10 at 30°C (see Section 2.1.5.3 and 2.1.5.5). The inocula were grown under semi-anaerobic (for anaerobic experiment) and aerobic (for aerobic experiments) conditions (30°C, 140rpm) until late exponential phase (Section 2.1.5.2). The anaerobic cultures were incubated in an anaerobic hood operating at 0% oxygen. For both anaerobic and aerobic conditions,

Figure 6.2 *S. cerevisiae* BY4742 (blue) and its 'low-ethanol' variants B2-c3-1s (green) and B2/1-c4-2s (red), and *S. cerevisiae* AWRI1628 (blue) and its 'low-ethanol' variant A3-c2-1s (purple) cultures were assayed for glycerol, ethanol and acetic acid concentrations. Experiments were conducted under aerobic conditions in YPD-10 at pH 4.5 or pH 8. Incubations were conducted at 30°C/140 rpm as described in Section 2.1.5.4. Experiments were conducted in triplicate and the error bars represent the standard deviation from the mean.

6.2i



6.2ii



6.2iii

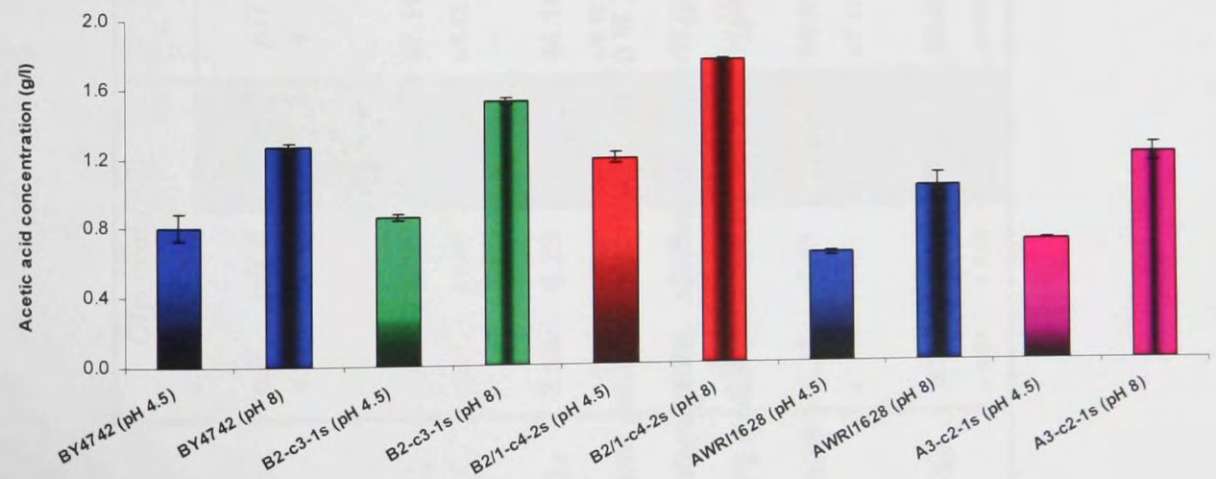


Table 6.1 Concentrations of glycerol, ethanol and acetic acid and biomass formation by parental strains BY4742 and AWRI1628 and their 'low-ethanol' variants B2-c3-1s, B2/1-c4-2s and A3-c2-1s, respectively, under alkaline or acidic pH conditions. Aerobic incubations were conducted in YPD-10 at 30°C/140 rpm, until glucose exhaustion .

	Glycerol (g/l)			*Ethanol (g/l)*			*Acetic acid (g/l)*			*OD_{600}*			*Dry Cell Weight (g)*		
	pH 4.5	***pH 8***	% decrease at pH 4.5	***pH 4.5***	***pH 8***	% increase at pH 4.5	***pH 4.5***	***pH 8***	% decrease at pH 4.5	***pH 4.5***	***pH 8***	% decrease at pH 4.5	***pH 4.5***	***pH 8***	% decrease at pH 4.5
BY4742	**2.61** ±0.01	**3.80** ± 0.02	**31.1**	**47.11** ± 0.17	**46.27** ± 0.17	**1.8**	**0.81** ± 0.08	**1.27** ± 0.02	**36.4**	**6.06** ± 0.12	**5.95** ± 0.09	**Not significant**	**5.67** ± 0.21	**5.80** ± 0.1	**Not significant**
B2-c3-1s	**3.54** ± 0.02	**5.23** ± 0.01	**32.2**	**46.19** ± 0.32	**44.90** ± 0.26	**2.9**	**0.86** ± 0.02	**1.53** ± 0.02	**43.9**	**5.62** ± 0.13	**5.85** ± 0.09	**Not significant**	**5.57** ± 0.06	**5.77** ± 0.15	**Not significant**
B2/1-c4-2s	**4.42** ± 0.02	**5.58** ± 0.01	**20.8**	**45.17** ± 0.15	**44.07** ± 0.21	**2.5**	**1.19** ± 0.03	**1.75** ± 0.01	**32.1**	**8.18** ± 0.16	**8.01** ± 0.07	**Not significant**	**6.50** ± 0.2	**6.87** ± 0.35	**Not significant**
AWRI1628	**3.35** ± 0.01	**4.11** ± 0.01	**18.4**	**44.99** ± 0.15	**44.56** ± 0.11	**1.0**	**0.63** ± 0.01	**1.01** ± 0.08	**37.9**	**10.38** ± 0.12	**11.38** ± 0.18	**8.8**	**10.37** ± 0.42	**11.03** ± 0.29	**Not significant**
A3-c2-1s	**4.74** ± 0.01	**5.04** ± 0.02	**5.9**	**43.84** ± 0.08	**43.58** ± 0.04	**0.6**	**0.69** ± 0.01	**1.20** ± 0.05	**42.0**	**8.99** ± 0.13	**10.57** ± 0.13	**15.0**	**10.07** ± 0.31	**10.50** ± 0.3	**Not significant**

samples were taken at regular intervals and used to determine absorbance at 600 nm (OD_{600}), cell counts, and metabolite analysis (for further details see Sections 2.2.1, 2.2.2 and 2.2.4 respectively).

6.3.2.1 *S. cerevisiae* BY4742 and variants B2-c3-1s and B2/1-c4-2s

(A) Aerobic conditions

Under aerobic conditions, BY4742 and the 'low-ethanol' variants B2-c3-1s and B2/1-c4-2s utilized all available glucose within approximately 27, 30 and 32 hours respectively (Figure 6.3). BY4742, with a final OD_{600} of 6.1, reached a final cell population of 2.3 x 10^8 cells/ml while B2-c3-1s reached an OD_{600} of 5.66 which corresponded to 2.0 x 10^8 cells/ml; B2/1-c4-2s had the highest biomass yield with a final OD_{600} of 7.98 and cell population of 2.7 x 10^8 cells/ml. Both of the 'low-ethanol' variants, B2-c3-1s and B2/1-c4-2s, had lower growth rates compared to the parent strain, which is reflected in their slightly lower glucose utilization rate.

Biosynthesis of glycerol, ethanol and acetic acid during aerobic incubation of BY4742, and its 'low-ethanol' variants is shown in Figure 6.4. The parent strain had a glycerol yield of 2.39 g/l, while B2-c3-1s and B2/1-c4-2s produced 3.5 g/l and 4.43 g/l of extracellular glycerol respectively. Increased glycerol production by B2-c3-1s and B2/1-c4-2s was accompanied by a slight decrease in final ethanol yield and productivity (Figure 6.4, Table 6.2). Acetic acid yields for the 'low-ethanol' variants had no obvious trend compared to the parent strain. B2-c3-1s had a lower acetic acid yield while B2/1-c4-2s had a higher acetic acid yield (Figure 6.4; Table 6.2).

(B) Anaerobic conditions

The glucose utilization rates and biomass profiles for BY4742 and 'low-ethanol' variants, B2-c3-1s and B2/1-c4-2s, were determined during incubation under anaerobic conditions

Figure 6.3 Growth and glucose concentration profiles of *S. cerevisiae* BY4742 (blue) and 'low-ethanol' variants B2-c3-1s (green) and B2/1-c4-2s (red) during growth in YPD-10 (pH 4.5) under anaerobic or aerobic conditions. Incubations were conducted at 30°C/140 rpm. Each fermentation was carried out in triplicate and the error bars represent the standard deviation from the mean.

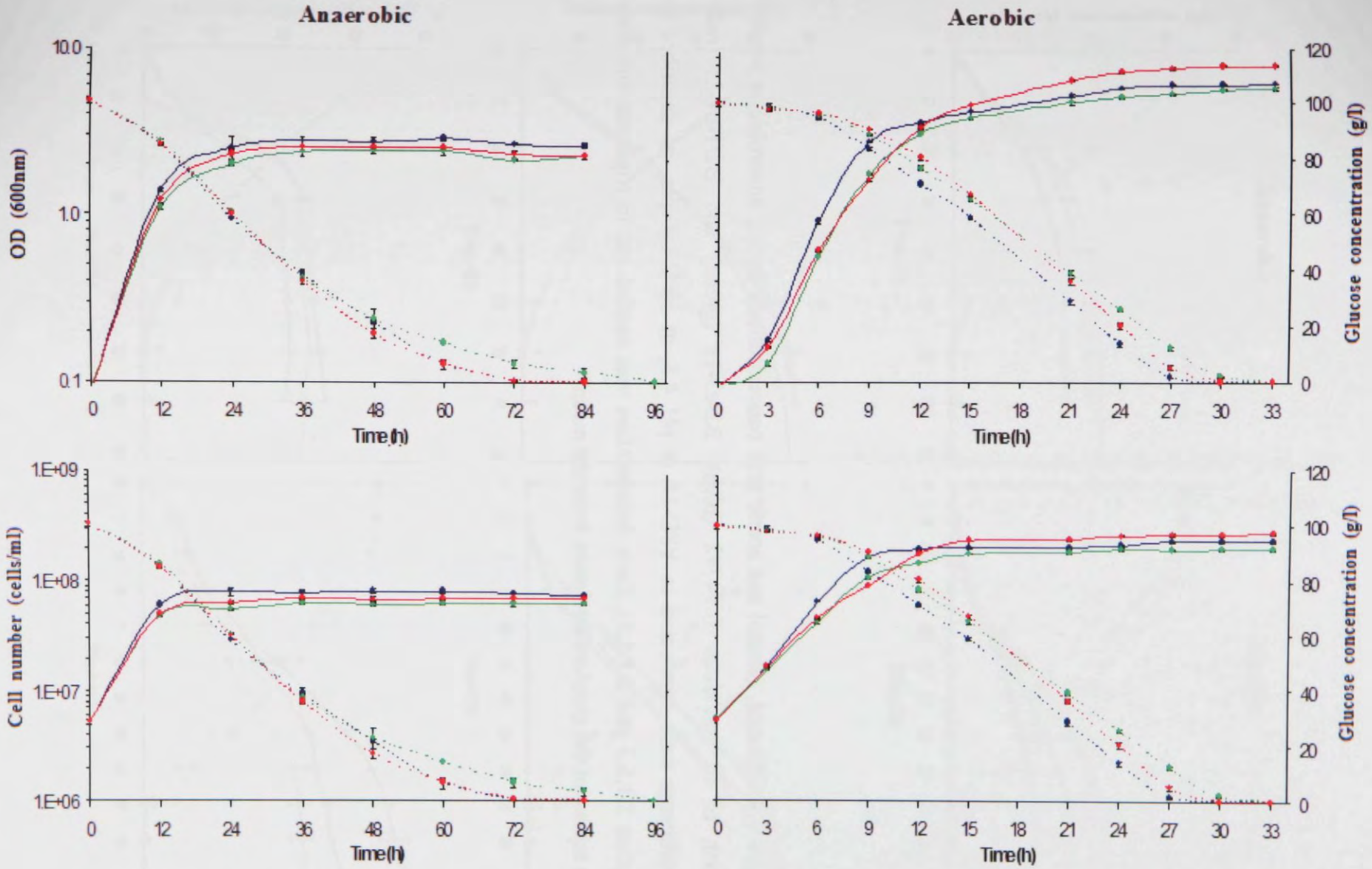

Anaerobic
Aerobic
OD (600nm)
10.0
1.0
0.1
Cell number (cells/ml)
1.E+09
1.E+08
1.E+07
1.E+06
Glucose concentration (g/l)
120
100
80
60
40
20
0
0 12 24 36 48 60 72 84 96
0 3 6 9 12 15 18 21 24 27 30 33
Time(h)

Figure 6.4 Glycerol, ethanol and acetic acid concentrations during anaerobic or aerobic growth of *S. cerevisiae* BY4742 (blue), B2-c3-1s (green) and B2/1-c4-2s (red). Experiments were conducted in YPD-10 at pH 4.5, at 30°C/140rpm, as described in (Section 2.1.5.3 and 2.1.5.5). Each fermentation was carried out in triplicate and error bars represent the standard deviation from the mean.

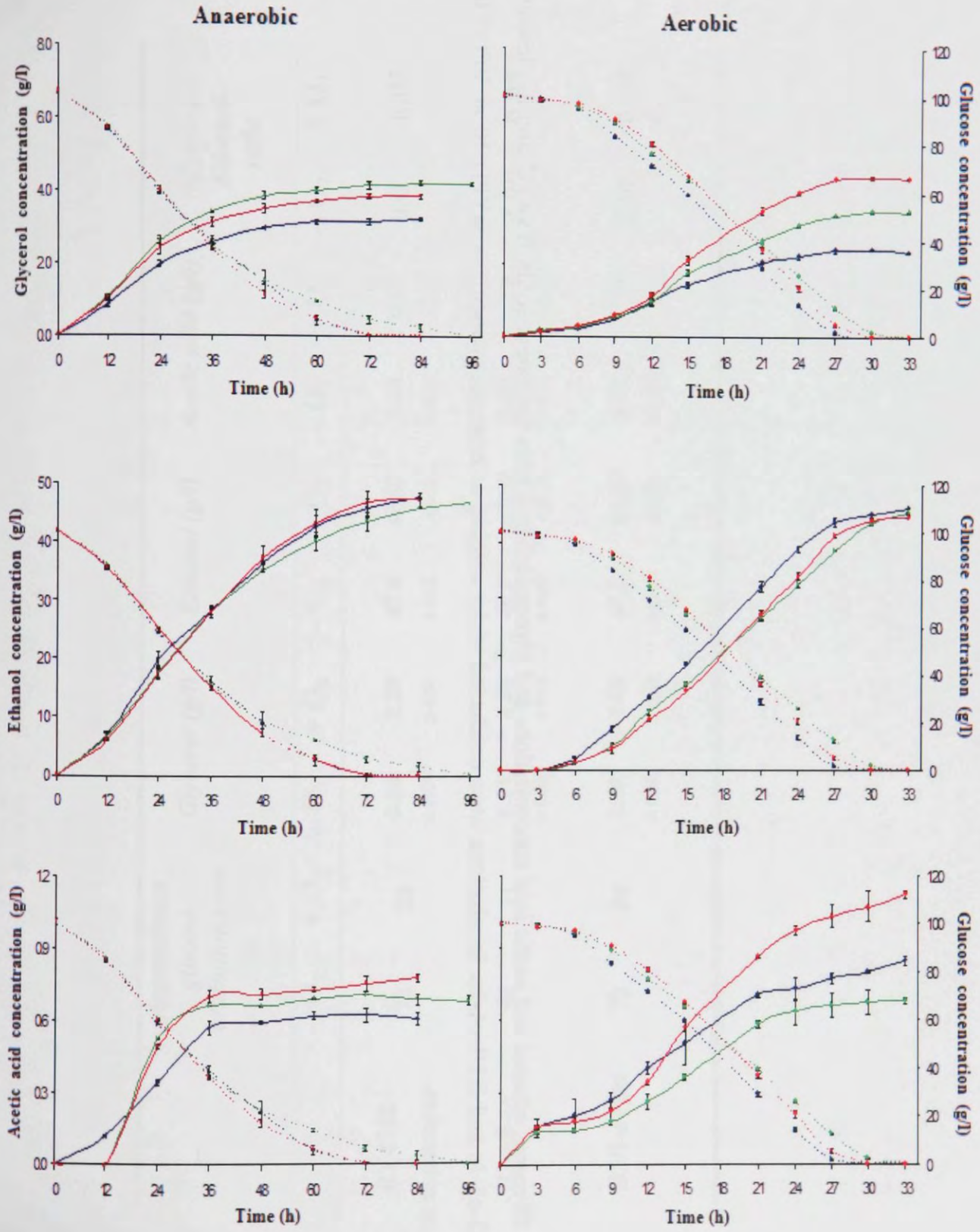

Anaerobic
Aerobic
Glycerol concentration (g/l)
Ethanol concentration (g/l)
Acetic acid concentration (g/l)
Glucose concentration (g/l)
Time (h)
Time (h)
Time (h)
Glucose concentration (g/l)
Time (h)
Glucose concentration (g/l)
Time (h)

Table 6.2 Glycerol, ethanol and acetic acid concentrations and glycerol/ethanol ratio for parent strain BY4742 and its 'low-ethanol' variants B2-c3-1s and B2/1-c4-2s. Incubations were conducted under aerobic or anaerobic conditions inYPD-10 at 30°C/140 rpm, until glucose exhaustion.

	Approximate glucose utilization time (h)		*Glycerol (g/l)*		*Ethanol (g/l)*		*Acetic acid (g/l)*		*Glycerol/ Ethanol ratio*	
	– O_2	**+ O_2**	**– O_2**	**+ O_2**	**– O_2**	**+ O_2**	**– O_2**	**+ O_2**	**– O_2**	**+ O_2**
BY4742	**72**	**28**	**3.24** ± 0.04	**2.39** ± 0.01	**47.6** ± 0.12	**46.07** ± 0.01	**0.61** ± 0.03	**0.87** ± 0.02	**0.07**	**0.05**
B2-c3-1s	**96**	**31**	**4.21** ± 0.05	**3.50** ± 0.03	**46.7** ± 0.20	**45.22** ± 0.15	**0.68** ± 0.02	**0.79** ± 0.01	**0.09**	**0.08**
B2/1-c4-2s	**72**	**30**	**3.86** ± 0.06	**4.43** ± 0.02	**47.2** ± 0.17	**44.50** ± 0.26	**0.77** ± 0.02	**1.14** ± 0.02	**0.08**	**0.10**

(Figure 6.3). Compared to their aerobic performance, the growth of all strains under anaerobic conditions was slower and biomass yields were significantly lower. Under anaerobic conditions, the parent strain BY4742 had a slightly higher biomass yield compared to both 'low-ethanol' variants, although the difference is very small (Figure 6.3, Table 6.2). Total fermentation times were much longer under anaerobic conditions compared to aerobic growth, with the time taken for the anaerobic cultures to reach glucose exhaustion being more than double the time taken under aerobic cultivation. The sugar utilization rate was similar for BY4742 and B2/1-c4-2s with these two strains consuming all the available glucose after approximately 72 hours of anaerobic incubation. B2-c3-1s needed an additional 24 hours to utilize the remaining glucose.

Glycerol, ethanol and acetic acid concentration profiles during anaerobic growth of BY4742 and 'low-ethanol' variants, B2-c3-1s, B2/1-c4-2s, are shown in Figure 6.4. Both 'low-ethanol' variants produced higher glycerol yields compared to BY4742. B2-c3-1s had the highest glycerol production with a glycerol yield of 4.2 g/l; B2/1-c4-2s produced 3.85 g/l of glycerol (Table 6.2). For and B2/1-c4-2s, There was very little difference in ethanol yields between all three strains with B2-c3-1s having the lowest ethanol yield at 46.7 g/l. BY4742 had an acetic acid yield of 0.6 g/l which was lower than both 'low-ethanol' variants B2-c3-1s and B2/1-c4-2s (see Figures 6.4 and 6.8, Table 6.2).

Overall, the time taken for all three strains to complete an anaerobic fermentation was more than double the fermentation time under aerobic conditions. Glycerol production was always higher in both variant strains compared to the parent strain, however, no other clear trends are evident in the results. Although acetic acid production was generally higher in the variants, it wasn't always the case. Aerobic conditions tended to produce higher acetic acid yields compared to anaerobic growth. Ethanol yields were slightly lower under aerobic conditions but there was very little difference in ethanol yield between all strains during anaerobic cultivation. The glycerol/ethanol ratios were always higher in the variants, with the greater difference between the parent and variant strains occurring under aerobic conditions (Table 6.2).

6.3.2.2 *S. cerevisiae* AWRI1628 and variant A3-c2-1s

(A) Aerobic conditions

Aerobic incubations of AWRI1628 and its 'low ethanol' variant, A3-c2-1s, were performed as described in Section 2.1.5.3. Growth profiles and sugar utilization for these strains incubated in presence of oxygen are shown in Figure 6.5. The glucose utilization rate was similar for AWRI1628 and A3-c2-1s; both strains consuming all of the available sugar in approximately 18 hours. Both strains had similar growth profiles over the 21 hour sampling period. When the glucose levels were exhausted, AWRI1628 reached an OD_{600} of 10.26 which corresponded to approximately 4.3×10^8 cells/ml, while A3-c2-1s had an OD_{600} of 9.1, corresponding to a cell population of 3.9×10^8 cells/ml.

Glycerol, ethanol and acetic acid profiles for AWRI1628 and A3-c2-1s under aerobic conditions are shown in Figures 6.6 and 6.8, and Table 6.3. Glycerol yield and productivity for A3-c2-1s were considerably higher compared to the parent strain, with a 53% increase in glycerol yield compared to AWRI1628 at the time of glucose exhaustion. Higher glycerol levels for A3-c2-1s were reflected by a slightly lower ethanol yield than AWRI1628, with an average ethanol yield of 42.1 g/l compared to 43.5 g/l for AWRI1628 i.e. around 3.2% decrease in ethanol yield for A3-c2-1s. A3-c2-1s also had higher acetic acid productivity and yields, with a final acetic acid yield of 0.7 g/l, compared to 0.55 g/l for AWRI1628, i.e. 27% increase in acetic acid yield for A3-c2-1s.

(B) Anaerobic Conditions

Anaerobic incubations of *S. cerevisiae* AWRI1628 and its 'low-ethanol' variant A3-c2-1s were conducted as described in Section 2.1.5.5. Biomass and glucose concentration profiles are shown in Figure 6.5. Under anaerobic conditions AWRI1628 used glucose more rapidly and had a higher biomass growth rate and yield compared to A3-c2-1s. AWRI1628 consumed all of the available glucose within 48 hours, while A3-c2-1s took

Figure 6.5 Growth and glucose utilization profiles of *S. cerevisiae* AWRI1628 (blue) and 'low-ethanol' variant A3-c2-1s (purple) during growth in YPD-10 (pH 4.5) under anaerobic and aerobic conditions. Incubations were conducted at 30°C/140 rpm. Each fermentation was carried out in triplicate and the error bars represent the standard deviation from the mean.

Aerobic
Anaerobic
Glucose concentration (g/l)
Glucose concentration (g/l)
OD (600nm)
Cell number (cells/ml)
Time(h)
Time(h)
Time(h)
Time(h)

Figure 6.6 Glycerol, ethanol and acetic acid concentrations during anaerobic and aerobic growth of *S. cerevisiae* AWRI1628 (blue) and 'low-ethanol' variant A3-c2-1s (purple). Experiments were conducted in YPD-10 at pH 4.5 (30°C/140rpm) as described in (Section 2.1.5.3 and 2.1.5.5). Each fermentation was carried out in triplicate and error bars represent the standard deviation from the mean.

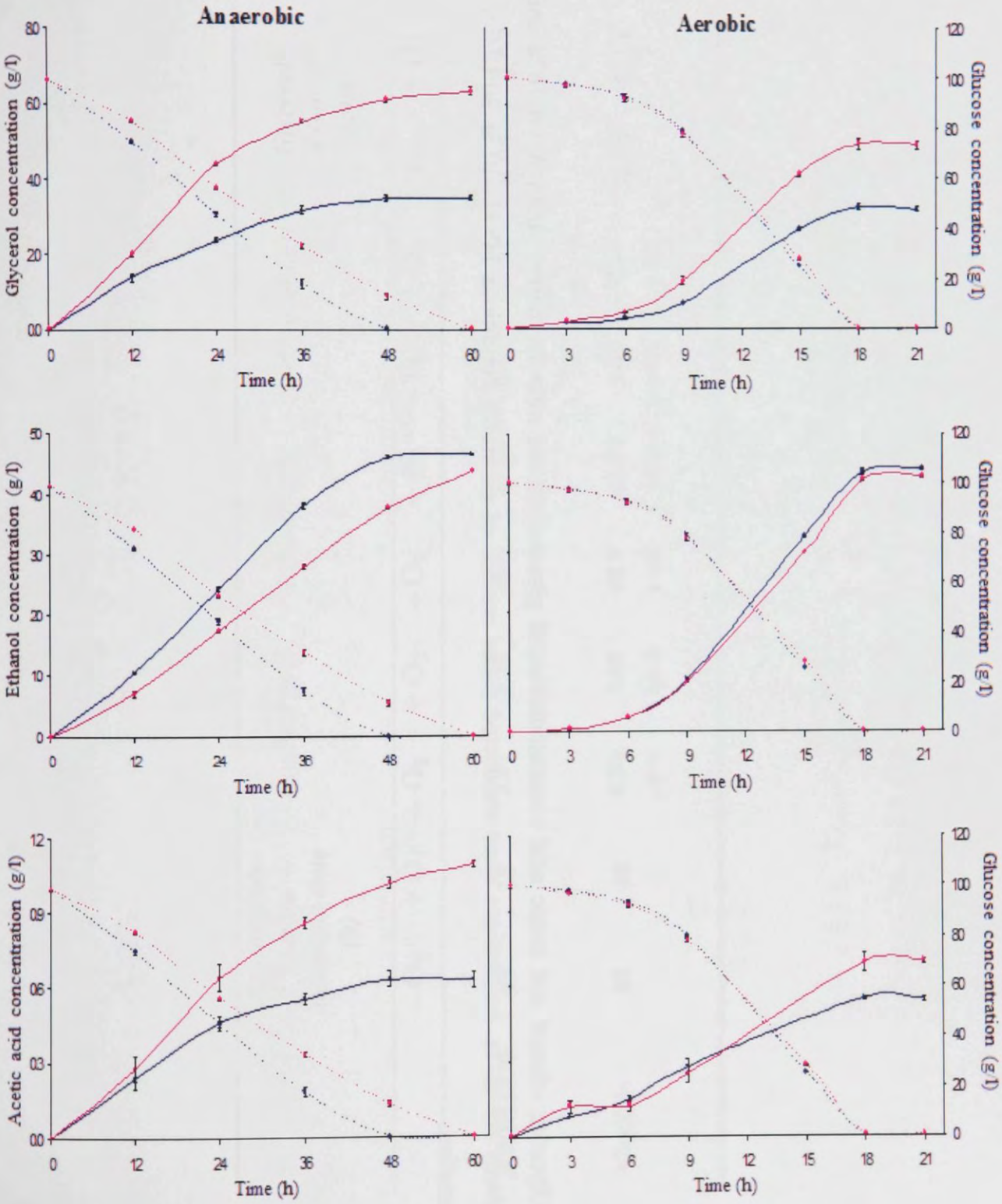
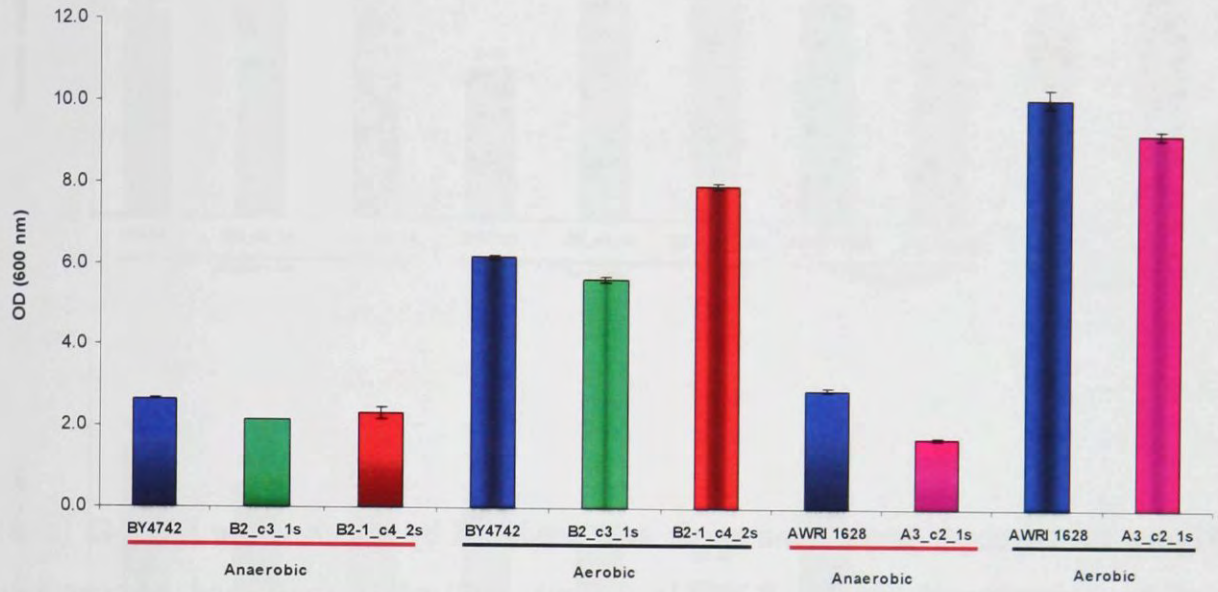

Anaerobic
Aerobic
Glycerol concentration (g/l)
Ethanol concentration (g/l)
Acetic acid concentration (g/l)
Glucose concentration (g/l)
Time (h)

Table 6.3 Glycerol, ethanol and acetic acid concentrations and glycerol/ethanol ratio for parent strain AWRI1628 and its 'low-ethanol' variants A3-c2-1s. Incubations were conducted under aerobic or anaerobic conditions inYPD-10 at 30°C/140 rpm, until glucose exhaustion.

	Approximate glucose utilization time (h)		*Glycerol (g/l)*		*Ethanol (g/l)*		*Acetic acid (g/l)*		*Glycerol/ Ethanol ratio*	
	– O_2	**+ O_2**	**– O_2**	**+ O_2**	**– O_2**	**+ O_2**	**– O_2**	**+ O_2**	**– O_2**	**+ O_2**
AWRI1628	**48**	**18**	**3.51** ± 0.08	**3.21** ± 0.07	**46.6** ± 0.16	**43.91** ± 0.02	**0.63** ± 0.03	**0.54** ± 0.01	**0.08**	**0.07**
A3-c2-1s	**60**	**18**	**6.32** ± 0.10	**4.90** ± 0.10	**43.7** ± 0.05	**42.79** ± 0.33	**1.09** ± 0.01	**0.69** ± 0.01	**0.14**	**0.11**

Figure 6.7 Absorbance (OD_{600}) and dry cell weight (g/l) for *S. cerevisiae* BY4742 (blue) and its 'low-ethanol' variants B2-c3-1s (green), B2/1-c4-2s (red), and *S. cerevisiae* AWRI1628 (blue) and its 'low-ethanol' variant A3-c2-1s (purple), were measured at the time of glucose exhaustion. Incubations were conducted in anaerobic and aerobic conditions as described in Section 6.2.2. All values are means of triplicate and error bars represent the standard deviation from the mean.


OD (600 nm)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
BY4742
B2_c3_1s
B2-1_c4_2s
BY4742
B2_c3_1s
B2-1_c4_2s
AWRI 1628
A3_c2_1s
AWRI 1628
A3_c2_1s
Anaerobic
Aerobic
Anaerobic
Aerobic

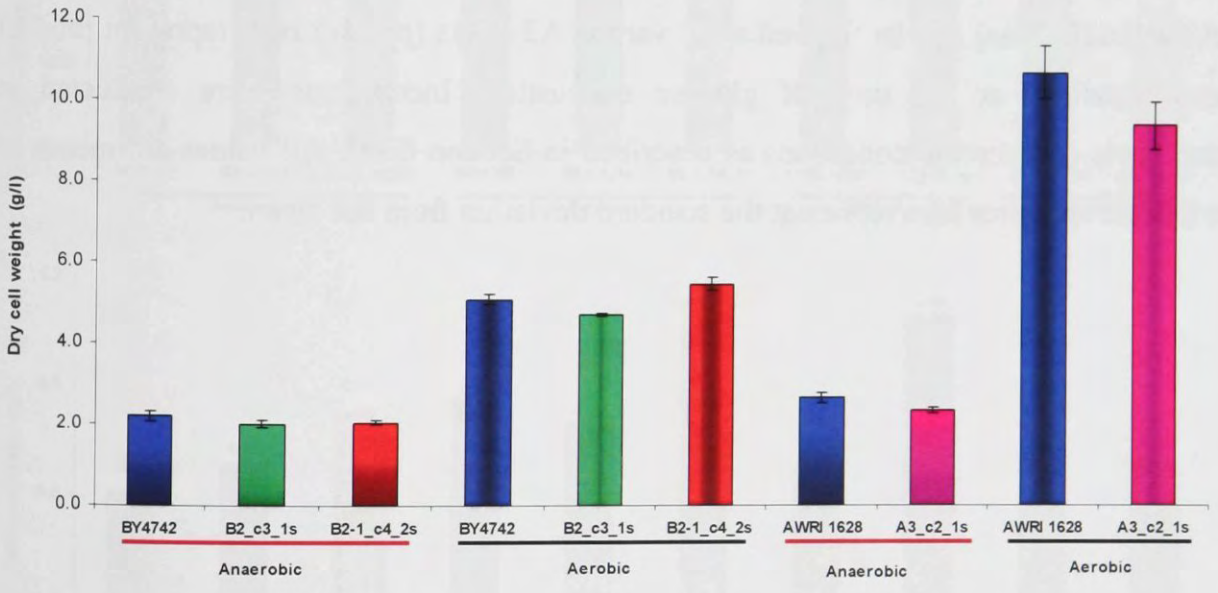
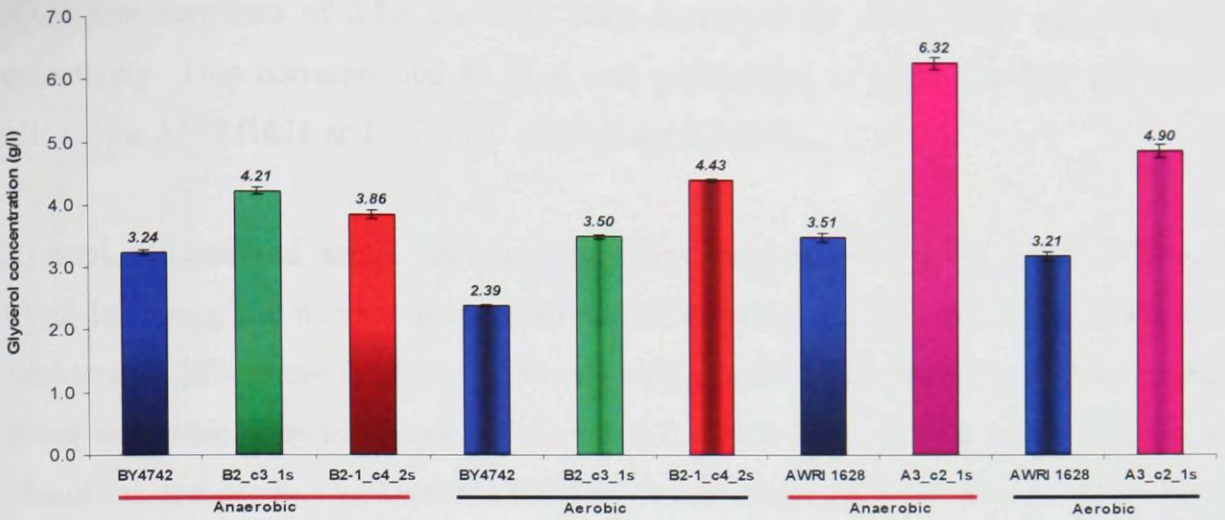
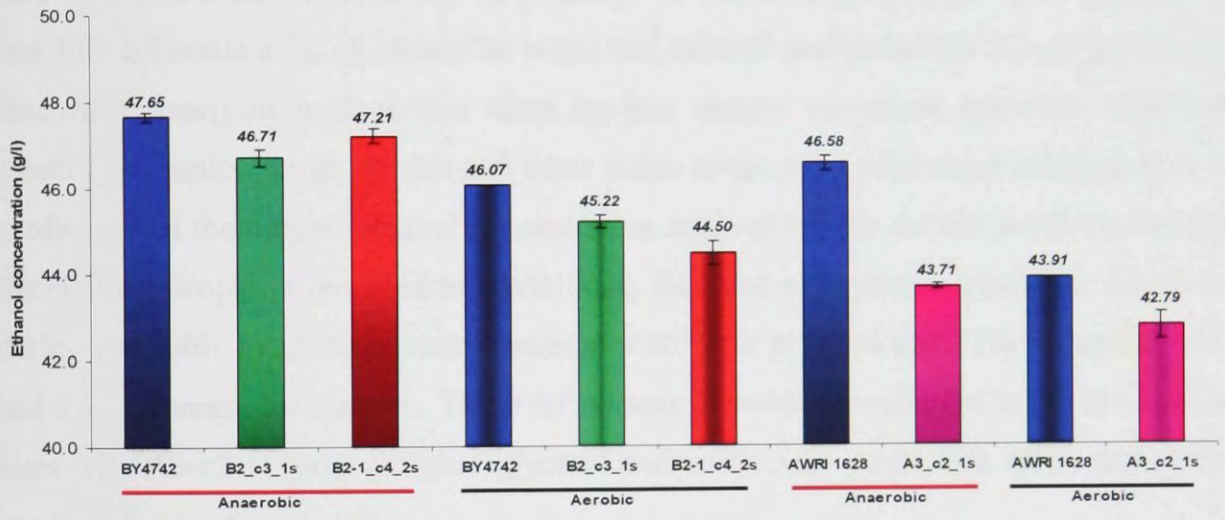
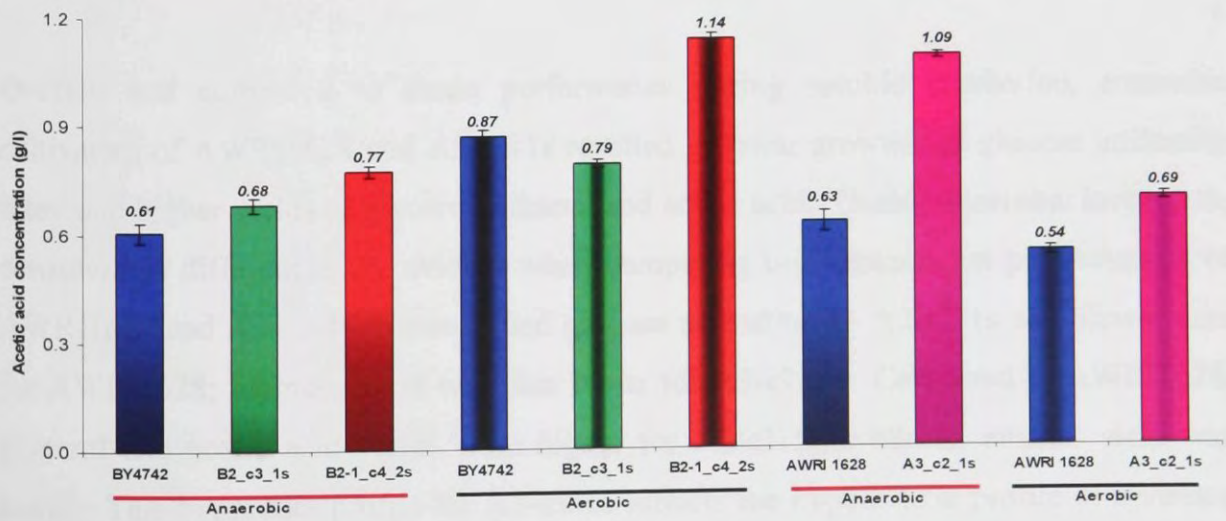

Dry cell weight (g/l)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
BY4742
B2_c3_1s
B2-1_c4_2s
BY4742
B2_c3_1s
B2-1_c4_2s
AWRI 1628
A3_c2_1s
AWRI 1628
A3_c2_1s
Anaerobic
Aerobic
Anaerobic
Aerobic

Figure 6.8 Glycerol, ethanol and acetic acid produced by *S. cerevisiae* BY4742 (blue) and its 'low-ethanol' variants B2-c3-1s (green), B2/1-c4-2s (red), and *S. cerevisiae* AWRI1628 (blue) and its 'low-ethanol' variant A3-c2-1s (purple) Bars represent product concentrations at the time of glucose exhaustion. Incubations were conducted in anaerobic and aerobic conditions as described in Section 6.2.2. All values are means of triplicate and error bars represent the standard deviation from the mean.


Glycerol concentration (g/l)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
3.24
4.21
3.86
2.39
3.50
4.43
3.51
6.32
3.21
4.90
BY4742
B2_c3_1s
B2-1_c4_2s
BY4742
B2_c3_1s
B2-1_c4_2s
AWRI 1628
A3_c2_1s
AWRI 1628
A3_c2_1s
Anaerobic
Aerobic
Anaerobic
Aerobic


Ethanol concentration (g/l)
50.0
48.0
46.0
44.0
42.0
40.0
47.65
46.71
47.21
46.07
45.22
44.50
46.58
43.71
43.91
42.79
BY4742
B2_c3_1s
B2-1_c4_2s
BY4742
B2_c3_1s
B2-1_c4_2s
AWRI 1628
A3_c2_1s
AWRI 1628
A3_c2_1s
Anaerobic
Aerobic
Anaerobic
Aerobic


Acetic acid concentration (g/l)
1.2
0.9
0.6
0.3
0.0
0.61
0.68
0.77
0.87
0.79
1.14
0.63
1.09
0.54
0.69
BY4742
B2_c3_1s
B2-1_c4_2s
BY4742
B2_c3_1s
B2-1_c4_2s
AWRI 1628
A3_c2_1s
AWRI 1628
A3_c2_1s
Anaerobic
Aerobic
Anaerobic
Aerobic

up to 60 hours to consume all of the available glucose. At the point of glucose exhaustion, OD_{600} measurements of 2.85 and 1.75 were measured for AWRI1628 and A3-c2-1s respectively. This corresponded to final cell populations of approximately 8.2 x 10^7 cells/ml for AWRI1628 and 3.7 x 10^7 cells/ml for A3-c2-1s.

Glycerol, ethanol and acetic acid concentrations were monitored for AWRI1628 and A3-c2-1s during the anaerobic incubations as described in Section 2.1.5.5. There are considerable differences in glycerol, ethanol and acetic acid formation by the two strains during anaerobic growth (Figures 6.6 and 6.7; Table 6.3). At the point of glucose exhaustion, end product yields for AWRI1628 were 3.4 g/l glycerol, 46.6 g/l ethanol and 0.63 g/l acetic acid, while for A3-c2-1s the yields were 6.3 g/l glycerol, 43.7 g/l ethanol and 1.09 g/l acetic acid. It should be noted that ethanol production for A3-c2-1s had not reached a clearly defined plateau when the last sample was taken, however, based on ethanol production trends by this and other stains at the point of glucose exhaustion, it is predicted that the highest ethanol concentration attained by this culture is represented by the 60 hour sample. Compared to AWRI1628, the trend in byproduct yields for A3-c2-1s during anaerobic incubation were increases of 85% for glycerol and 73% for acetic acid, and a 6% decrease for ethanol. These differences in yields are reflected in the production rates with A3-c2-1s having higher glycerol and acetic acid production rates, and lower production rates for ethanol.

Overall, and compared to strain performance during aerobic incubation, anaerobic cultivation of AWRI1628 and A3-c2-1s resulted in lower growth and glucose utilization rates, and higher yields of glycerol, ethanol and acetic acid. During anaerobic incubation, considerable differences are evident when comparing the fermentation performances of AWRI1628 and A3-c2-1s. Growth and glucose utilization by A3-c2-1s was slower than for AWRI1628; biomass yield was also lower for A3-c2-1s. Compared to AWRI1628, glycerol and acetic acid yields were higher for A3-c2-1s, while its ethanol yield was lower. This byproduct profile for A3-c2-1s reflects the hypothetical profile of a variant that has evolved from long-term incubation with sulfite as a selection pressure. The increase in acetic acid yield was not expected, however, given that sulfite was presumably

maintaining low intracellular acetaldehyde concentrations during the adaptive evolution experiment.

6.4 Discussion

The main objective of this chapter was to characterize the phenotypes of 'low-ethanol' variants created during the work described in Chapters 4 and 5. The results revealed some unique variant phenotypes under various environmental conditions. Given that the adaptive evolution experiments were conducted under aerobic conditions at pH 8, and that wine incubations are normally anaerobic at acidic pH, it was of particular interest to document the response of all strains to a pH shift from 8 to 4.5, and from aerobic to anaerobic conditions.

6.4.1 Effect of pH on fermentation profile of 'low-ethanol' variants

The incubation of parent and variant strains in acidic or alkaline conditions had a significant impact on their metabolism. All of the strains produced more glycerol at pH 8, compared to pH 4.5. This effect of alkaline conditions on glycerol production by yeast has previously been observed (Freeman and Donald, 1957). The magnitude of metabolic redirection towards glycerol production at pH 8 varied for different strains, the largest increase in glycerol yield was observed for 'low-ethanol' variant B2-c3-1s. For all strains, increased glycerol production at high pH was accompanied by a decrease in ethanol production. All strains also had elevated acetic acid production at pH 8. Generally, increased glycerol production rates were accompanied by lower ethanol production rates and higher acetic acid production rates, providing evidence of the metabolic links between the pathways of these metabolic products; NAD^+ or NADH availability most likely governing the metabolic flux of the respective pathways. Although there is no clear explanation in literature on how pH influences redox balance of the cell, it could be associated with the higher intracellular pH levels that exist at high external pH and, as a consequence, a low availability of protons for NADH production from NAD^+ (Imai and Ohno, 1995a; Imai and Ohno, 1995b).

Importantly, the relative differences in glycerol/ethanol ratios between parent and variant strains were preserved in switching culture pH from alkaline to acidic. This demonstrates that the variants were stable since they did not lose their 'low-ethanol' phenotype at low pH. This observation supports the use of adaptive evolution experiments using sulfite at pH 8, by demonstrating that variants created by this process will retain their phenotype when used in wine-like pH conditions.

6.4.2 Adaptive evolution and sulfite exposure

Although sulfite has been used to increase glycerol production during batch cultivation (Kalle and Naik 1987, Petrovska et al. 1999), there is no evidence in the literature relating to sulfite being used as a selection pressure to create mutants that would constitutively produce higher glycerol yields. Glycerol production for all 'low-ethanol' variants described in this chapter was higher than their respective parent strains, supporting the concept that adaptive evolution experiments using sulfite as a selection pressure can force the evolution of higher glycerol-producing strains. The biggest increase in glycerol production was for the wine strain variant, A3-c2-1s, with an 85% increase in glycerol yield compared to its parent strain.

Glycerol production in most cases was higher under anaerobic conditions compared to aerobic conditions, the exception being for incubations using B2/1-c4-2s. This could be attributable to NADH oxidation by mitochondrial-linked NADH dehydrogenase, or other mitochondrial mechanisms, which would only have a significant contribution under aerobic conditions. Mitochondrial-based contributions to NADH oxidation are, however, normally low during aerobic incubation under high sugar concentrations, due to glucose repression of a number NADH-oxidising mitochondrial mechanisms (see, for review, Bakker et al. 2001). Nevertheless, aerobic adaptive evolution of the parent strains to produce 'low-ethanol' variants, as used in this project, would have provided selection pressure for the creation of mutants that are less affected by glucose repression of mitochondrial-based NADH oxidation. Thus, under aerobic conditions, mitochondrial activity in these variants would more efficiently oxidize cytostolic NADH to NAD^+, which reduces glycerol production by either NAD^+ inhibition of glycerol-3-P

dehydrogenase activity or limiting the cystolic supply of NADH. Under anaerobic conditions, the contribution of mitochondrial activity to the cellular NAD^+/NADH ratio could be considered negligible placing greater demand on glycerol production for maintaining intracellular redox balance. With this proposed mechanism of adaptation in mind, the impact of mitochondrial activity on NAD^+/NADH ratio could be significant when aerobic conditions are used for the production of 'low-ethanol' variants. This needs to be considered in the design of future adaptive evolution experiments for the purpose of generating high glycerol-producing variants.

The phenotype of 'low-ethanol' variant B2/1-c4-2s supports the concept of sulfite-generated adaptation conferring behavioral changes in mitochondrial-based activities. Yeast producing elevated glycerol yields tend to have lower biomass yields, as was the case for 'low-ethanol' variants B2-c3-1s and A3-c3-1s, as well as for a number of strains genetically engineered for glycerol over-production, (see for example, Michnick et al 1996, Remize et al. 1999). However, variant B2/1-c4-2s attained high biomass yields as well as high glycerol yields under aerobic conditions. One explanation for this phenotype is that, in this strain, mitochondria had a greater role in cell metabolism, leading to higher energy and biomass production.

Ethanol production for all strains was higher under anaerobic conditions, and for most strains ethanol yields were statistically lower in the variants compared to their parent strains, although in most cases the difference was small. The decrease in ethanol yield in the variants never reached the high level of reductions observed in sulfite-containing batch cultures of the parent strains (see Section 3.3.3). While disappointing, the reason may be due to the role played by sulfite in redirecting metabolism. In keeping with the proposed mechanism for the effect of sulfite on central carbon metabolism (Section 1.3.5.3), sulfite as a selection pressure favors the evolution of higher glycerol producing strains (as evidenced in the results of this chapter), but not necessarily lower ethanol-producing strains. It is entirely possible that a competitive variant would have increased activity and metabolic flux in its glycerol pathway, but no noticeable changes in the activity and capacity of the ethanol pathway. By depriving the ethanol pathway of acetaldehyde, the presence of sulfite does not provide any competitive advantage to

variants with alterations in their ethanol pathway. Rather, competitive advantage is provided to variants that can increase NAD^+ production, such as via changes in the glycerol pathway. In the absence of sulfite, ethanol production in the high glycerol-producing variant may well return to similar levels observed in the parent strain. Despite this scenario, the premise for using sulfite as a selection pressure is still valid (i.e. in the absence of sulfite, higher glycerol-producing variants may produce less ethanol because of the increased NAD^+ supply and the consequences of this on alcohol dehydrogenase activity). It depends on the evolutionary path taken by the strain during the adaptive evolution process.

The question remains about the required scale of increase in glycerol production by the sulfite-selected variants in order to achieve a useful (for the wine industry) decrease in ethanol production. Lower ethanol production in the variants is expected to occur due to changes in the redox balance as a result of higher glycerol production. Taking into account the stoichiometry of the glycerol-ethanol connection and a simplistic view of the redox-based relationship, it can be assumed that a 1 mole increase in glycerol yield would result in a 1 mole decrease in ethanol yield under anaerobic conditions (so that contributions from mitochondrial activities can be discounted). Using the results of A3-c2-1s under anaerobic conditions as an example, this variant increased its glycerol yield by 2.81 g/l, or 30.5 mMol/l. The equivalent decrease in ethanol yield (assuming a 1 mole increase in glycerol yield results in a 1 mole decrease in ethanol yield) would equate to 1.4 g/l; the actual decrease in ethanol yield was 2.9 g/l. The difference between the expected and actual decrease in ethanol yield is probably due to limitations of the simplistic model used to predict the ethanol yield which does not account for intracellular accumulation of glycerol or the impact of changes in NAD^+/NADH ratio on pathways other than ethanol in the cell. Nonetheless, it predicts that the magnitude of change in glycerol production in the variants cannot be expected to result in large reductions in ethanol yield. The small changes in ethanol yield observed in the variants are theoretically in keeping with the magnitude of change occurring in their glycerol yield. Under aerobic conditions the effect of the increase in glycerol yield on ethanol yield could be expected to be even less, due to the contribution of mitochondrial-based NADH oxidation activity on the NAD^+/NADH ratio.

Taking the above argument further, a reduction in ethanol concentration of wine of 2 % (v/v) (assuming an ethanol density of 0.79 g/ml) is equivalent to a 31.6 g/l, or 343.5 mMol/l, yield decrease which, based on the above model, would require a 343.5 mMol/l increase in glycerol yield, or 30.9 g/l. Given the limitations of the calculations (i.e. the assumptions made above), these figures are supported by observed changes in glycerol and ethanol yields in recombinant strains (Table 1.2). For example, a *S. cerevisiae* recombinant mutant with *GPD1* overexpression and *TPI1* and *ADH1* deletions increased glycerol yield by 44 g/l resulting in a decrease of 33 g/l in ethanol yield (Cordier et al. 2007).

Although this project has demonstrated that sulfite can be used in adaptive evolution experiments to generate mutants with increased glycerol production, the increase in glycerol yield in the variants was relatively small, given that such increases will need to be of the order 20-30 g/l to achieve subsequent reductions in ethanol concentration of around 2% (v/v); the variant with the highest glycerol production in this project had an increase in glycerol yield of 2.81 g/l. This is well short of the required increase in glycerol yield but a number of improvements could be made to the adaptive evolution experiments conducted under sulfite pressure in order to achieve variants with higher glycerol yields. Using a chemostat for evolutionary experiments, for example, would provide better 'control' of the experimental system (allowing continuous fine tuning of sulfite concentration in the feed) and ensure that the cell population is more rapidly dominated by variants with the greatest competitive edge. Selection of high glycerol-producing isolates could be improved by plating evolving populations on YPD agar plates containing sulfite; with selection based on rapidly-growing colonies. Adaptive evolution experiments could be conducted under anaerobic conditions and/or in the presence of inhibitors of mitochondrial activity, such as rotenone or adriamycin, limiting contributions to NADH oxidation by mitochondria. Such treatment would favor adaptations within pathways directly involved in balancing of NAD^+/NADH redox balance, such as the glycerol pathway.

Different strains of *S. cerevisiae* have been reported to produce different levels of acetic acid under the same fermentation conditions (Remize et al. 2000). Differences in acetic acid production appear to be dependent on strain origin also for the strains used in this study. For BY4742 and its variants, acetic acid yields were always higher under aerobic conditions. The reverse was true for AWRI1628 and its variants, which had higher acetic acid yields under anaerobic conditions. In most cases, the variants produced more acetic acid than their parent strains, the exception being B2-c3-1s, which produced less acetic acid than parent BY4742 only under aerobic conditions.

6.4.3 Mitochondrial influence on the relationship between glycerol/ethanol/acetic acid ratios.

The wine strain can be expected to have lower mitochondrial activity due to a history of selection under anaerobic fermentation, whereas the lab strain possibly has higher mitochondrial activity. This could explain many of the observations made in this chapter. High mitochondrial activity in the lab strain means higher NAD^+ production (by mitochondrial oxidation of NADH) and therefore the role of ethanol in producing NAD^+ is lessened - this is reflected in the small effect of increased glycerol production on ethanol yield and large effect on acetic acid production (which uses NAD^+ as a cofactor) in the lab strains. The more significant influence of increased glycerol production on reduced ethanol yields in the wine strain variants most likely reflects the lesser role by mitochondrial activity in this strain. Being more dependent on ethanol production for NAD^+ supply, ethanol yield is likely to be more responsive to changes in glycerol yield. This is most noticeable under anaerobic conditions where the biggest decrease in ethanol yield was observed. Acetic acid production by the wine strain is also lower under aerobic conditions (compared to the lab strain), possibly due to lower mitochondrial-based NAD^+ production.

Adaptive evolution using sulfite to provide selection pressure was shown in this project to be a promising method for the generation of non-recombinant yeast that produce less ethanol. The experimental design used in this work, however, was rudimentary and, as discussed earlier, there are a number of ways of improving the experimental approach to

achieve more successful outcomes in variant phenotypes. Although the use of sulfite as a selection pressure for the evolution of 'low ethanol' phenotypes was shown in principle to work, other adaptive evolution-based approaches could be used in tandem, or exclusively, for the generation of 'low-ethanol' yeast. One such approach would be to use inhibitors in the chemostat feed that specifically reduce or abolish the activity of particular enzymes. For example, inhibitors targeting pyruvate decarboxylase (PDC) activity may provide a selection pressure that favors the evolution of 'low-ethanol' yeast. As with sulfite removal of acetaldehyde, impairment of PDC activity could reduce the flow of metabolites to the fermentation pathway providing stimulation for glycerol production (Nevoigt and Stahl 1996). There are a number of PDC inhibitors reported in the literature that could be used for the purposes of adaptive evolution of 'low-ethanol' variants. Kuo and Jordan (1983) describe inactivation of yeast PDC activity by (E)-4-(4-chlorophenyl)-2-oxo-3-butenoic acid (CPB). Pyruvate decarboxylase was also shown to be inhibited by 2-oxo-3-alkynoic acids that harbor a phenyl substituent at carbon 4 (Brown et al. 1997). Using sub lethal concentrations of such inhibitors in an adaptive evolution setting could create a selection pressure that would favor adaptations conferring better NADH oxidation rates via pathways other than ethanol production. Inhibition of PDC activity would also reduce the availability of acetaldehyde for acetic acid production, which could be an additional benefit for 'low-ethanol' yeast development. Alternatively, PDC inhibition could be connected with sulfite and anaerobic conditions to generate considerable selection pressure for the evolution of 'low ethanol' phenotypes.

CHAPTER 7

GENETIC CHARACTERIZATION OF 'LOW-ETHANOL' YEAST VARIANTS

7.1 Aims

The main objectives of the work described in this chapter were to characterize and identify the genes conferring a 'low-ethanol' phenotype in the 'best' 'low-ethanol' isolates, B2-c3-1s and A3-c2-1s, from the first round of adaptive evolution, and B2/1-c4-2s derived from B2-c3-1s in the second round of adaptive evolution. To achieve this goal, 'low-ethanol' variants were backcrossed to their parent and the segregation patterns of the phenotypes assessed. Comparative Genomic Hybridization (CGH) and sequencing potential 'low-ethanol' genes were used in an attempt to identify 'low-ethanol' conferring genes.

7.2 Introduction

Following the use of adaptive evolution in the laboratory to generate novel phenotypes, the researcher typically attempts to characterize mutations that confer traits of interest. One of the first steps in this characterization is assessment of the dominance/recessiveness of the trait, knowledge of which is important for further assessment of mutations; for instance, if a mutated gene is dominant, it may easily be isolated from a gene library.

The phenotype of heterozygous diploids derived from backcrosses to the parental strain will show whether a mutation is dominant or recessive. The heterozygous diploid progeny can then be sporulated and the segregation patterns of the genes of interest determined; this will give some indication of the number of genes involved in conferring the mutant phenotype. More detailed characterization of the mutations, however, should benefit from the adoption of more advanced molecular techniques, such as the application of DNA microarrays.

Over recent years, a number of techniques have been developed to enable the identification of mutations at a genomic level. Of these techniques, Comparative Genomic Hybridization (CGH) has proven to be particularly powerful for accurate detection of DNA copy number differences. One of the first applications of CGH was to scan genomes of cancer cells to screen for cytogenetic changes (Kallioniemi et al. 1992). Recently, DNA arrays and CGH have been used to characterize nucleotide variation in wide a range of organisms, including yeast (Gresham et al. 2006). In general, CGH is based on hybridization of fluorescently labeled genomic DNA from a reference organism (control) and the mutant to be characterized to a tiling array containing probes that cover the genome sequence of interest. The two genomic DNA pools are isolated and labeled with different fluorochromes, which emit light at different wavelengths. After hybridization of the reference and mutant genomic DNA to the array, the array scanned and visualized to determine intensities of light emission of the hybridized DNA. For genomic regions that are the same in the mutant and the reference, hybridizations will be equal and emission of light from both fluorochromes will be the same. However, in places where amplification or deletion has occurred in the genome of the mutant, the intensity of light emitted by the fluorochrome that was used to label the parental (reference) strain will predominate, and this is detected and quantified using appropriate scanners and software. CGH can also allows identification of DNA changes at single nucleotide resolution; so called Single Nucleotide Polymorphisms (SNPs) (Zhao et al. 2004, Gresham et al. 2006). Such arrays are usually used for comparisons of closely related genomes, for instance an adaptively evolved mutant and its parent, for genomic alternations conferring phenotypic traits of the mutants. CGH was used in this study in attempt to identify mutation(s) that confer 'low-ethanol' phenotypes in variants B2-c3-1s and B2/1-c4-2s.

7.3 Results of backcrossing experiments

Backcrossing of the 'low-ethanol' variants B2-c3-1s and B2/1-c4-2s to the original parent strain, BY4742, was performed to assess whether 'low-ethanol' genes were dominant or recessive. In addition the segregation patterns of the 'low-ethanol' conferring genes were

used to estimate how many genes might be involved in shaping the phenotype. A2-c3-1s was not included in backcrossing experiments because it failed to sporulate.

7.3.1 Mating type inter-conversion and phenotypic assessment of homozygous diploids

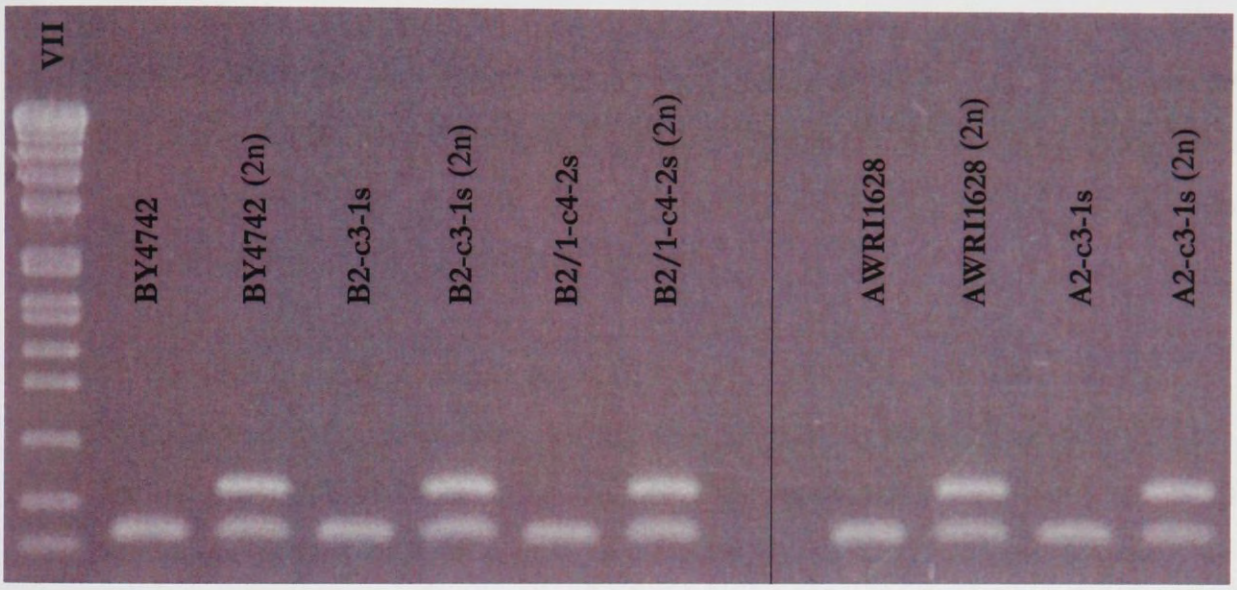
Mating of heterothallic[1] haploid yeast, such as BY4742, requires that the two strains involved are of opposite mating types; 'a' and 'α'. The 'low-ethanol' mutants isolated in work described in Chapters 4 and 5 were of the same mating type as their parents, and therefore could not be backcrossed without first switching their mating type, or that of the parent. Mating type switching is readily achieved in heterothallic laboratory strains of *S. cerevisiae* by introducing the wild type HO gene into the HO locus (see Haber 1998, for full description of the yeast mating type locus).

The approach used to switch mating type in parent and mutant strains for work described in this thesis utilized a plasmid carrying a copy of the HO gene, pHO-NAT and the CloNAT antibiotic resistance marker. HOp is an endonuclease required for production of a double strand cut within the *MAT* locus, enabling a mating-type switch. When activity of pHO endonuclease is induced a mating type inter-conversion can occur in which some cells switch mating-type (see Jensen et al. 1983). Following induction of a switch some of the resultant mixed population of 'a' and 'α' cells fuse to form 'a/α' diploids.

Potential diploids were initially identified and assessed as described in Section 2.3.3 (Figure 7.1). Stock cultures of the diploids chosen for further assessment were kept at -80°C.

[1] Heterothallic organisms are those which have sexes that reside stably in different individuals. In the context of *S. cerevisiae* this means that mating types do not readily switch; 'a' does not switch to 'α' and *vice versa*.

Figure 7.1 Mating-type PCR of haploid and diploid (2n) strains of the parental BY4742 and AWRI1628, and 'low-ethanol' variants B2-c3-1s, B2/1-c4-2s and A3-c2-1s.


VII
BY4742
BY4742 (2n)
B2-c3-1s
B2-c3-1s (2n)
B2/1-c4-2s
B2/1-c4-2s (2n)
AWRI1628
AWRI1628 (2n)
A2-c3-1s
A2-c3-1s (2n)

7.3.2 Phenotypic assessment of homozygous diploids and their derivative segregants

To determine whether ploidy had any impact on the 'low-ethanol' phenotype of mutants, homozygous diploids generated as discribed above (Section 7.3.1), and their haploid progenitors were assessed for glycerol, ethanol and acetic acid production. Triplicate fermentations were inoculated from inoculum cultures prepared as described in Section 2.1.5.2, and conducted as described in Section 2.1.5.4. When glucose was exhausted, samples of medium were analyzed using HPLC (see Section 2.2.4). In all cases there was decreased glycerol production in diploids relative to their haploid progenitors, although this was marginal in the case of BY4742 (Figure 7.2). Decreases in glycerol production for diploids BY4742(2n) and B2-c3-1s(2n), were not associated with either increased ethanol production or decreased acetic acid yields. However, there was a linkage between lowered glycerol, elevated ethanol and decreased acetic acid levels for diploids B2/1-c4-2s(2n), AWRI1628(2n) and A3-c2-1s(2n).

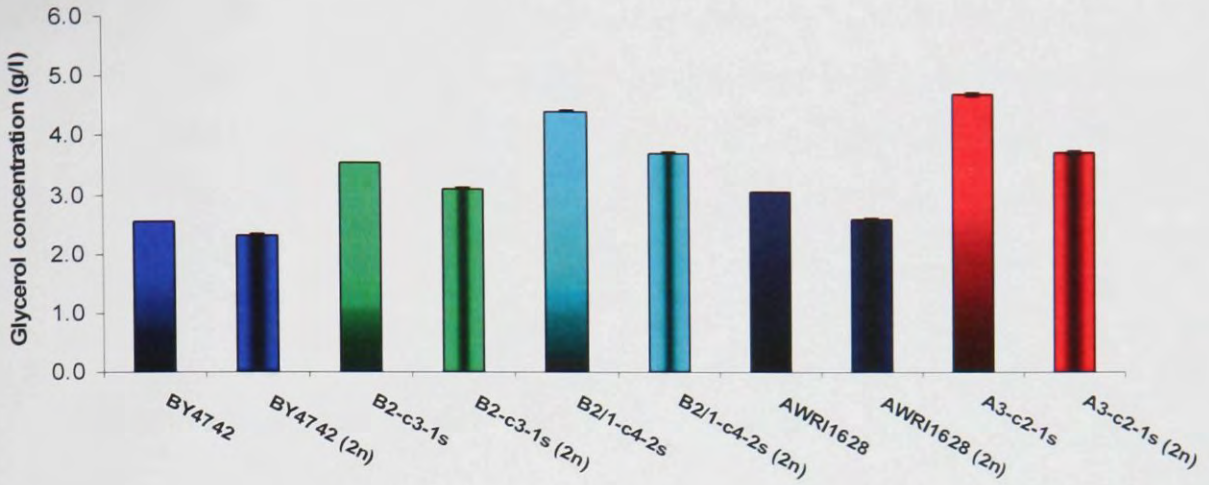
Haploids derived from diploidized parental strains were also assessed for glycerol, ethanol and acetic acid production as above. Sporulated cultures of BY4742(2n), B2-c3-1s(2n) and B2/1-c4-2s(2n) microdissected as described in section 2.3.6. YPD plates containing groups of four spores were incubated at 30°C for approximately 48 hours and random tetrads were tested for mating type using mating-type PCR (Section 2.3.7.2). Tetrads displaying a 2:2 ('a':'α') mating type segregation ratio (see Figure 7.3), were selected from each homozygous diploid and tested for parentage using Ty1 transposon PCR (data not shown) (Section 2.3.7.1). All spore-derived haploids displayed the same DNA fingerprint pattern as the parent haploids of BY4742, B2-c3-1s and B3/1-c4-2s. Stock cultures of all spore-derived haploids were prepared as described in Section 2.1.5.1, and kept frozen at -80°C for future work.

Haploids derived from single spores, following sporulation of BY4742(2n), B2-c3-1s(2n) and B2/1-c4-2s(2n), were tested for glycerol, ethanol and acetic acid production. Triplicate fermentations were inoculated from inoculum cultures prepared as described in

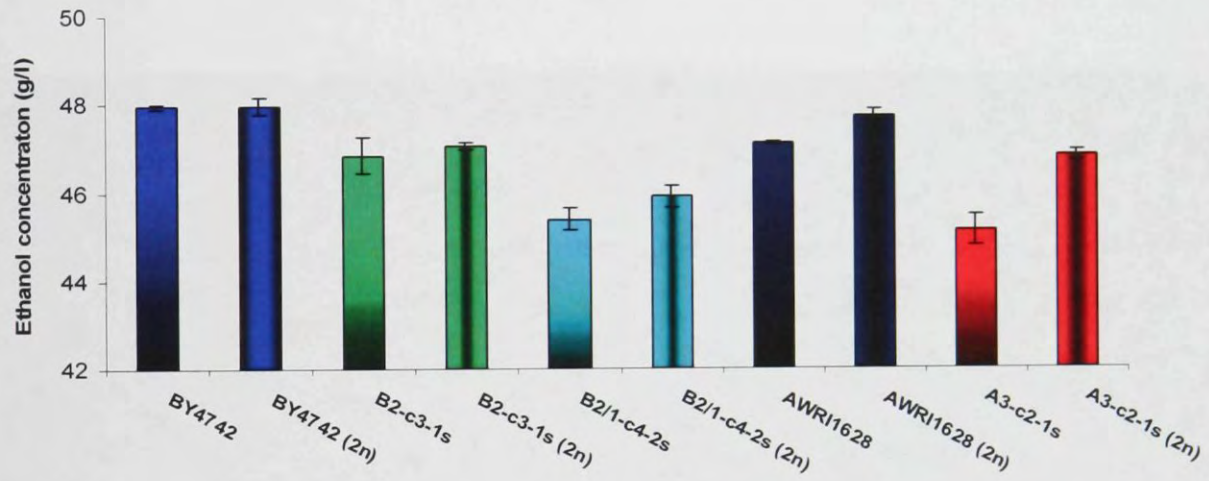
Figure 7.2 Glycerol, ethanol and acetic acid production for haploids and homozygous diploids of the parental strains *S. cerevisiae* BY4742 and AWRI1628 and 'low-ethanol' variants B2-c3-1s, B2/1-c4-2s and A3-c2-1s.

Haploids and homozygous diploids of BY4742 (blue), AWRI1628 (dark blue), B2-c3-1s (green), B2/1-c4-2s (light blue) and A3-c2-1s (red) were tested for glycerol (7.2i), ethanol (7.2ii) and acetic acid (7.2iii) production. Assays were performed as described in Section 2.1.5.4. Fermentations were carried out in triplicate and values are means ± SD.

7.2i



7.2ii



7.2iii

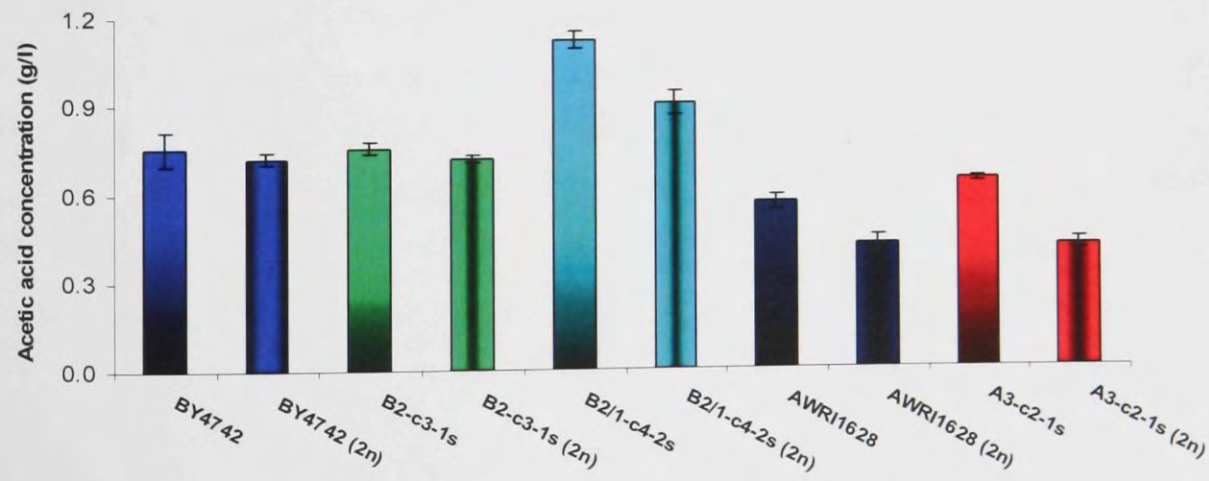
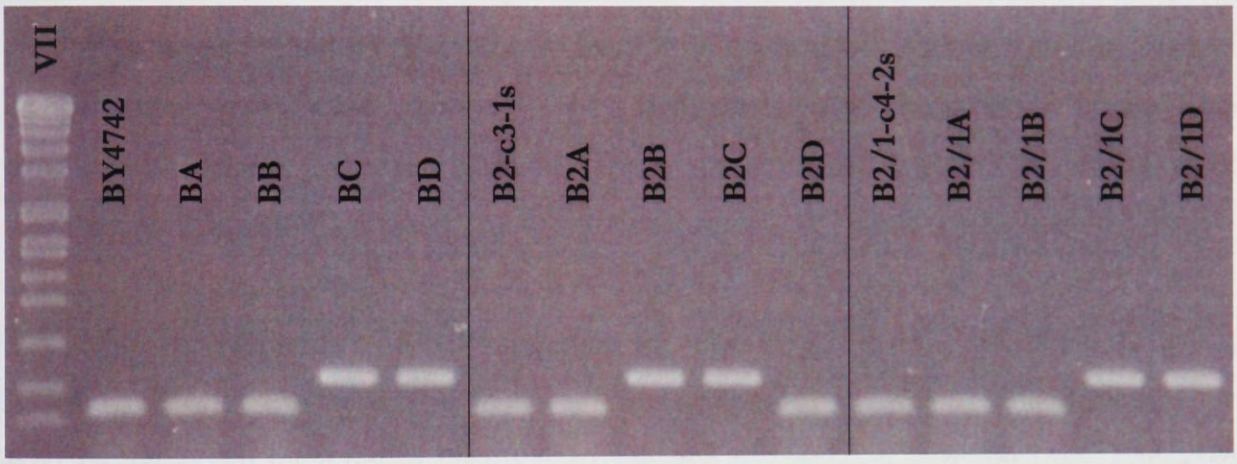


Figure 7.3 Mating-type PCR for tetrads derived from homozygous diploids BY4742(2n), B2-c3-1s(2n) and B2/1-c4-2s(2n).


VII
BY4742
BA
BB
BC
BD
B2-c3-1s
B2A
B2B
B2C
B2D
B2/1-c4-2s
B2/1A
B2/1B
B2/1C
B2/1D

Section 2.1.5.2, and conducted as described in Section 2.1.5.4. When glucose was exhausted, samples of medium were analyzed using HPLC (see Section 2.2.4). All spore-derived haploids displayed identical phenotypes to the haploid strains that homozygous diploid strains were derived from (Figure 7.4), producing the same amounts of glycerol, ethanol and acetic acid, thus validating the homozygous state of the diploids generated as described in Section 7.3.1.

7.3.3 Assessment of dominance/recessiveness of mutations conferring 'low-ethanol' phenotypes

To determine the dominance characteristics of mutations conferring 'low-ethanol' phenotypes, variants B2-c3-1s and B2/1-c4-2s were backcrossed to the original parental strain BY4742[2] (see Section 2.3.4), as illustrated in Figure 7.5. Isolation of diploid progeny was performed as described in Section 2.3.4, and the diploid status confirmed using mating-type PCR (see Section 2.3.7.2). Individuals possessing a double mating-type band were picked (data not shown), frozen at -80°C and kept for future work (see Section 2.1.5.1).

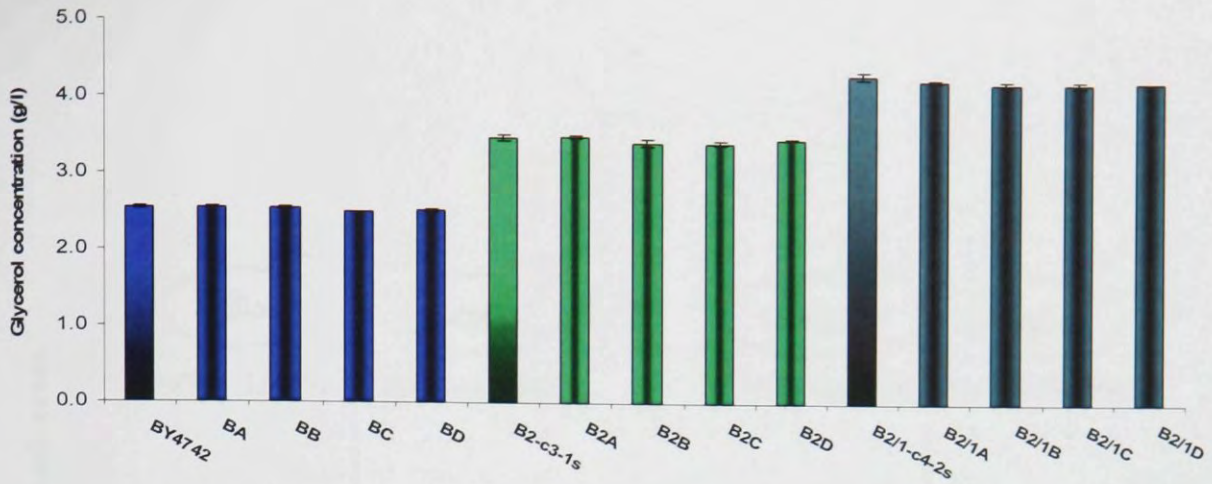
Heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)], [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)], [BY4742(*MAT*α) x B2/1-c4-2s(*MAT*a)] and [BY4742(*MAT*a) x B2/1-c4-2s(*MAT*α)] were tested for glycerol, ethanol and acetic acid production. Triplicate fermentations were inoculated from inoculum cultures prepared as described in Section 2.1.5.2, and conducted as described in Section 2.1.5.4. When glucose was exhausted, samples of medium were analyzed using HPLC (see Section 2.2.4). The heterozygous diploids had produced glycerol, ethanol and acetic acid levels similar to that of the homozygous diploid BY4742(2n) (Figure 7.6), which is consistent with the 'low-ethanol' phenotype being a recessive trait.

[2] Reciprocal back-crosses were conducted such that both, BY4742 (*MAT*α) and BY4742 (*MAT*a), were used for mating with the 'low-ethanol' variants of opposite mating types.

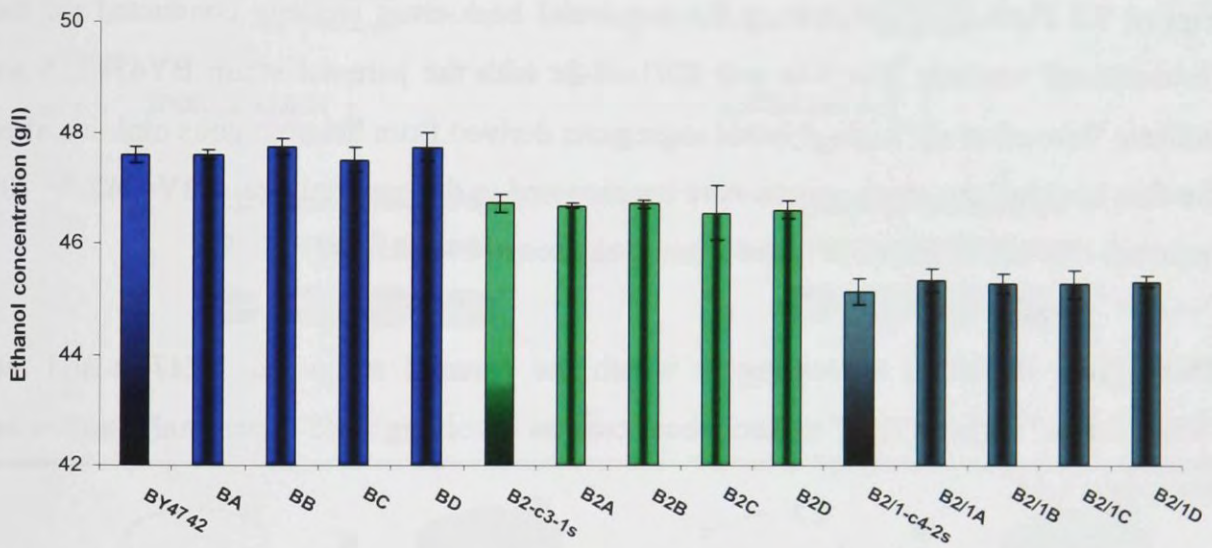
Figure 7.4 Glycerol, ethanol and acetic acid production for tetrads derived from homozygous diploids BY4742(2n), B2-c3-1s(2n) and B2/1-c4-2s(2n).

Spores derived from tetrads, derived from sporulation of homozygous diploids BY4742(2n) (blue), B2-c3-1s(2n) (green), B2/1-c4-2s(2n) (gray), were tested for glycerol (7.4i), ethanol (7.4ii) and acetic acid (7.4iii) production. The original haploids BY4742, B2-c3-1s and B2/1-c4-2s were used as controls. Assays were performed as described in Section 2.1.5.4. Fermentations were carried out in triplicate and values are means ± SD.

7.4i



7.4ii



7.4iii

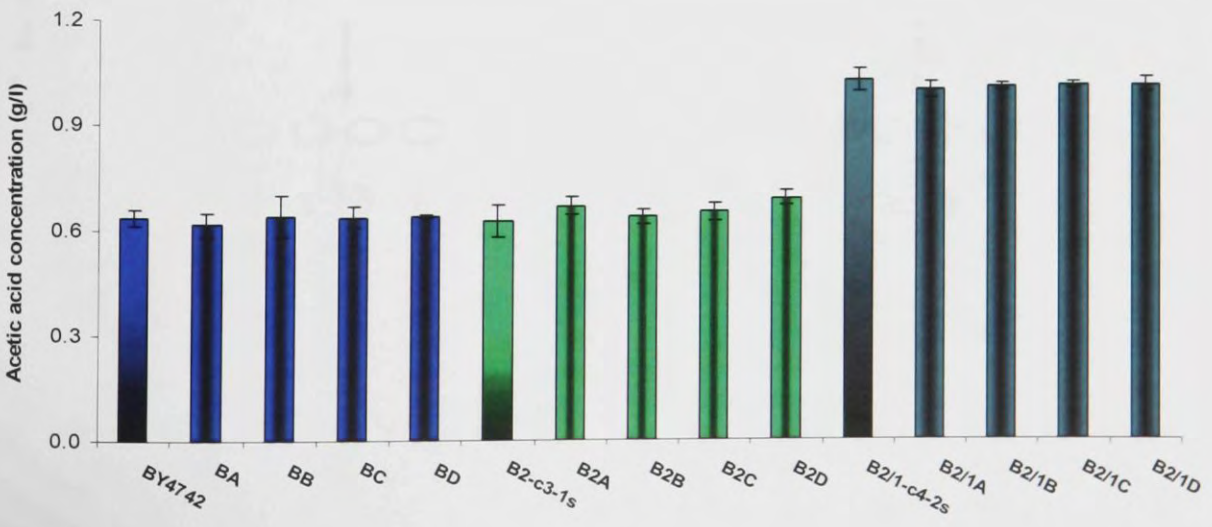


Figure 7.5 Flow chart illustrating the sequential back-cross strategy conducted for the 'low-ethanol' variants B2-c3-1s and B2/1-c4-2s with the parental strain BY4742. Stars indicate 'low-ethanol'/'high-glycerol segregants derived from heterozygous diploids after the first backcrosses; these spores were backcrossed to the parental strain BY4742. 'x 10' indicates that 10 tetrads were tested from each second back-cross.

This Figure illustrates a crossing in which the parental strain was *MAT* α and the 'low-ethanol' variants *MAT* a. Reciprocal crosses involving *MAT* a parental strain were also performed.

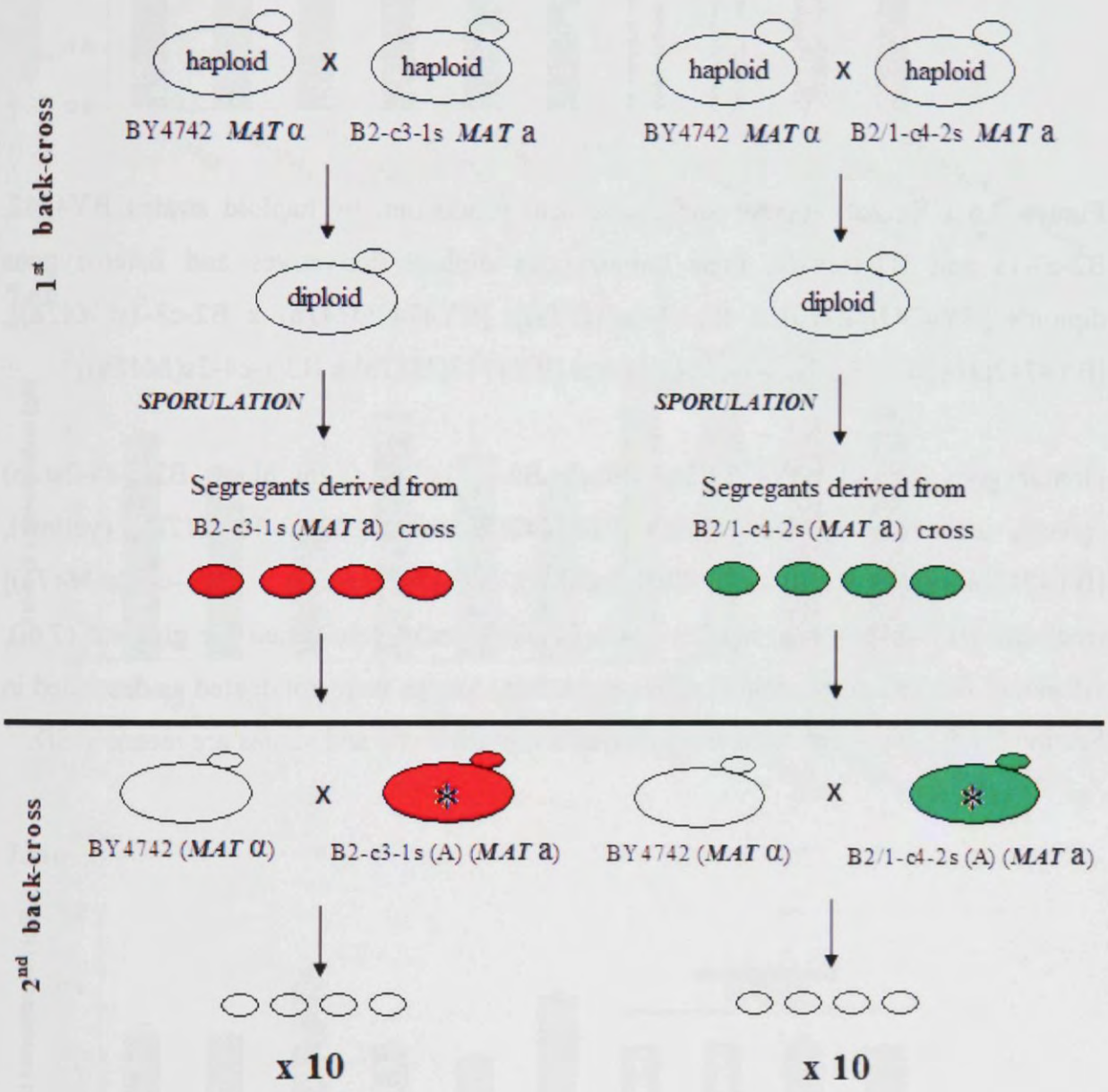
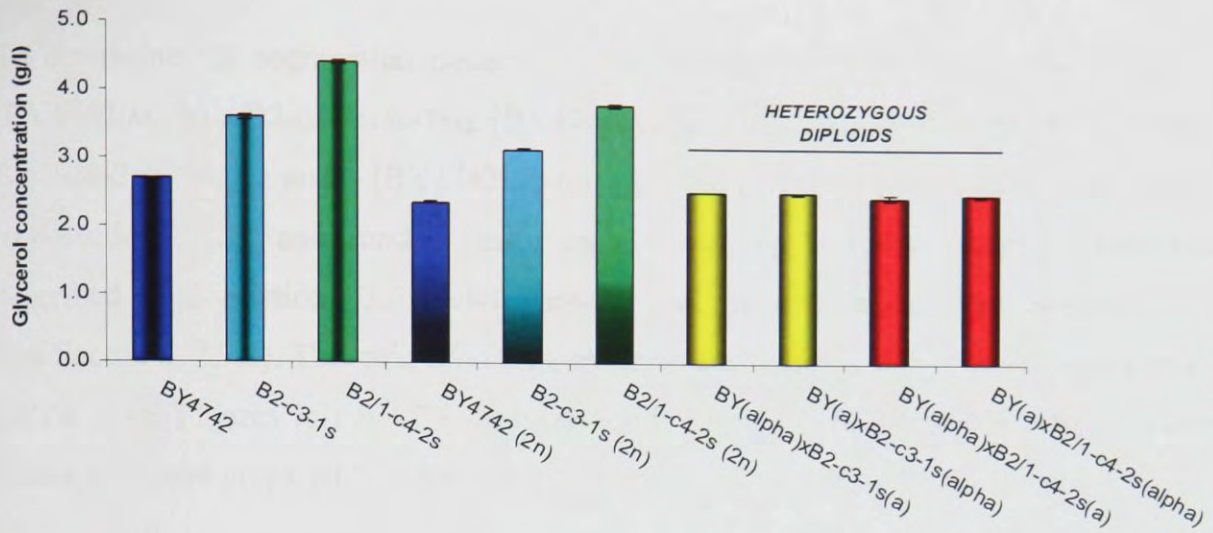

1st back-cross
haploid
x
haploid
BY4742 MAT α
B2-c3-1s MAT a
diploid
SPORULATION
Segregants derived from
B2-c3-1s (MAT a) cross
haploid
x
haploid
BY4742 MAT α
B2/1-c4-2s MAT a
diploid
SPORULATION
Segregants derived from
B2/1-c4-2s (MAT a) cross
2nd back-cross
x
BY4742 (MAT α)
B2-c3-1s (A) (MAT a)
x 10
x
BY4742 (MAT α)
B2/1-c4-2s (A) (MAT a)
x 10

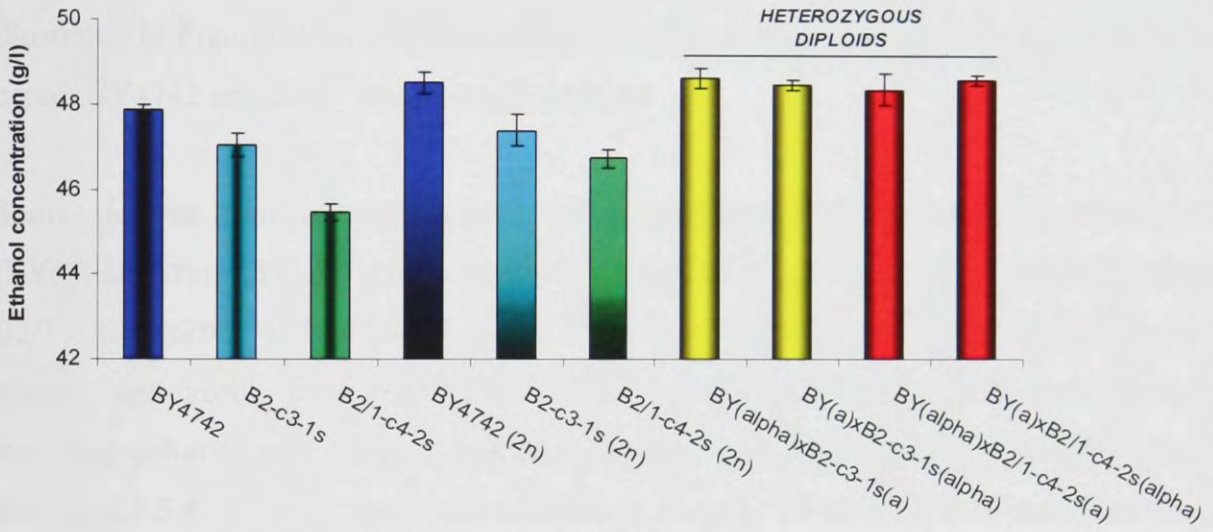
Figure 7.6 Glycerol, ethanol and acetic acid production by haploid strains BY4742, B2-c3-1s and B2/1-c4-2s, their homozygous diploid derivatives and heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)], [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)], [BY4742(*MAT*α) x B2/1-c4-2s(*MAT*a)] and [BY4742(*MAT*a) x B2/1-c4-2s(*MAT*α)]

Homozygous diploids BY4742(2n) (blue), B2-c3-1s(2n) (light blue), B2/1-c4-2s(2n) (green), and heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)] (yellow), [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)] (yellow), [BY4742(*MAT*α) x B2/1-c4-2s(*MAT*a)] (red) and [BY4742(*MAT*a) x B2/1-c4-2s(*MAT*α)] (red), were tested for glycerol (7.6i), ethanol (7.6ii) and acetic acid (7.6iii) production. Assays were conducted as described in Section 2.1.5.4. Fermentations were carried out in triplicate and values are means ± SD.

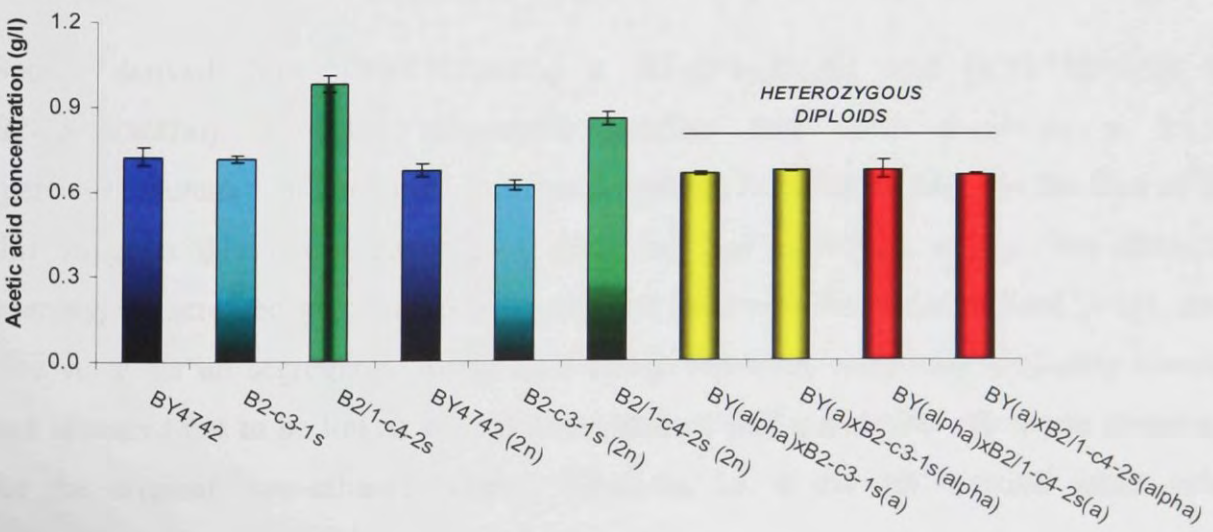
7.6i



7.6ii



7.6iii



7.3.4 Segregation of phenotypes of the 'low-ethanol' variants

To determine the segregation pattern of the 'low-ethanol' trait, heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)], [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)], [BY4742(*MAT*α) x B2/1-c4-2s(*MAT*a)] and [BY4742(*MAT*a) x B2/1-c4-2s(*MAT*α)], were sporulated (see Section 2.3.5), and random asci, each containing 4 spores, were dissected as described in Section 2.3.6, and tested for ploidy using mating-type PCR (see Section 2.3.7.2). The ratio of mating types for the 4 spores within each ascus was 2:2 ('a':'α') (see Figures 7.7i and 7.8i, respectively). Frozen stocks of cultures prepared from each spore were prepared (see Section 2.1.5.1), and kept at -80°C for future use.

Ty1 transposon PCR was used to check the parentage of segregants (Section 2.3.7.1). As illustrated in Figures 7.7ii and 7.8ii all individuals had the same DNA fingerprints as the parent BY4742 and both 'low-ethanol' variants.

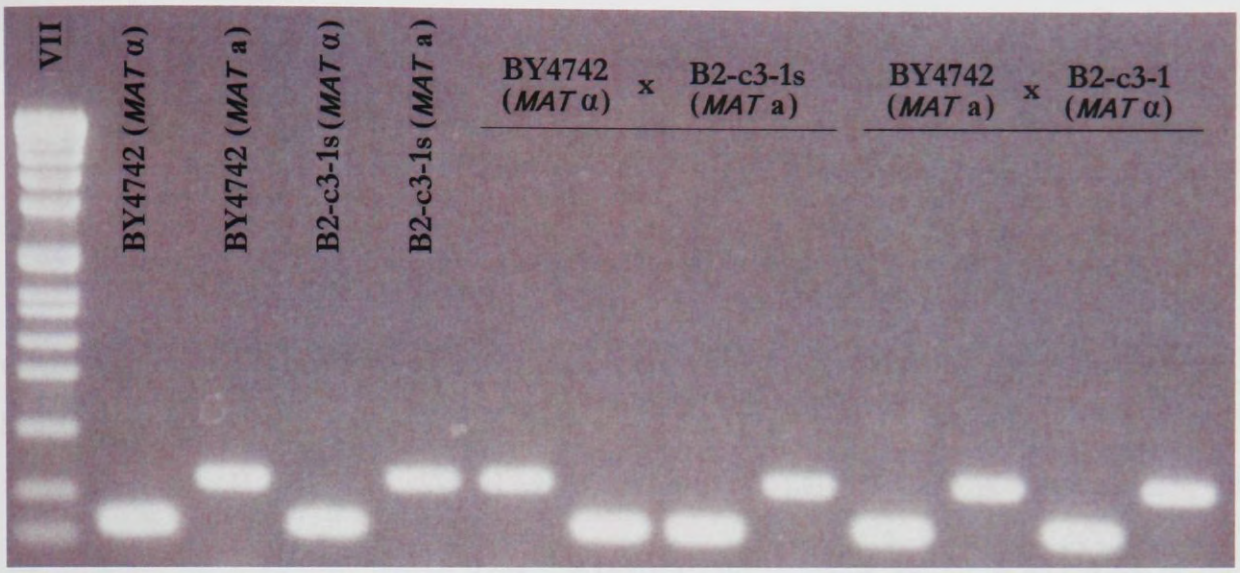
Spores derived from four tetrads, each one derived from different heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)], [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)], [BY4742(*MAT*α) x B2/1-c4-2s(*MAT*a)] and [BY4742(*MAT*a) x B2/1-c4-2s(*MAT*α)], were tested for glycerol, ethanol and acetic acid production. Triplicate fermentations were inoculated from inoculum cultures prepared as described in Section 2.1.5.2, and conducted as described in Section 2.1.5.4. When glucose was exhausted, samples of medium were analyzed using HPLC (see Section 2.2.4).

Spores derived from [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)] and [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)] displayed phenotypic profiles that were close to a 1:1:2 (parental : mutant : intermediate) segregation pattern (see Figure 7.9). On the face of it, this suggests that two independently assorting loci contribute to the 'low-ethanol' phenotype. Increased glycerol levels were associated with decreased ethanol yields, and vice versa for all segregants. Acetic acid levels, however, were only negligibly altered and appeared not to be linked with glycerol/ethanol shifts; a similar effect was observed for the original 'low-ethanol' variant B2-c3-1s, i.e. it did not increase acetic acid

Figure 7.7 Mating type and Ty1 transposon fingerprints for parental *S. cerevisiae* BY4742, 'low-ethanol' variant B2-c3-1s, and two groups of four segregants derived from heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)], [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)]

Spores from two tetrads, derived from sporulation of heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)] and [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)] were tested for mating type segregation (7.7i) and Ty1 transposon fingerprints(7.7ii). Parental *S. cerevisiae* BY4742 (*MAT*α and *MAT*a) and 'low-ethanol' variant B2-c3-1s (*MAT*a and *MAT*a) were used as controls.

7.7i


VII
BY4742 (MAT α)
BY4742 (MAT a)
B2-c3-1s (MAT α)
B2-c3-1s (MAT a)
BY4742 (MAT α) x B2-c3-1s (MAT a)
BY4742 (MAT a) x B2-c3-1 (MAT α)

7.7ii

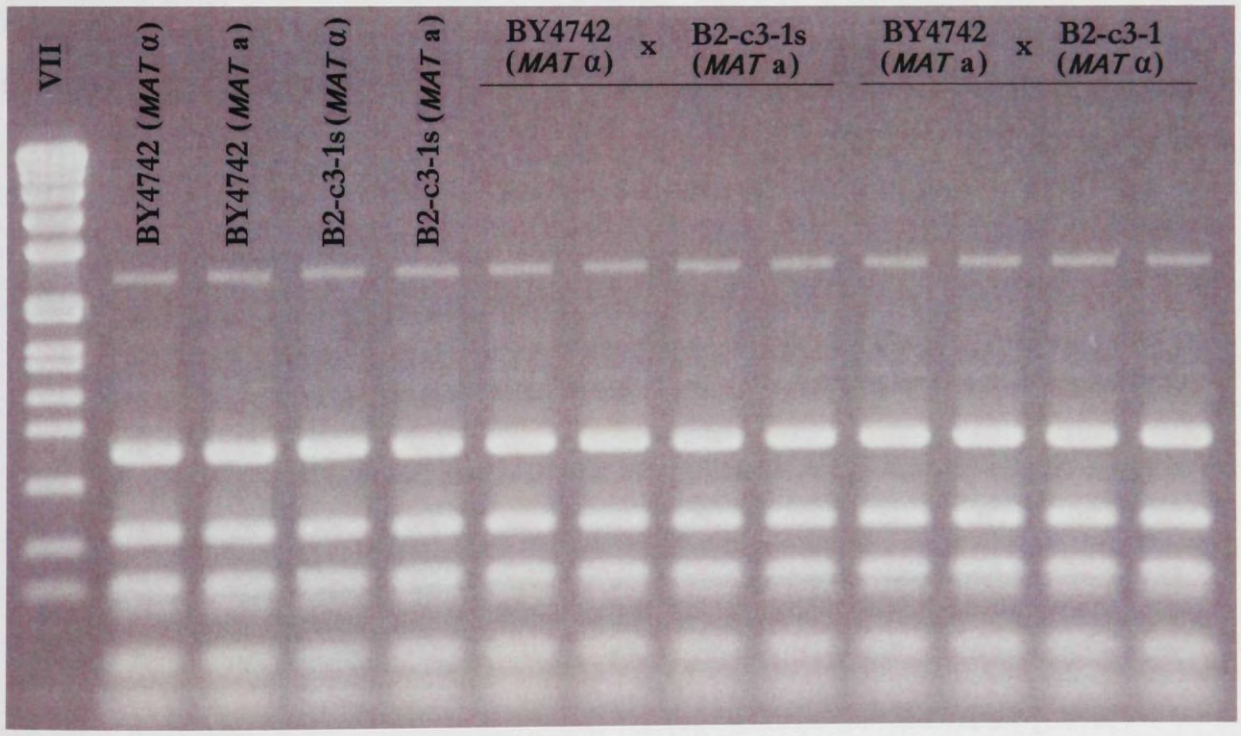
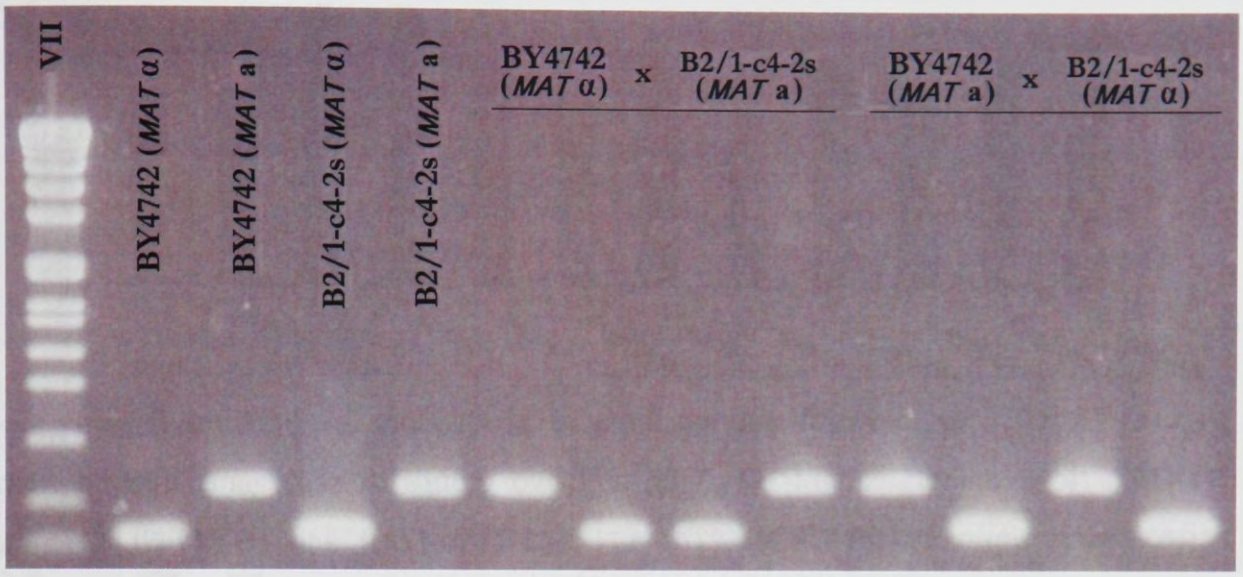

VII
BY4742 (MAT α)
BY4742 (MAT a)
B2-c3-1s (MAT α)
B2-c3-1s (MAT a)
BY4742 (MAT α) x B2-c3-1s (MAT a)
BY4742 (MAT a) x B2-c3-1 (MAT α)

Figure 7.8 Mating types and Ty1 transposon fingerprints for parental *S. cerevisiae* BY4742, 'low-ethanol' variant B2/1-c4-2s, and two groups of four segregants derived from heterozygous diploids [BY4742(*MAT*α) x B2/1-c4-2s(*MAT*a)], [BY4742(*MAT*a) x B2/1-c4-2s(*MAT*α)]

Spores from two tetrads, derived from sporulation of heterozygous diploids [BY4742(*MAT*α) x B2/1-c4-2s(*MAT*a)] and [BY4742(*MAT*a) x B2/1-c4-2s(*MAT*α)] were tested for mating-type segregation (7.8i) and Ty1 transposon fingerprints (7.8ii). Parental *S. cerevisiae* BY4742 and 'low-ethanol' variant B2/1-c4-2s of both mating types were used as controls.

7.8i


VII
BY4742 (MAT α)
BY4742 (MAT a)
B2/1-c4-2s (MAT α)
B2/1-c4-2s (MAT a)
BY4742 (MAT α) x B2/1-c4-2s (MAT a)
BY4742 (MAT a) x B2/1-c4-2s (MAT α)

7.8ii

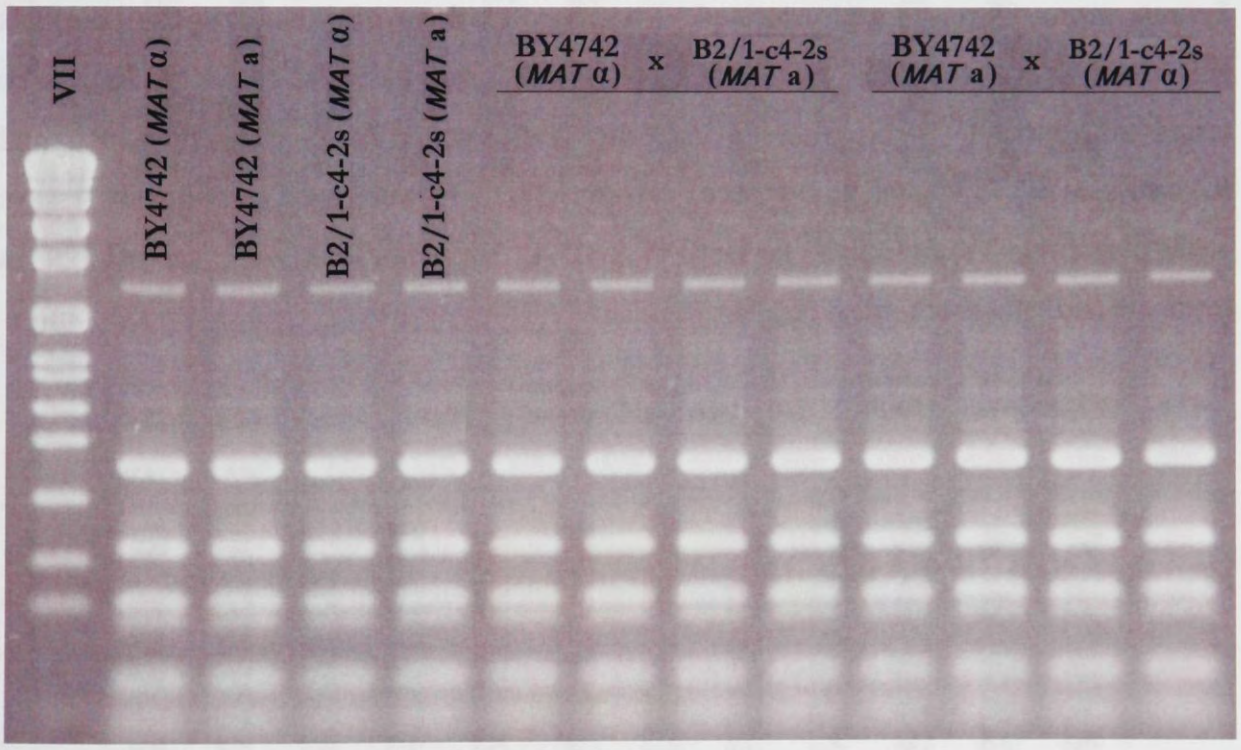
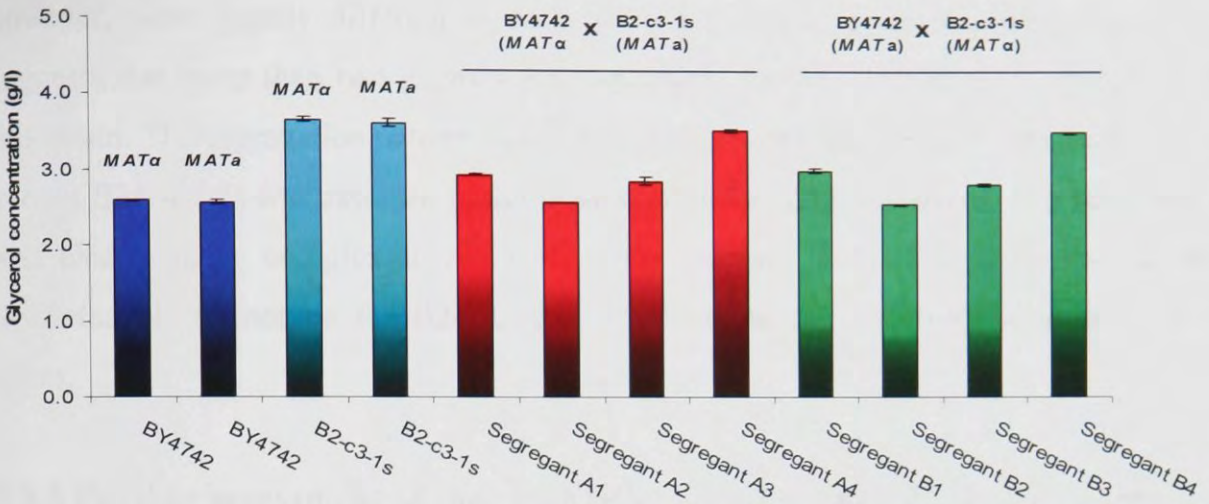

VII
BY4742 (MAT α)
BY4742 (MAT a)
B2/1-c4-2s (MAT α)
B2/1-c4-2s (MAT a)
BY4742 (MAT α) x B2/1-c4-2s (MAT a)
BY4742 (MAT a) x B2/1-c4-2s (MAT α)

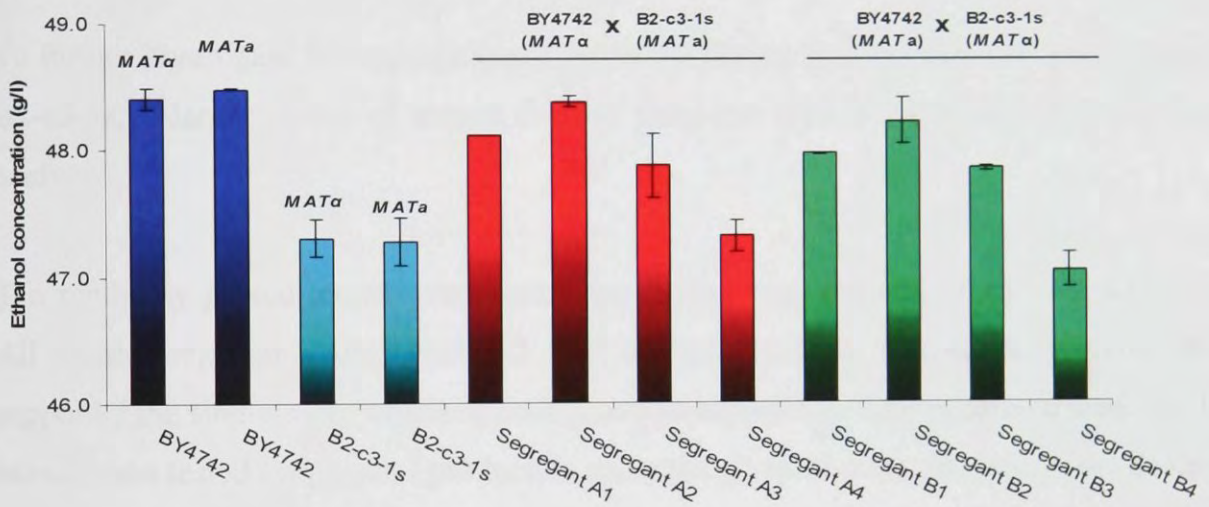
Figure 7.9 Glycerol, ethanol and acetic acid production for parental *S. cerevisiae* BY4742, 'low-ethanol' variant B2-c3-1s, and two groups of segregants derived from heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)] and [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)]

Spores from two tetrads, derived from sporulation of heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)] and [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)] were tested for glycerol (7.9i), ethanol (7.9ii) and acetic acid (7.9iii) production. Parental *S. cerevisiae* BY4742 and 'low-ethanol' variant B2-c3-1s were used as controls. Assays were performed as described in Section 2.1.5.4. Fermentations were carried out in triplicate and values are means ± SD.

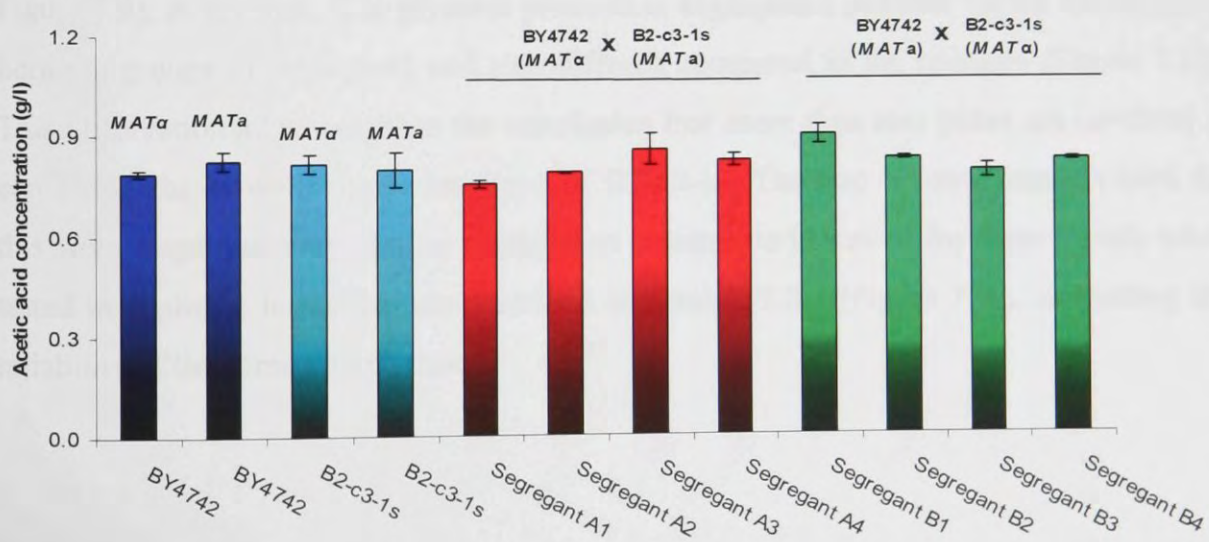
7.9i



7.9ii



7.9iii



production in a response to elevated glycerol yields (Section 4.3.4). The intermediates, however, were slightly different to each other in terms of glycerol production, which suggests that more than two genes were responsible for the 'low-ethanol' phenotype of this strain. The segregation pattern from heterozygous diploids derived from backcrossed variant B2/1-c4-2s was assessed in the same way as for B2-c3-1s above. The segregation was clearly more complex than for B2-c3-1s (Figure 7.10). This suggests that the 'low-ethanol' phenotype for B2/1-c4-2s variant might be conferred by at least three genes.

7.3.5 Further assessment of the segregation of 'low-ethanol' phenotype in spores derived from [BY4742 x B2-c3-1s]

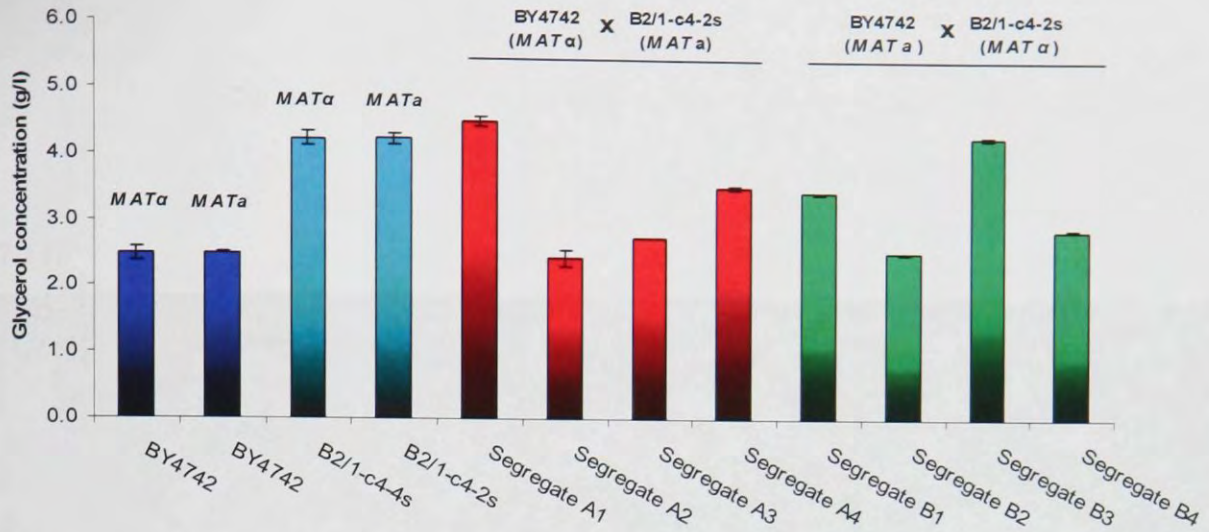
To further investigate the segregation of genes conferring the 'low-ethanol' phenotype in B2-c3-1s, a larger group of tetrads derived from the BY4742 x B2-c3-1s cross were analyzed.

Ten randomly picked tetrads were tested for mating type segregation (see figure 7.11). All of the segregants displayed 2:2 ('a':'α') mating type segregation patterns that supported the single-ascus origin of each group of segregants. Spores derived from the 10 tetrads were tested for glycerol production using the glycerol-screening assay (see Section 4.3.3), including two tetrads that were tested earlier, (see previous Section and Figure 7.9), as controls. The glycerol production segregation patterns varied substantially between groups of segregants and also differed compared to the controls (Figure 7.12). This observation adds weight to the conclusion that more than two genes are involved in conferring the 'low-ethanol' phenotype of B2-c3-1s. The two 'control' tetrads used for this work displayed very similar segregation patterns as observed for these tetrads when tested in triplicate fermentations described in Section 7.3.4 (Figure 7.9i), supporting the reliability of the screening method.

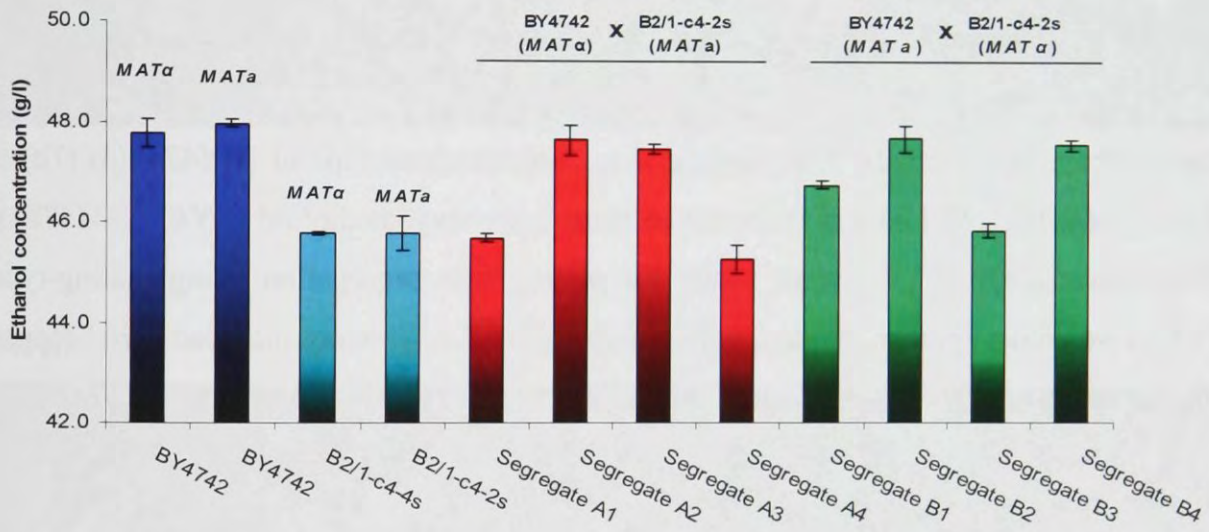
Figure 7.10 Glycerol, ethanol and acetic acid production for parental *S. cerevisiae* BY4742, 'low-ethanol' variant B2-c3-1s, and two groups of segregants derived from heterozygous diploids [BY4742(*MAT*α) x B2/1-c4-2s(*MAT*a)], [BY4742(*MAT*a) x B2/1-c4-2s(*MAT*α)]

Spores from two tetrads, each derived from sporulation of heterozygous diploids [BY4742(*MAT*α) x B2/1-c4-2s(*MAT*a)] and [BY4742(*MAT*a) x B2/1-c4-2s(*MAT*α)] were tested were tested for glycerol (7.10i), ethanol (7.10ii) and acetic acid (7.10iii) production. Parental *S. cerevisiae* BY4742 and 'low-ethanol' variant B2-c3-1s of both mating types were used as controls. Assays were performed as described in Section 2.1.5.4. Fermentations were carried out in triplicate and values are means ± SD.

7.10i



7.10ii



7.10iii

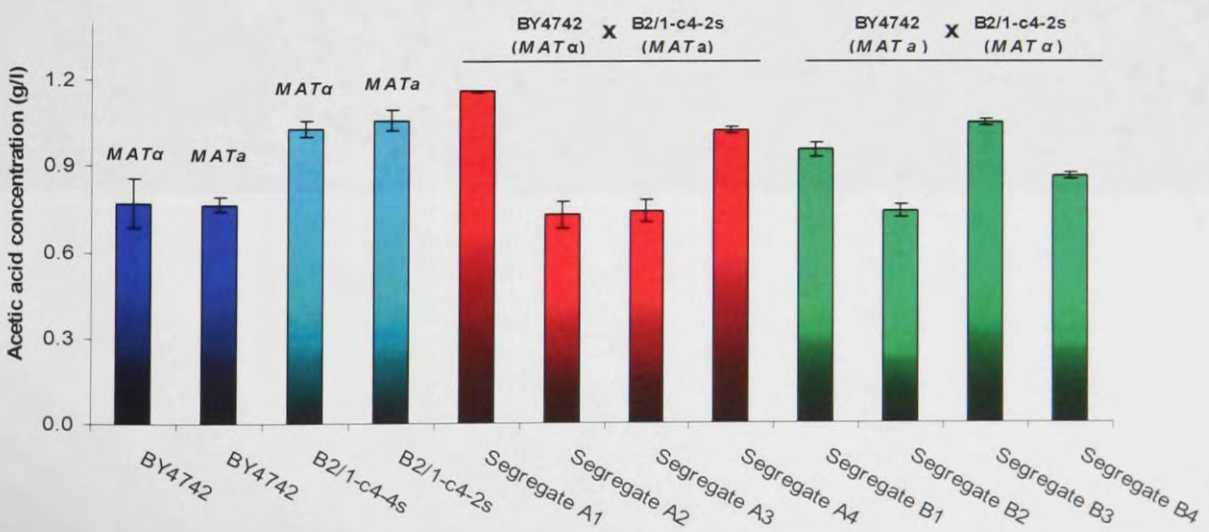
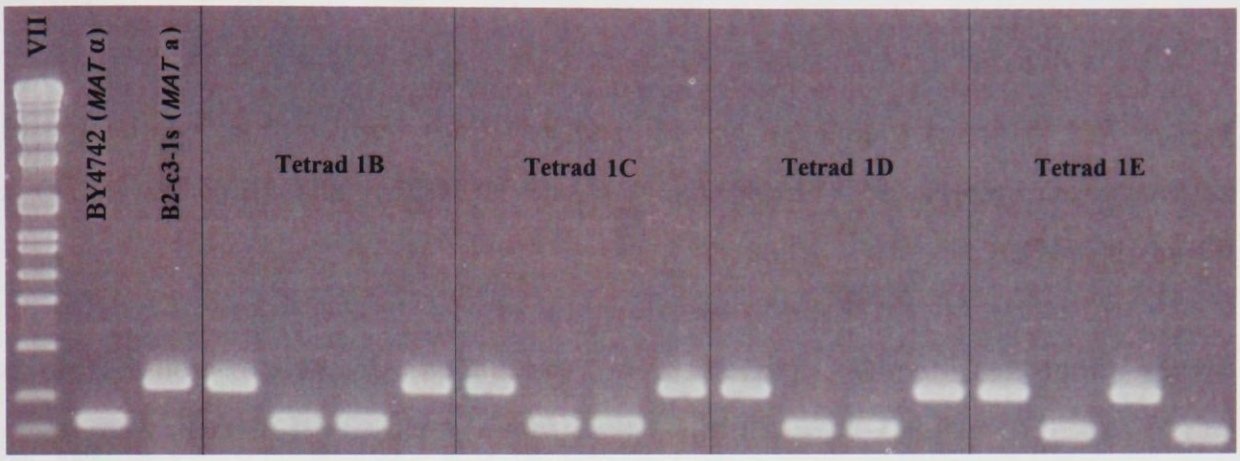


Figure 7.11 Mating type PCR for spores from eight tetrads, derived from heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)] and [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)]

Spores from eight tetrads, four derived from heterozygous diploid [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)] (7.11i) and four derived from heterozygous diploid [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)] (7.11ii) were tested for mating-type segregation using mating-type PCR. Two control groups, marked with star in Figure 7.5, were not included here because they were assayed earlier (see Figure 7.7i)

7.11i


VII
BY4742 (MAT α)
B2-c3-1s (MAT a)
Tetrad 1B
Tetrad 1C
Tetrad 1D
Tetrad 1E

7.11ii

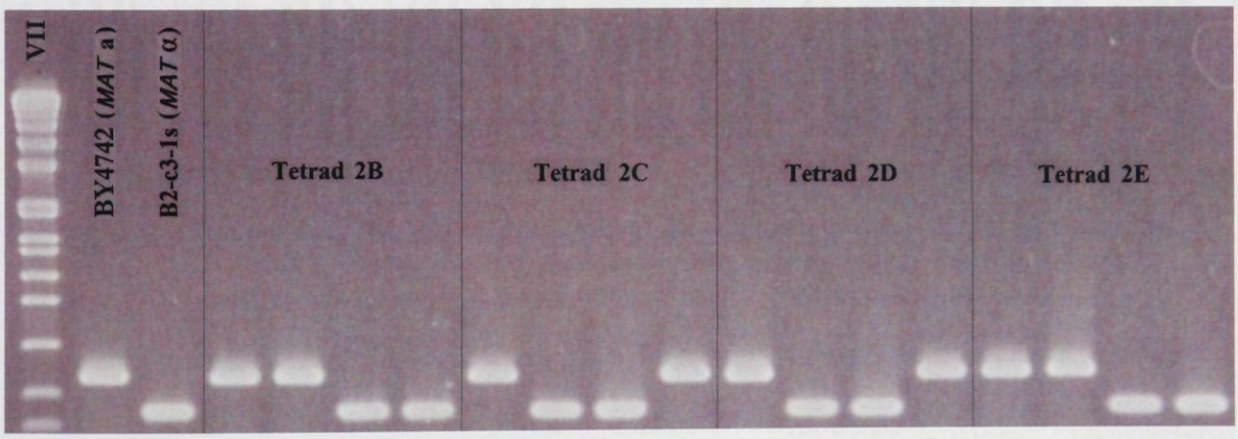
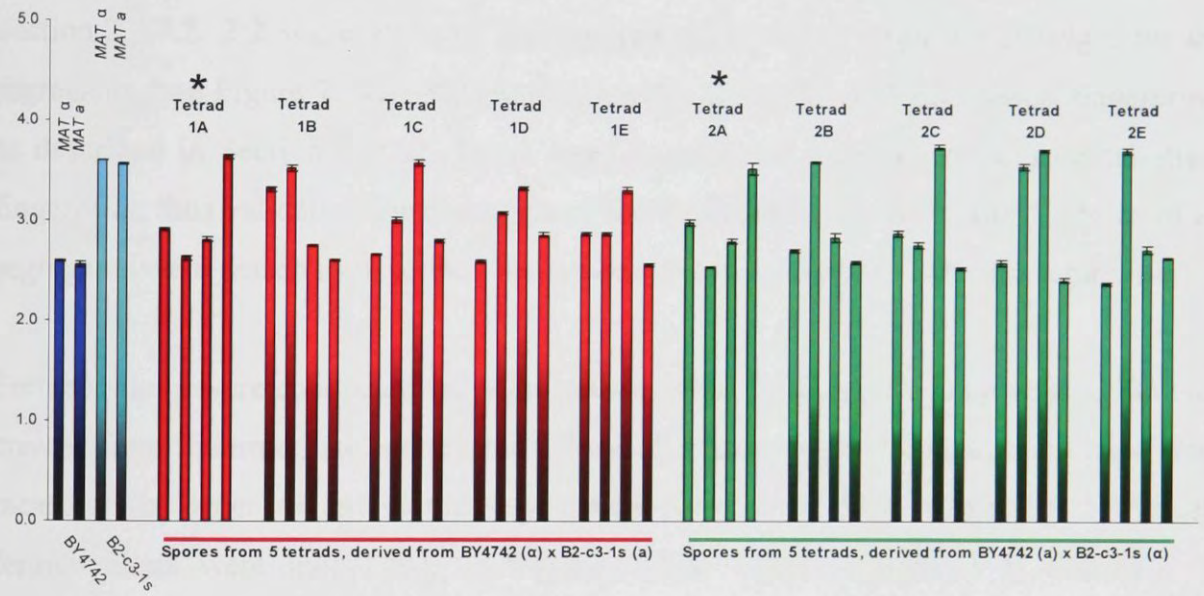

VII
BY4742 (MAT a)
B2-c3-1s (MAT α)
Tetrad 2B
Tetrad 2C
Tetrad 2D
Tetrad 2E

Figure 7.12 Glycerol production for 10 random tetrads derived from sporulation of heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)] and [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)]

Spores from ten tetrads, derived from sporulation of heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)], [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)], were tested for glycerol production. Parental *S. cerevisiae* BY4742 and 'low-ethanol' variant B2-c3-1s of both mating types were used as controls. Assays were performed as described in Section 4.3.3. Fermentations were carried out in triplicate and values are means ± SD.

Stars indicate tetrads that were previously tested (see Figure 7.8), and were used as controls in this experiment.

7.12


5.0
4.0
3.0
2.0
1.0
0.0
MAT α
MAT a
MAT α
MAT a
*
Tetrad 1A
Tetrad 1B
Tetrad 1C
Tetrad 1D
Tetrad 1E
*
Tetrad 2A
Tetrad 2B
Tetrad 2C
Tetrad 2D
Tetrad 2E
BY4742
B2-c3-1s
Spores from 5 tetrads, derived from BY4742 (α) x B2-c3-1s (a)
Spores from 5 tetrads, derived from BY4742 (a) x B2-c3-1s (α)

7.3.6 Segregation of the 'low-ethanol' phenotype from crosses between segregants with intermediate 'low-ethanol' status.

If two independently assorting loci are required to confer the 'low-ethanol' phenotype, a cross between segregants that display an intermediate phenotype should yield a segregation pattern of 1:1:2 (parental : mutant : intermediate), (see Figure 7.13). A cross between segregants of intermediate phenotype derived from heterozygous diploid [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)] was therefore conducted to assess the segregation pattern from such a diploid. Intermediate segregants, A1 and A3, were mated as described in Section 2.3.4 and sporulated as described in Section 2.3.5.

Spores derived from two tetrads, were tested for mating-type, as described in Section 2.3.7.2. 2:2 segregation of mating type suggested a single ascus origin for the segregants (see Figure 7.14i). All segregants were tested for Ty1 transposon fingerprints as described in Section 2.2.7.1. These were found to be identical to the parental strain fingerprint, thus validating the parentage of the segregants (Figure 7.14ii). Cultures of all segregants were frozen as described in Section 2.1.5.1 and kept at -80°C for future use.

Fermentations were conducted for segregants derived from the above sporulated diploids created from 'intermediate' segregants. Glycerol, ethanol and acetic acid production were measured in order to assess the segregation patterns of these segregants. Triplicate fermentations were inoculated from preinoculum cultures prepared as described in Section 2.1.5.2, and conducted as described in Section 2.1.5.4. When glucose was exhausted, samples of medium were analyzed using HPLC (see Section 2.2.4). The segregation pattern of the 'low-ethanol' phenotype is complex, and clearly not 1:1:2 (parental : mutant : intermediate) (Figure 7.15). This supports the assumption that mutations within more that two independently assorting loci are involved in shaping the 'low-ethanol' phenotype of B2-c3-1s.

Figure 7.13 Hypothetical segregation pattern of 'low-ethanol' phenotype conferred by two recessive genes, and segregation pattern after cross of the intermediates

Hypothetical segregation pattern for heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)] and [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)]. Blue structures represent parental strain BY4742 chromosomes and carry dominant traits, red structures represent chromosomes from 'low-ethanol' variant and carry recessive traits.

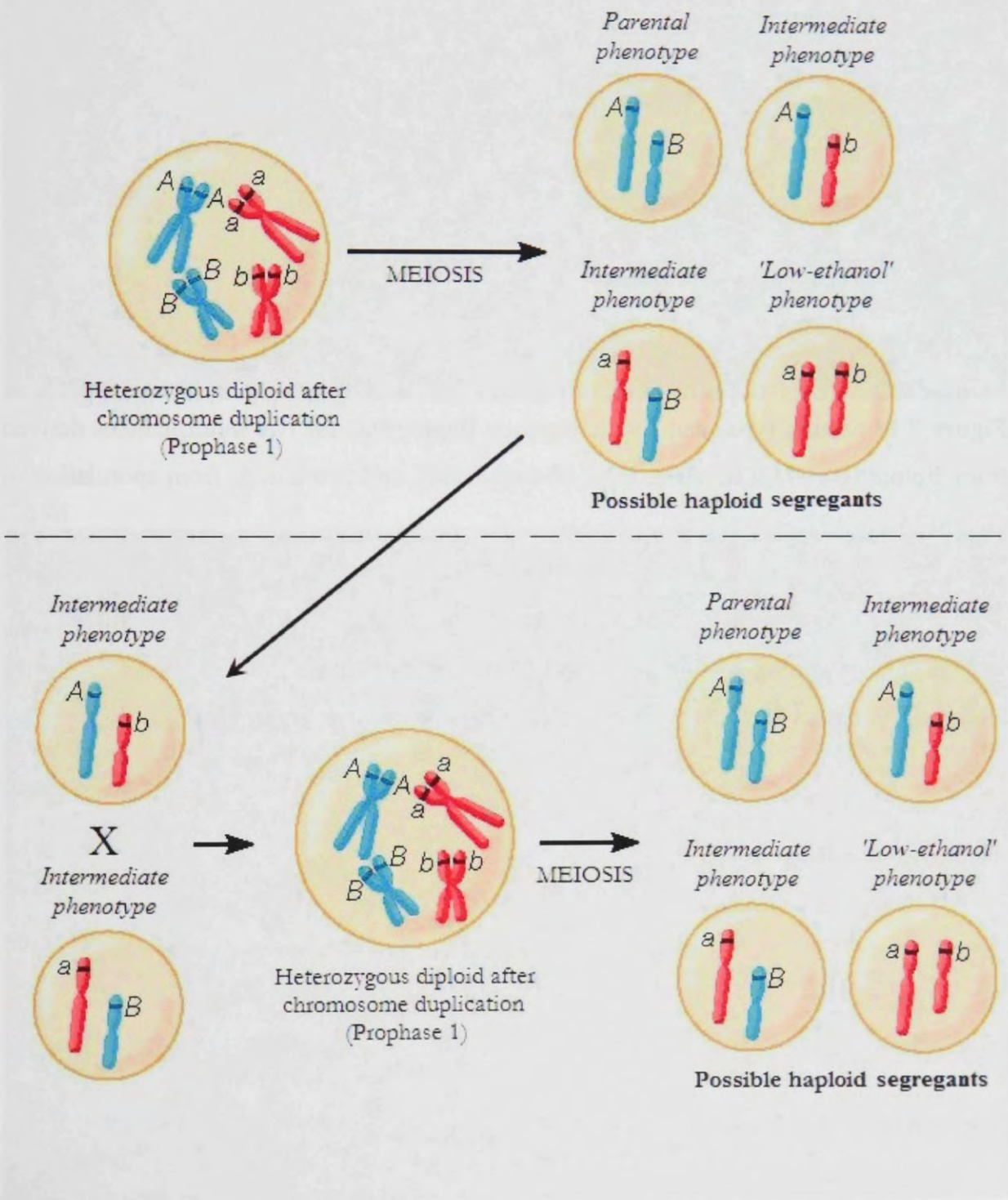
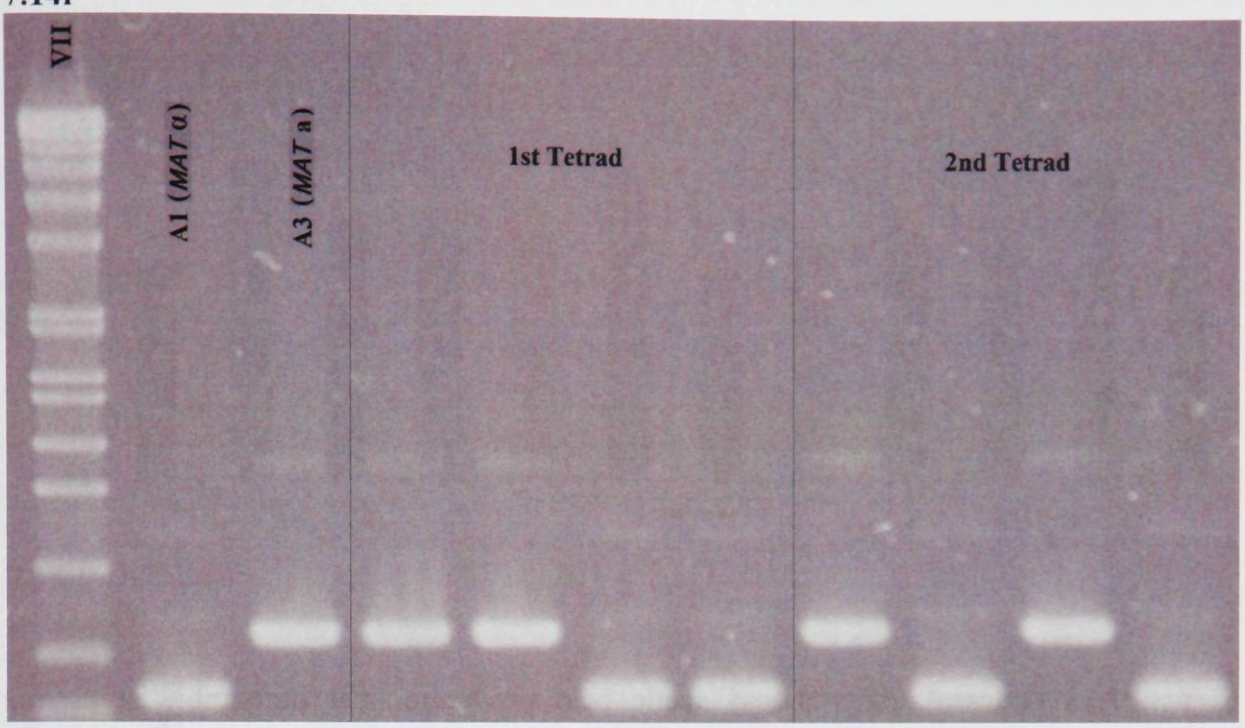

Parental phenotype
Intermediate phenotype
A B
A b
MEIOSIS
Intermediate phenotype
'Low-ethanol' phenotype
a B
a b
Possible haploid segregants
A A a a B B b b
Heterozygous diploid after chromosome duplication (Prophase 1)
Intermediate phenotype
A b
X
Intermediate phenotype
a B
Heterozygous diploid after chromosome duplication (Prophase 1)
MEIOSIS
Parental phenotype
Intermediate phenotype
A B
A b
Intermediate phenotype
'Low-ethanol' phenotype
a B
a b
Possible haploid segregants

Figure 7.14 Mating types and Ty1 transposon fingerprints for two intermediates derived from diploid [BY4742(*MAT*α) x B2/1-c4-2s(*MAT*a)], and two tetrads from sporulation of diploid [A1 x A3]

Spores from two tetrads, derived from heterozygous diploid [A1(*MAT*α) x A3(*MAT*a)], were tested for mating type segregation (7.14i) and Ty1 transposon fingerprints (7.14ii). Intermediate haploids A1(*MAT*α) and A3(*MAT*a)were used as controls.

7.14i


VII
A1 (MAT α)
A3 (MAT a)
1st Tetrad
2nd Tetrad

7.14ii

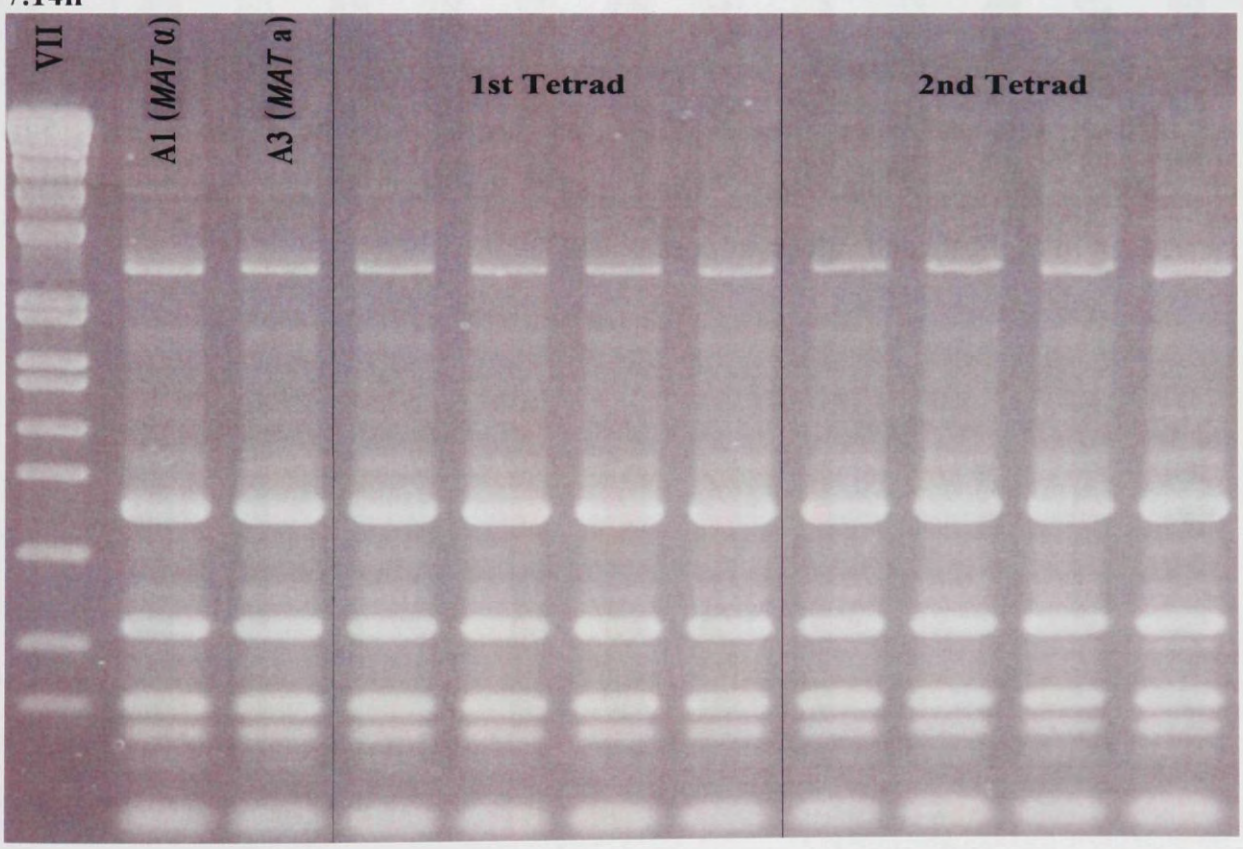
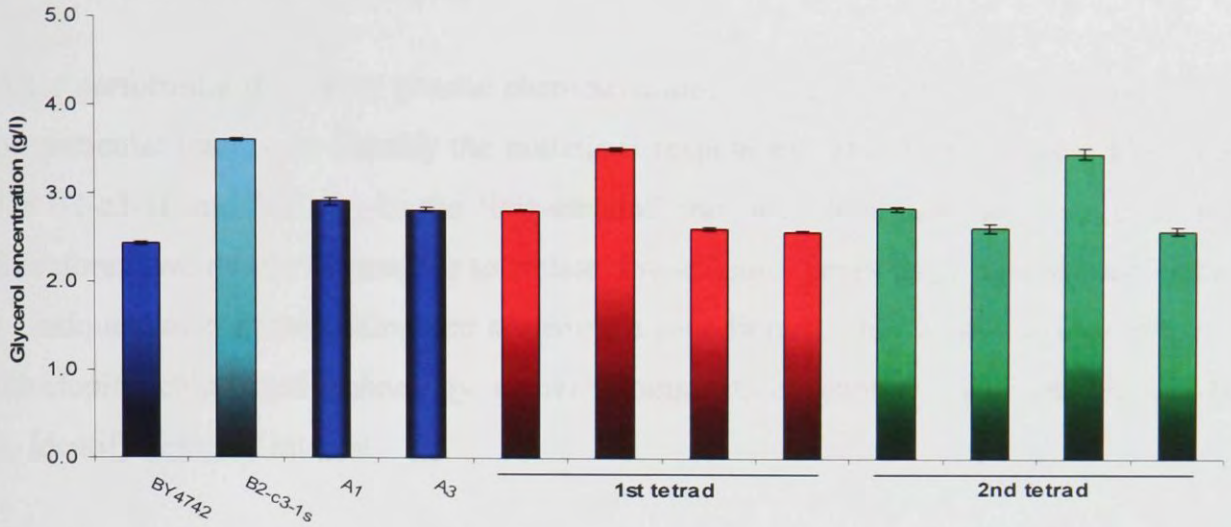

VII
A1 (MAT α)
A3 (MAT a)
1st Tetrad
2nd Tetrad

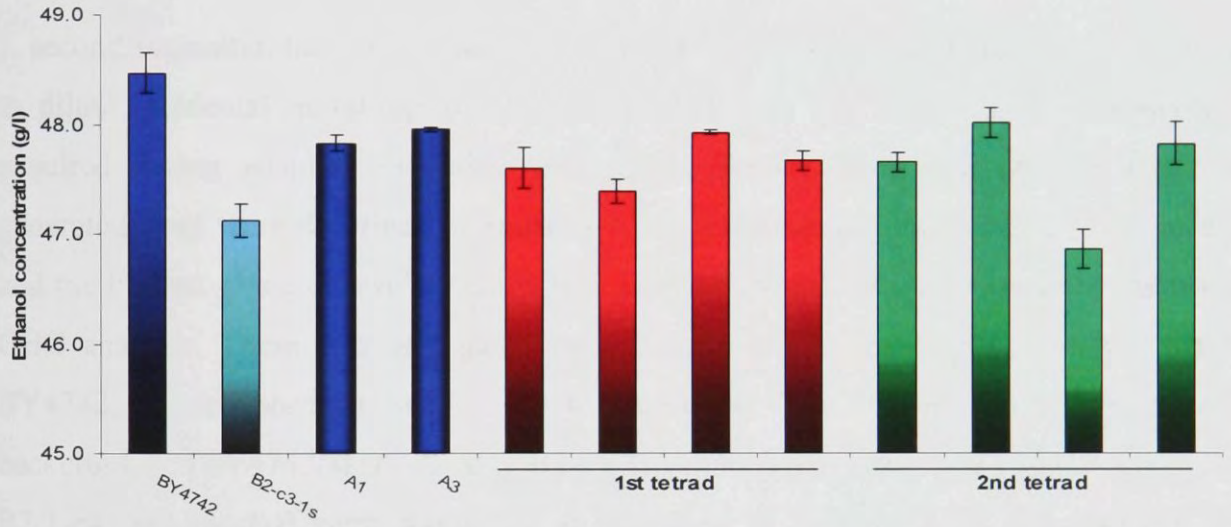
Figure 7.15 Glycerol, ethanol and acetic acid production for parental *S. cerevisiae* BY4742, 'low-ethanol' variant B2-c3-1s, two intermediates A1 and A3 and two groups of segregants derived from heterozygous diploid [A1(*MAT*α) x A3(*MAT*a)]

Spores from two tetrads, derived from sporulation of heterozygous diploid [A1(*MAT*α) x A3(*MAT*a)], were tested for glycerol (7.15i), ethanol (7.15ii) and acetic acid (7.15iii) production. Assays were performed as described in Section 2.1.5.4. Fermentations were carried out in triplicate and values are means ± SD.

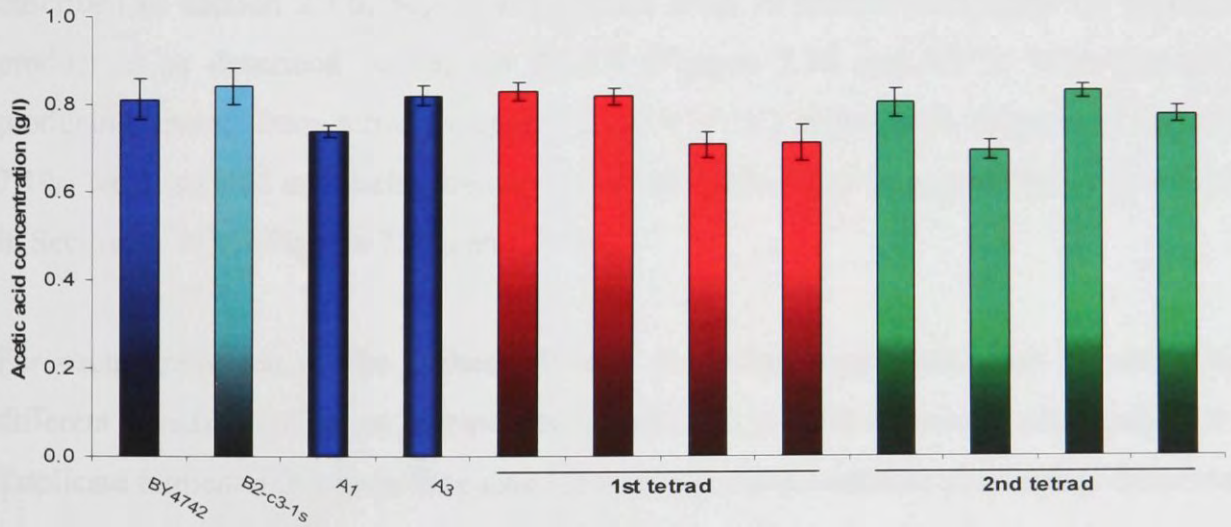
7.15i



7.15ii



7.15iii



7.4 Results to CGH analysis

After performing the above genetic characterization of the 'low-ethanol' variants, it was of particular interest to identify the mutations responsible. However, it was evident that for B2-c3-1s and B2/1-c4-2s the 'low-ethanol' trait was multigenic and recessive, and therefore it would not be possible to isolate 'low-ethanol' genes using standard molecular techniques, such as generating and screening a gene library. Thus it was decided to trial a developing chip-based technology, namely Comparative Genomic Hybridization (CGH), to identify genes of interest

7.4.1 Genome 'clean up' for CGH arrays

A second sequential backcross was performed for both B2-c3-1s and B2/1-c4-2s in order to dilute incidental mutations (i.e. not associated with the 'low-ethanol' phenotype), acquired during adaptive evolution. Segregants, derived from heterozygous diploids generated from work described in Sections 7.3.4, that produced the lowest ethanol levels and the highest glycerol levels, named B2-c3-1s(A) and B2/1-c4-2s(A), were chosen for CHG analysis. These two segregants were backcrossed to the original parental strain BY4742, as described in Section 2.3.4 (see Figure 7.5). Diploids from this second backcross, [BY4742(*MAT*α) x B2-c3-1s(A)(*MAT*a)] and [BY4742(*MAT*α) x B2/1-c4-2s(A)(*MAT*a)] were sporulated as described in Sections 2.3.5 and dissected as described in Section 2.3.6. Segregants derived from 10 tetrads were tested for glycerol production as described in Section 2.1.5.4 (Figures 7.16 and 7.17). 'High-glycerol' producing spores from tetrads displaying 2:2 ('α':'a') segregation (Figures 7.18i and 7.19i) were isolated and their parentage confirmed using Ty1 fingerprinting as described in Section 2..3.7.1 (Figures 7.18ii and 7.19ii).

For each strain, ten of the highest glycerol producing segregants, each chosen from different tetrads, were tested in triplicate for glycerol, ethanol and acetic acid production. Triplicate fermentations were inoculated from preinoculum cultures prepared as described in Section 2.1.5.2, and conducted as described in Section 2.1.5.4. When glucose was exhausted, samples of medium were analyzed using HPLC (see Section 2.2.4). Nine out

Figure 7.16 Glycerol production for spores from ten randomly chosen tetrads, derived from heterozygous diploid [BY4742(*MAT*α) x B2-c3-1s(A)(*MAT*a)]

Spores from ten tetrads, derived from sporulation of heterozygous diploid [BY4742(*MAT*α) x B2-c3-2s(A)(*MAT*a)], were tested for glycerol production. Parental *S. cerevisiae* BY4742 (dark blue) and 'low-ethanol' variant B2-c3-1s (light blue) were used as controls. Assays were performed as described in Section 4.3.3. Fermentations were carried out in triplicate and values are means ± SD. 'High-glycerol' segregants marked with stars were chosen for further assessment.

7.16

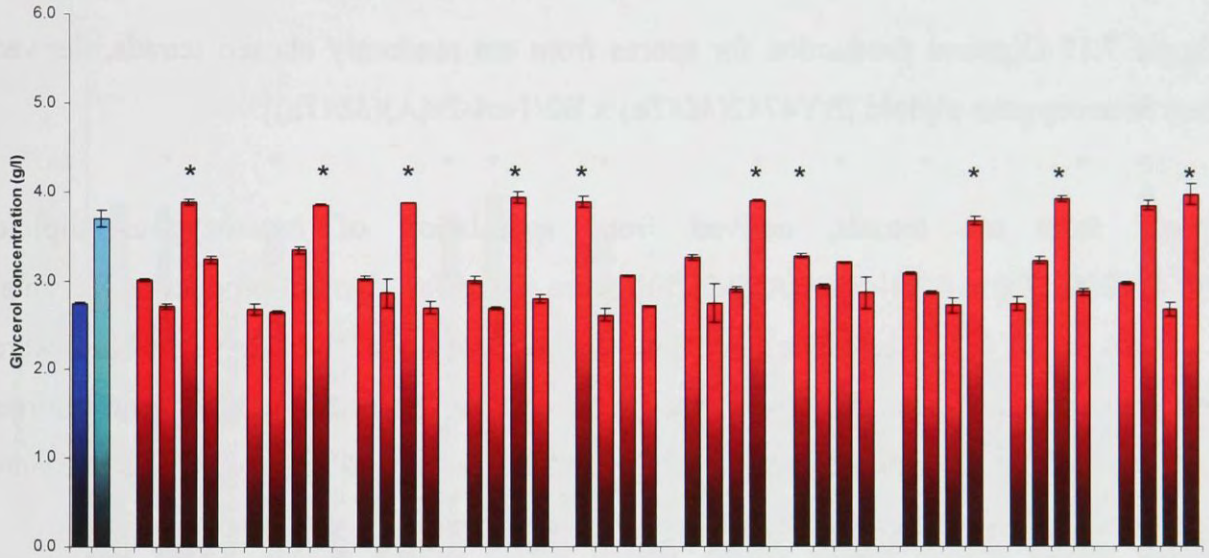


Figure 7.17 Glycerol production for spores from ten randomly chosen tetrads, derived from heterozygous diploid [BY4742(*MAT*α) x B2/1-c4-2s(A)(*MAT*a)]

Spores from ten tetrads, derived from sporulation of heterozygous diploid [BY4742(*MAT*α) x B2/1-c4-2s(A)(*MAT*a)], were tested for glycerol production. Parental *S. cerevisiae* BY4742 (dark blue) and 'low-ethanol' variant B2/1-c4-2s (light blue) were used as controls. Assays were performed as described in Section 4.3.3. Fermentations were carried out in triplicate and values are means ± SD. 'High-glycerol' segregants marked with stars were chosen for subsequent assessment.

7.17

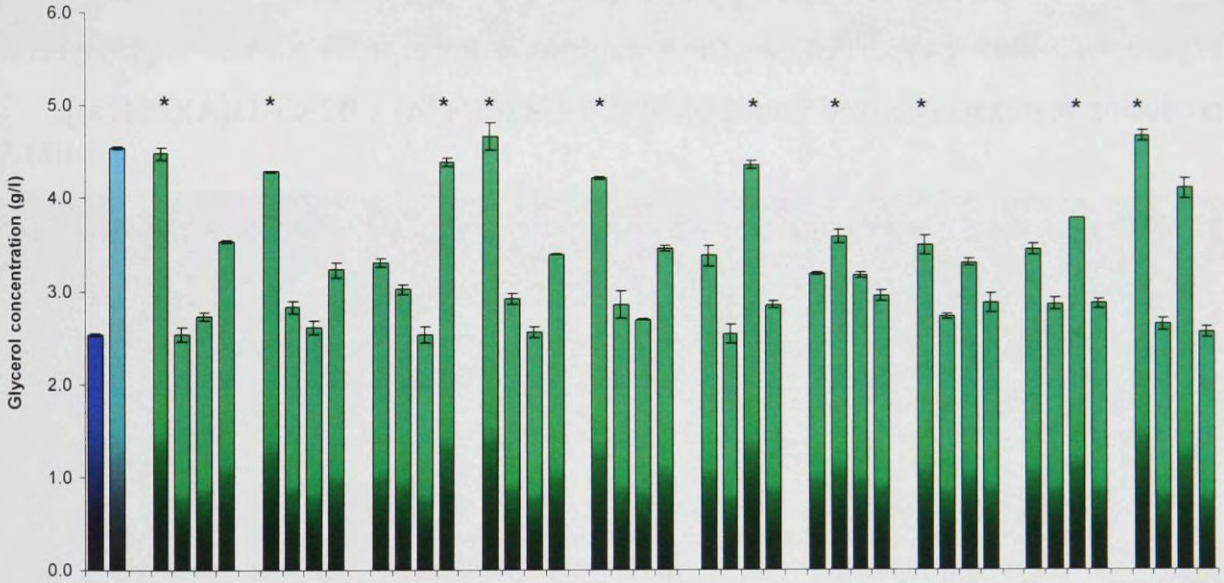
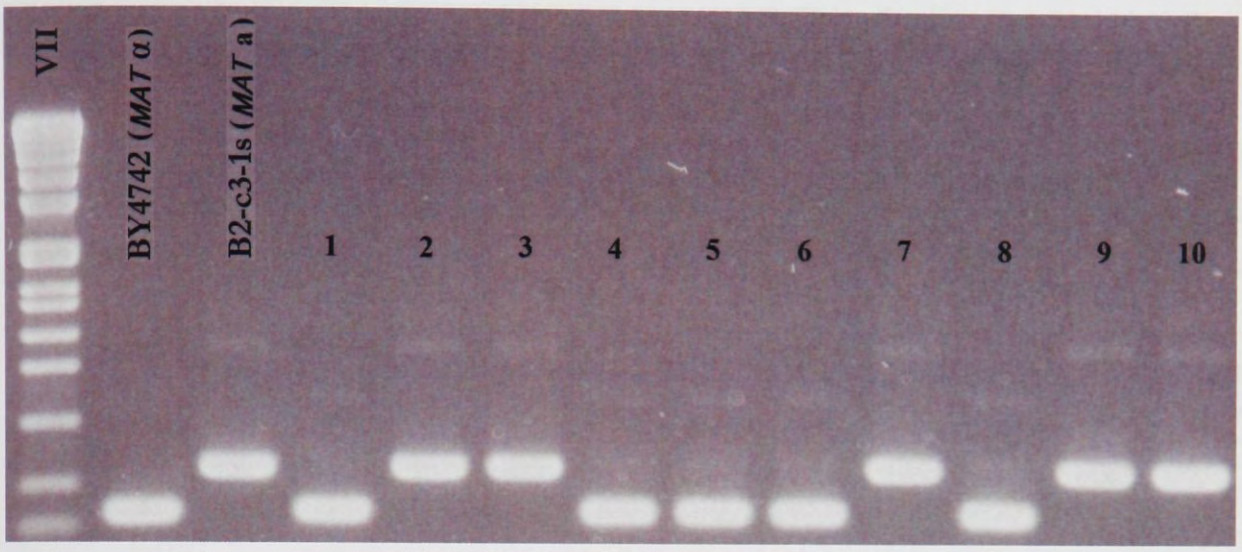


Figure 7.18 Mating-type PCR and Ty1 transposon fingerprints for ten 'high-glycerol' producing segregants derived from diploid [BY4742(*MAT*α) x B2-c3-1s(A)(*MAT*a)]

Ten 'high-glycerol' segregants derived from heterozygous diploid [BY4742(*MAT*α) x B2-c3-1s(A)(*MAT*a)], were tested for mating-type to check their ploidy (7.18i), and Ty1 transposon fingerprints to check their parentage (7.18ii). Haploids BY4742(*MAT*α) and B2-c3-1s(*MAT*a) were used as controls.

7.18i


VII
BY4742 (MAT α)
B2-c3-1s (MAT a)
1
2
3
4
5
6
7
8
9
10

7.18ii

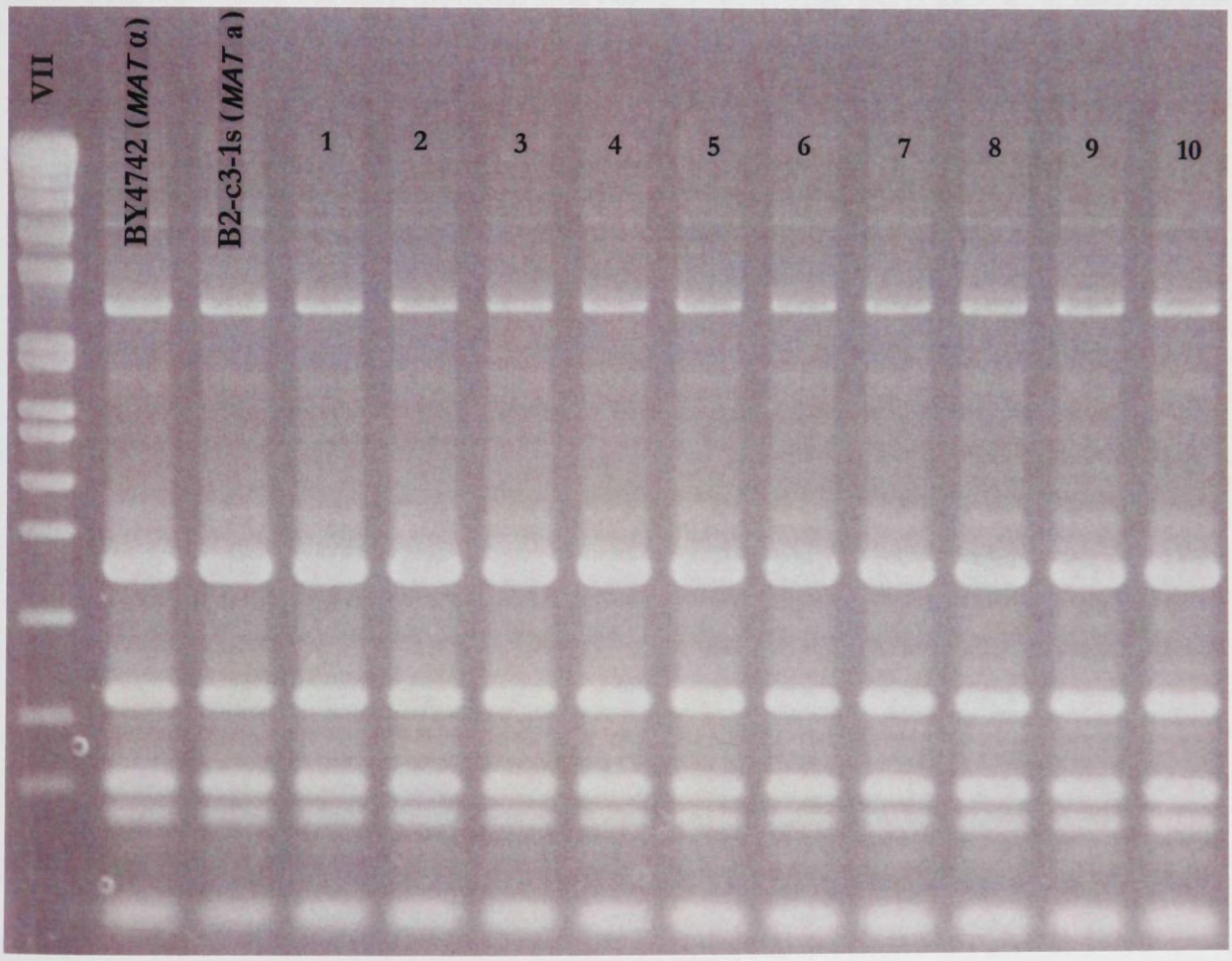
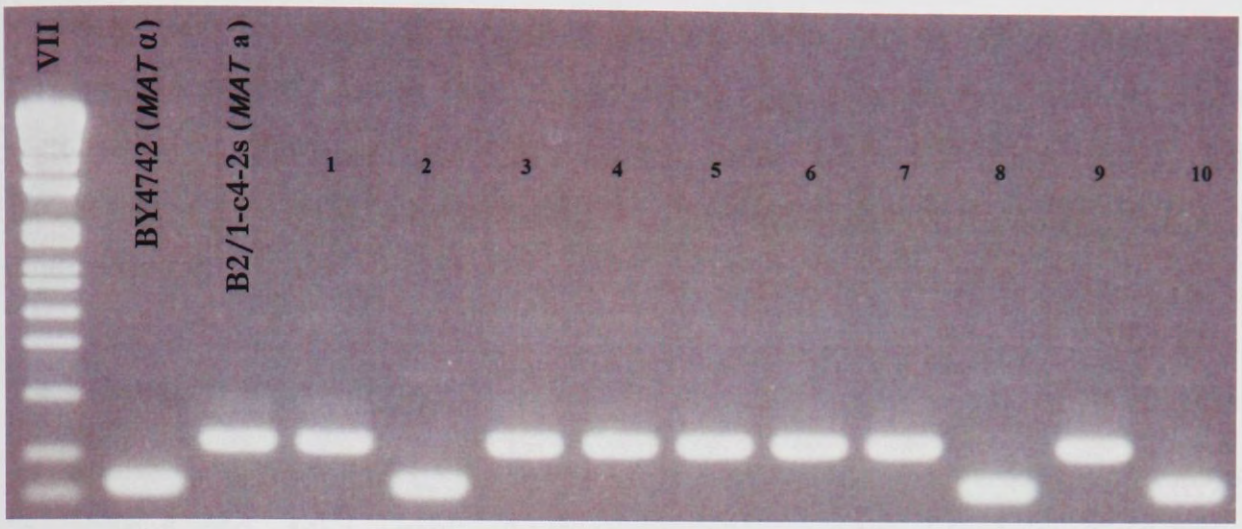

VII
BY4742 (MAT α)
B2-c3-1s (MAT a)
1
2
3
4
5
6
7
8
9
10

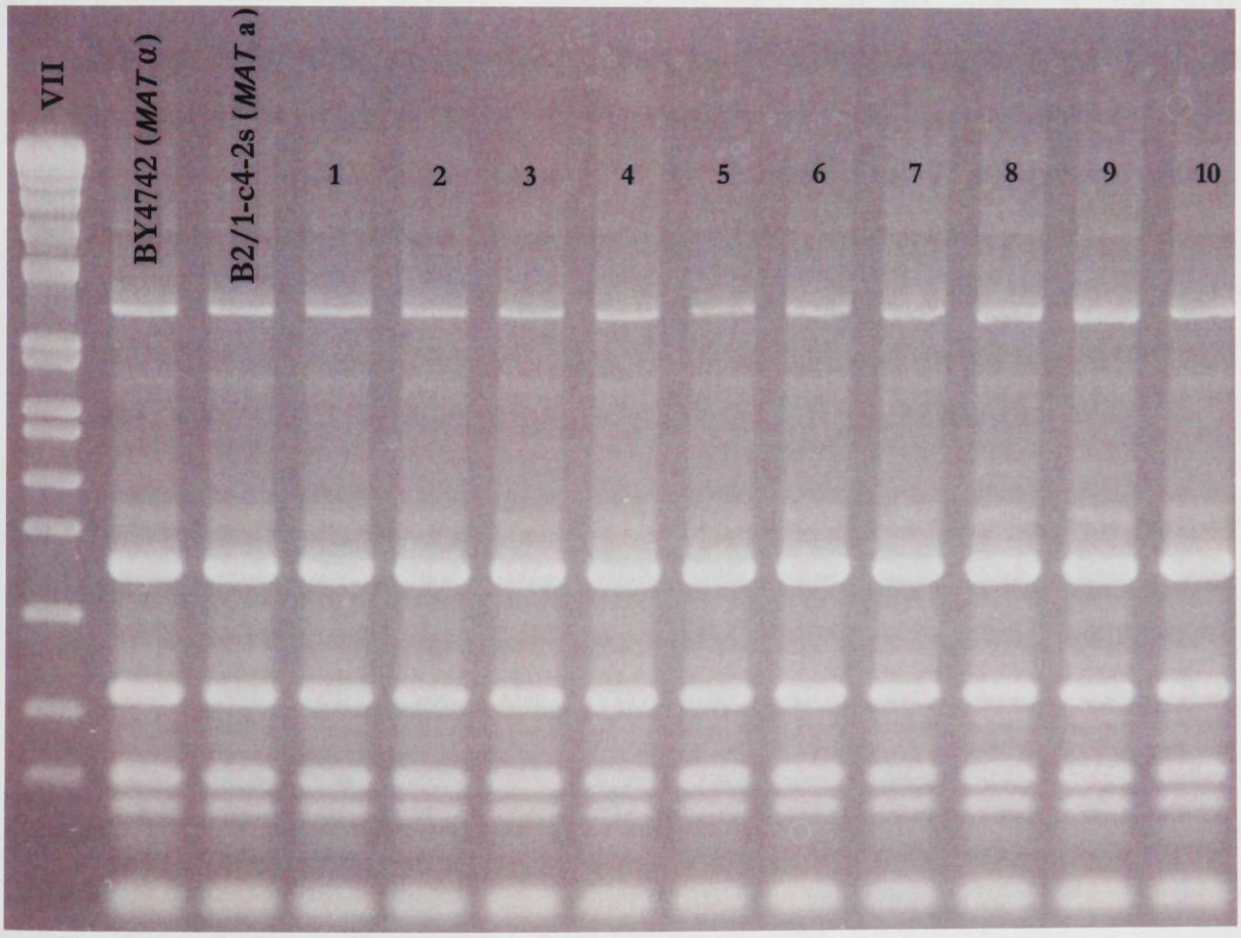
Figure 7.19 Mating types and Ty1 transposon fingerprints for ten 'high-glycerol' producing segregants derived from diploid [BY4742(*MAT*α) x B2/1-c4-2s(A)(*MAT*a)]

Ten 'high-glycerol' segregants derived from heterozygous diploid [BY4742(*MAT*α) x B2/1-c4-2s(A)(*MAT*a)], were tested for mating-type to check their ploidy (7.21i), and Ty1 transposon fingerprints to check their parentage (7.21ii). Haploids BY4742(*MAT*α) and B2/1-c4-2s(A)(*MAT*a) were used as controls.

7.19i


VII
BY4742 (MAT α)
B2/1-c4-2s (MAT a)
1
2
3
4
5
6
7
8
9
10

7.19ii


VII
BY4742 (MAT α)
B2/1-c4-2s (MAT a)
1
2
3
4
5
6
7
8
9
10

of ten 'high-glycerol' segregants derived from diploid [BY4742(*MAT*α) x B2-c3-1s(A)(*MAT*a)] retained their 'low-ethanol' phenotypes; they produced elevated glycerol levels that were associated with reduced ethanol yields (see Figure 7.20). However, in the case of segregants derived from diploid [BY4742(*MAT*α) x B2/1-c4-2s(A)(*MAT*a)], segregation of phenotypes was very diverse, in most cases segregants were dissimilar to their 'low-ethanol' parent, B2/1-c4-2s (Figure 7.21). This further supports the earlier conclusion that the 'low-ethanol' phenotype in B2/1-c4-2s is a multigenic trait.

7.4.2 Genomic Tiling arrays

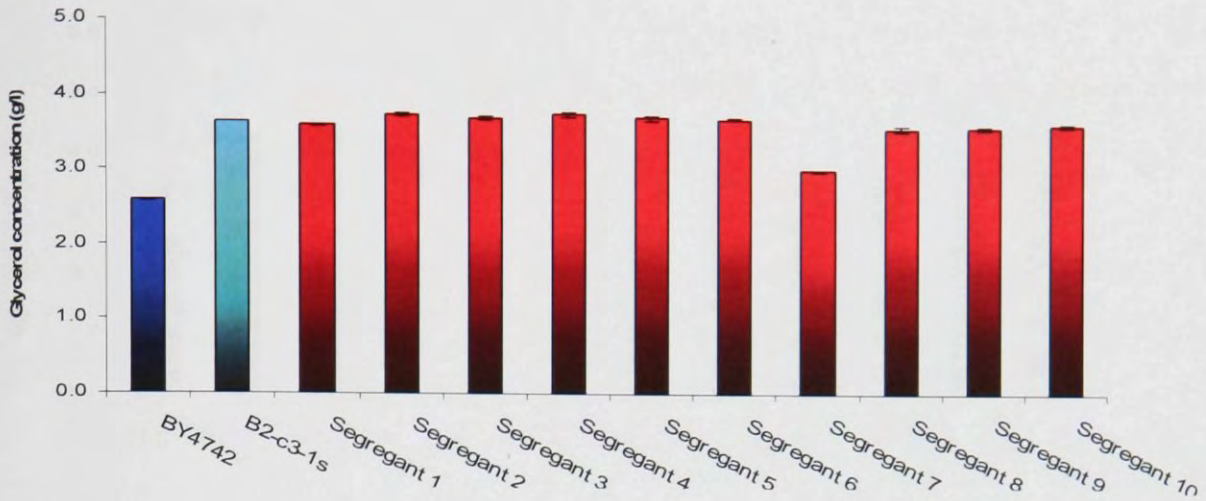
As discussed in section 7.2 chip-based CGH requires the use of arrays of probes immobilized onto a solid support, and there are numerous types of such arrays available. For a whole genome analysis it is necessary to cover entire coding and non-coding regions, and for maximum coverage tiling arrays are the preferable option. A tiling array is a subtype of microarray, in which labeled target DNA is hybridized to unlabeled probes that are designed to cover the entire genome sequence. *S. cerevisiae* 1.0R Array, which was used in this study, is a single array comprised of over 3.2 million perfect match/mismatch probe pairs tiled to cover the complete *S. cerevisiae* genome. Genome sequence information for the design of the *S. cerevisiae* Tiling 1.0R Array was drawn from the October 2003 Stanford Yeast Genome Database files (www.yeastgenome.org), http://www.affymetrix.com/products/arrays/specific/scerevisiae_tiling.affx.

For array analysis it was decided to use a pool of genomic DNA from the 'low-ethanol' segregants derived from the second back-cross described in the previous section. This approach should have quantitatively enhanced the occurrence of the 'low-ethanol' mutations relative to incidental genetic changes. Such a strategy was implemented for 'low-ethanol' segregants derived from [BY4742(*MAT* α) x B2-c3-1s(A)(*MAT*a)] diploid. Genomic DNA from 9 'low-ethanol' segregants, derived from the above diploid, was isolated as described in Section 2.3.1, and equal aliquots of DNA from these segregates was pooled and sent to Australian Genome Research Facility Ltd., Melbourne (AGRF) for CGH analysis.

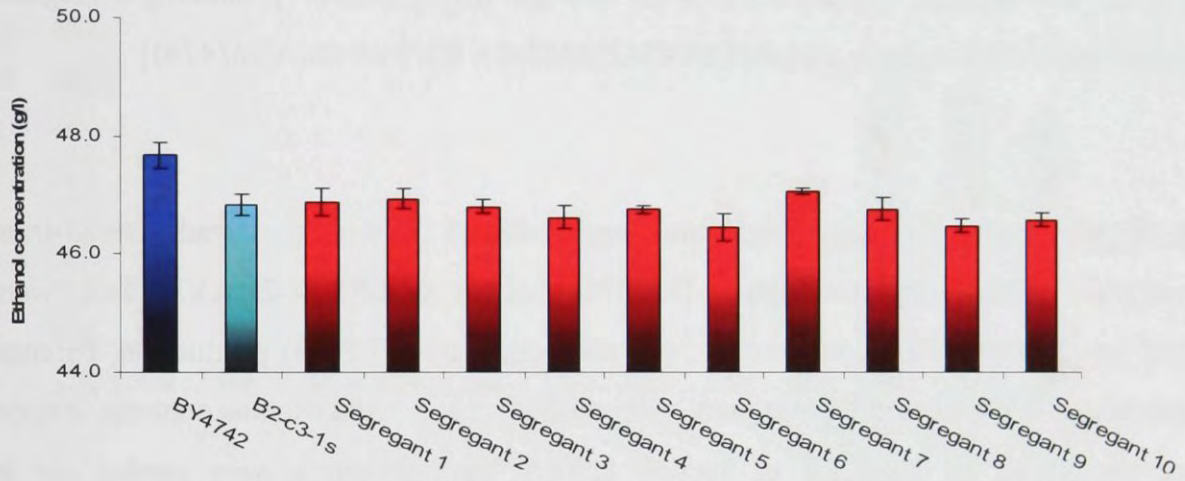
Figure 7.20 Glycerol, ethanol and acetic acid production for parental *S. cerevisiae* BY4742, 'low-ethanol' variant B2-c3-1s, and ten 'high-glycerol' producing segregants, derived from heterozygous diploid [BY4742(*MAT*α) x B2-c3-1s(A)(*MAT*a)]

Ten 'high-glycerol' producing segregants, each selected from single tetrad derived from sporulation of heterozygous diploid [BY4742(*MAT*α) x B2-c3-1s(A)(*MAT*a)], were tested for glycerol (7.17i), ethanol (7.17ii) and acetic acid (7.17iii) production. Parental *S. cerevisiae* BY4742 and 'low-ethanol' variant B2-c3-1s were used as controls. Assays were performed as described in Section 2.1.5.4. Fermentations were carried out in triplicate and values are means ± SD.

7.20i



7.20ii



7.20iii

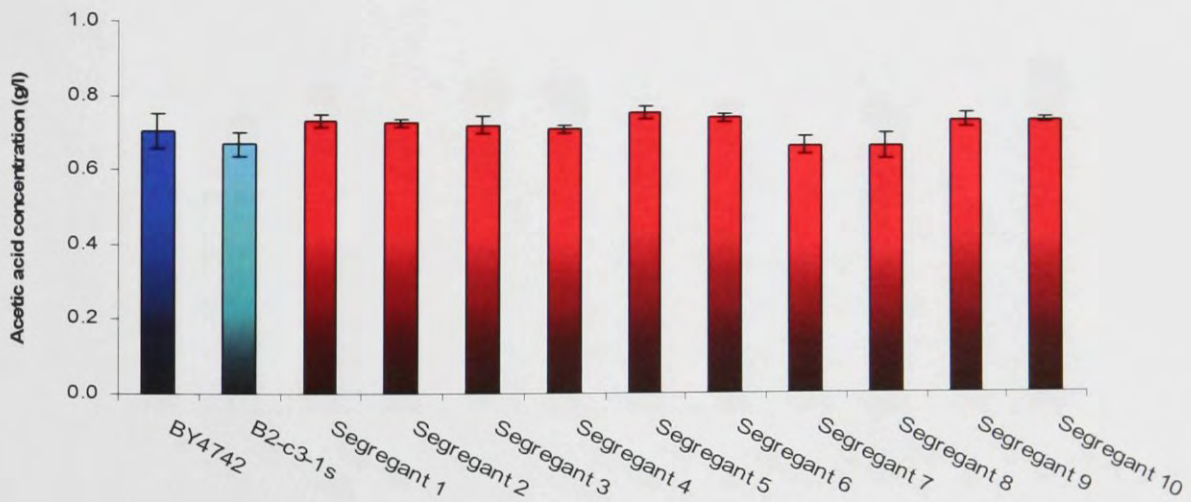
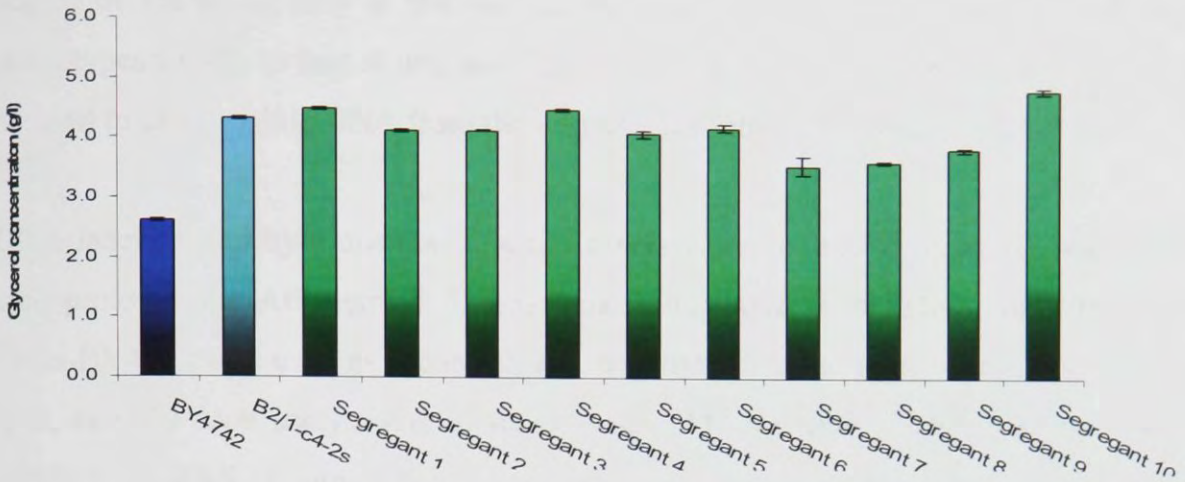


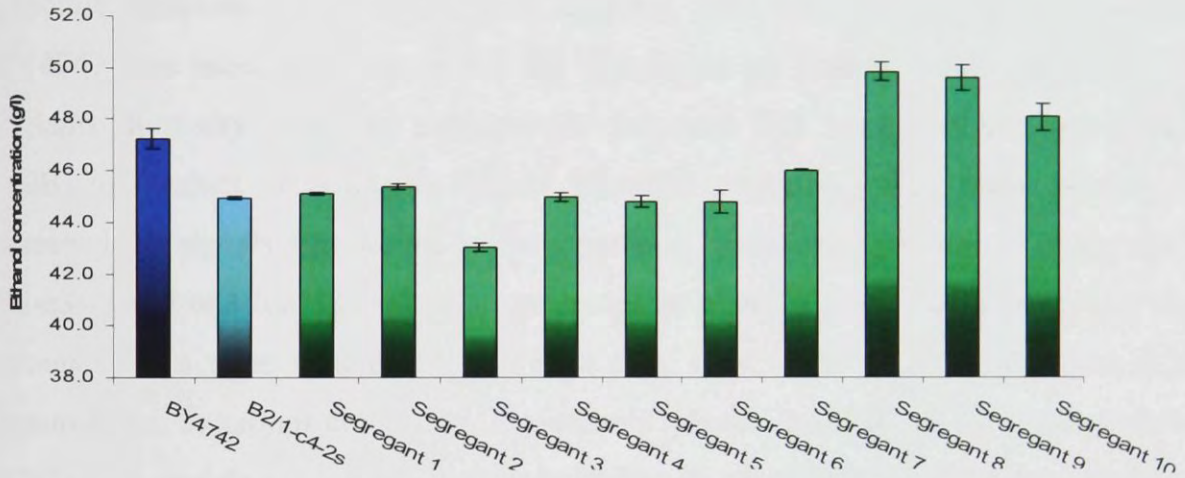
Figure 7.21 Glycerol, ethanol and acetic acid production for parental *S. cerevisiae* BY4742, 'low-ethanol' variant B2/1-c4-2s, and ten 'high-glycerol' producing segregants selected from heterozygous diploid [BY4742(*MAT*α) x B2/1-c4-2s(A)(*MAT*a)]

Ten 'high-glycerol' producing segregants, each selected from single tetrad derived from sporulation of heterozygous diploid [BY4742(*MAT*α) x B2/1-c4-2s(A)(*MAT*a)], were tested for glycerol (7.20i), ethanol (7.20ii) and acetic acid (7.20iii) production. Parental *S. cerevisiae* BY4742 and 'low-ethanol' variant B2/1-c4-2s were used as controls. Assays were performed as described in Section 2.1.5.4. Fermentations were carried out in triplicate and values are means ± SD.

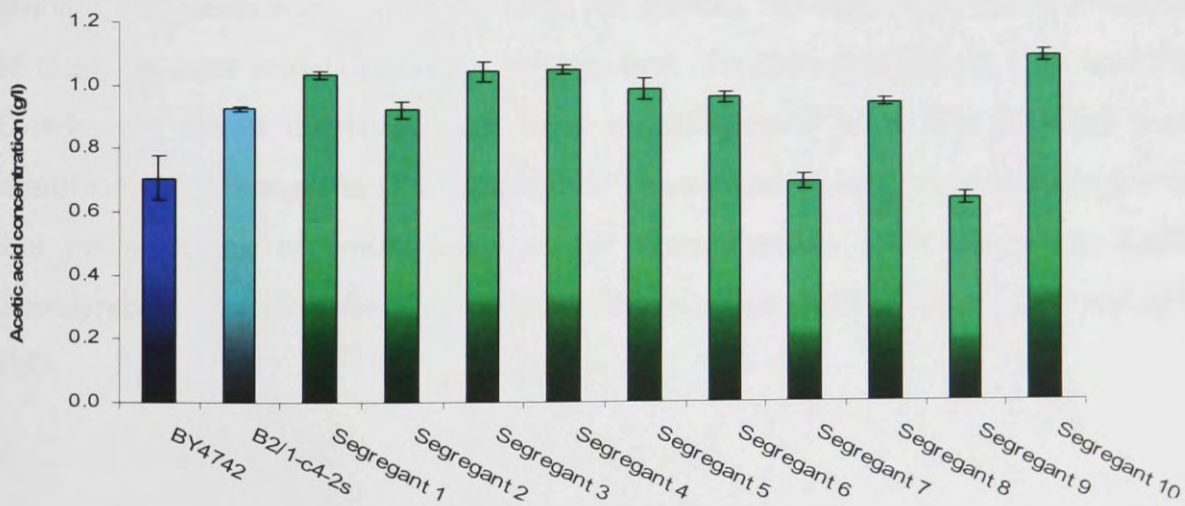
7.21i



7.21ii



7.21iii



The segregation pattern of the 'low-ethanol' phenotype of B2/a-c4-2s displayed a high degree of variation; only a few out of the total pool of ten tetrads tested displayed phenotypes similar to that of original B2/a-c4-2s (see Figure 7.21). Because of this, it was decided to use genomic DNA from the original 'low-ethanol' variant B2/1-c4-2s.

DNA labeling and hybridization to CGH arrays were conducted at AGRF according to their protocols for Affimetrix® *S. cerevisiae* Tiling Array 1.0R. DNA from the parental strain BY4742 was used as a control, and was hybridized to a separate array. CEL data files, one for each array, were analyzed using Affymetrix® Tiling Analysis Software (TAS) v1.1. TAS feature-intensity data files were used to produce signal- and p-values for each interrogated genomic probe position on the arrays that represented overlapping genomic positions within entire yeast genome. Data obtained for the parental strain BY4742 was used as a control for the assessment of both B2-c3-1s and B2/1-c4-2s variants. Intensity data was subsequently imported into Integrated Genome Browser (IGB) to conduct visualization against genomic annotations. The entire genome was screened for signals that would indicate potential mutations. Two sets of data, one for B2-c3-1s and one for B2/1-c4-2s, were compared simultaneously and only points where strong signals were visible for both data sets, were taken into consideration; it was assumed that mutations conferring 'low-ethanol' phenotype of B2-c3-1s were inherited in B2/1-c4-2s, and thus should be present in both analyzed signals[3] (see, for example, Figure 7.22). Several loci were identified that matched the above criteria, and corresponding genomic fragments were sequenced (data not shown). However, sequencing revealed all of these apparent mutations to be artifacts (see, for example, Figures 7.22 and 7.23). Clearly, the arrays displayed high level of background noise that disabled precise identification of changes in DNA sequences. The method, although potentially a powerful tool for screening of whole genomes for alternations in DNA sequences, requires considerable refinement. Because of time constrains this could not be done as part of this PhD.

[3] Reminder: B2-c3-1s was isolated in the first round of adaptive evolution (Chapter 4). B2/1-c4-2s was derived from B2-c3-1s in the second round of adaptive evolution (Chapter 5). Thus, B2/1-c4-2s is a descendant of B2-c3-1s.

Figure 7.22 Example of a screen from Integrated Genome Browser (IGB) distinguishing potential, common mutations in B2-c3-1s and B2/1-c4-2s. Red arrows show potential position place of mutation within *GSH1* gene

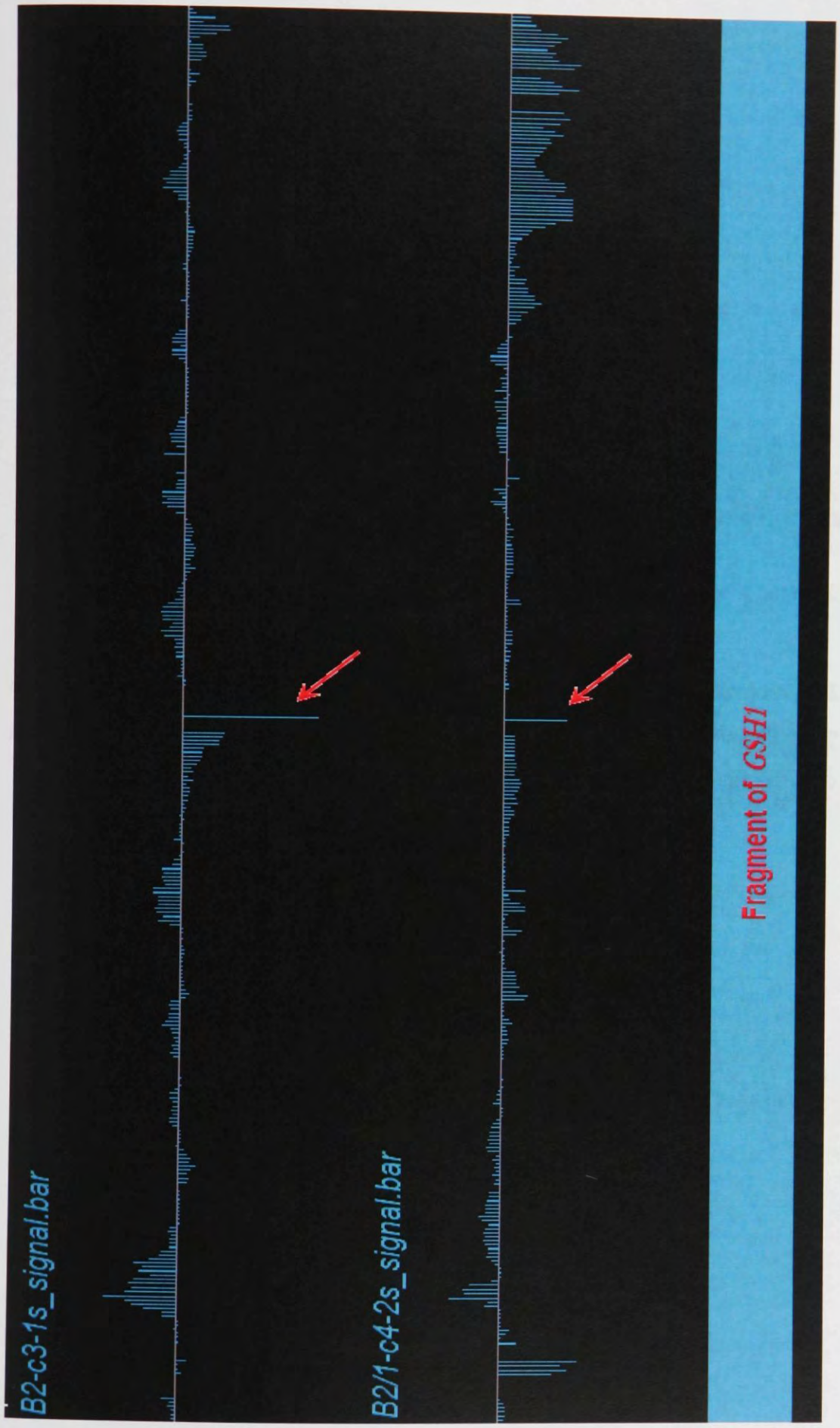
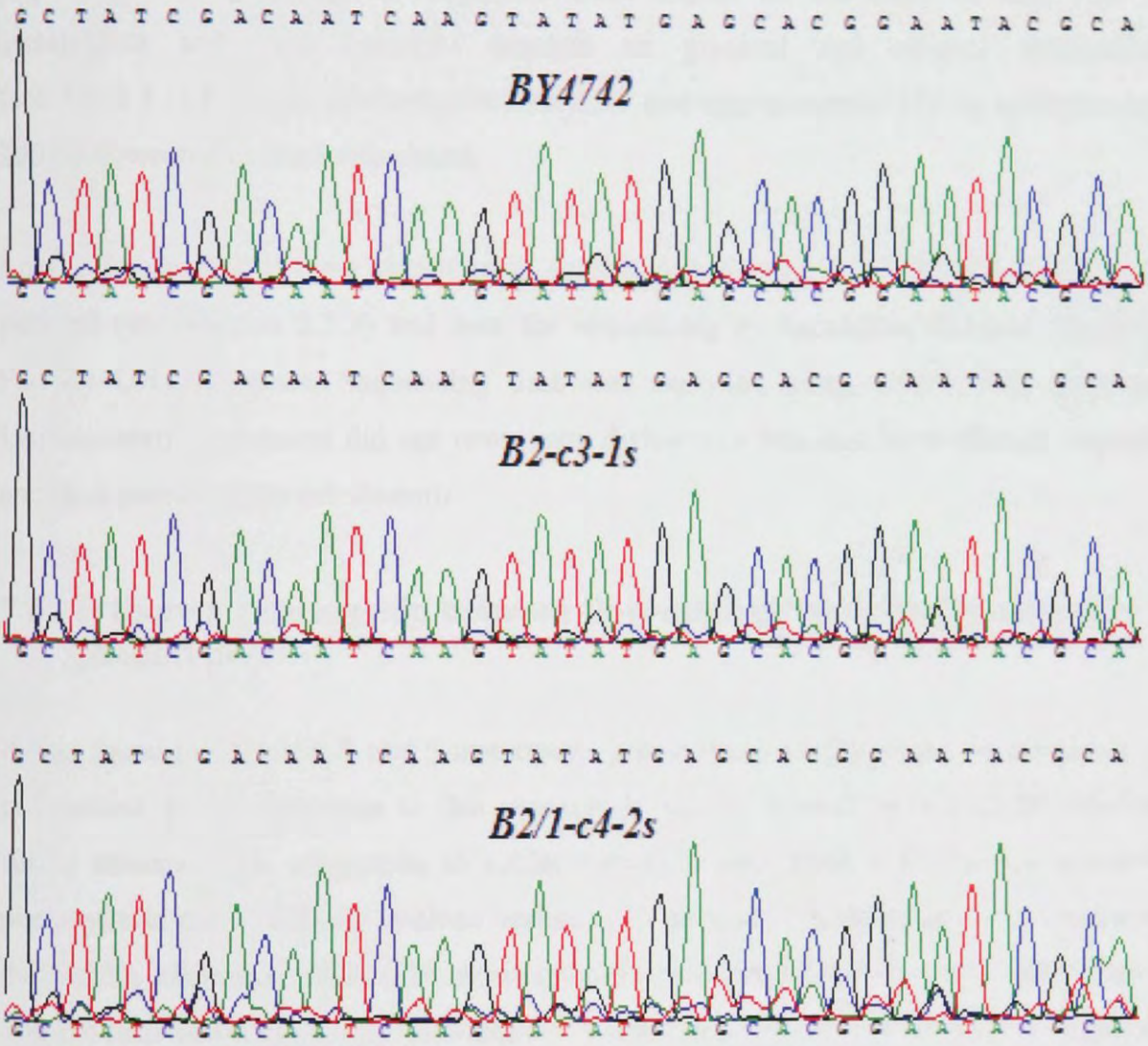

B2-c3-1s_signal.bar
B2/1-c4-2s_signal.bar
Fragment of GSH1

Figure 7.23 Chromatograms of the genomic equence, encompassing the position within the *GSH1* gene that data from tiling array analysis (see Figure 7.22) indicated may carry a mutation. Clearly, there was no mutation in this region.


G C T A T C G A C A A T C A A G T A T A T G A G C A C G G A A T A C G C A
BY4742
G C T A T C G A C A A T C A A G T A T A T G A G C A C G G A A T A C G C A
B2-c3-1s
G C T A T C G A C A A T C A A G T A T A T G A G C A C G G A A T A C G C A
B2/1-c4-2s

7.5 Results to sequencing genes with potential to confer the 'low-ethanol' phenotype

CGH analysis failed to resolve the mutations conferring a 'low-ethanol' phenotype. Therefore an alternative strategy was trialed. Candidate genes which might have an effect in redirecting of carbon flow away from ethanol and favoring glycerol synthesis were sequenced. The genes to be sequenced were chosen on the basis of their role in metabolism and their potential impacts on glycerol and ethanol metabolism (see Table 7.1) For each of these genes the ORF and approximately 350 bp upstream and 200 bp downstream were sequenced.

Loci to be sequenced were amplified as described in Section 2.3.7. PCR products were purified (see Section 2.3.9) and sent for sequencing to Australian Genome Research Facility Ltd., Brisbane. Sequencing data was analyzed using Vector NTI software. Unfortunately, sequences did not reveal any differences between 'low-ethanol' variants and their parents (data not shown).

7.6 Is there a relationship between 'low-ethanol' and 'sulfite-tolerance' phenotypes?

It was found in Chapters 4 and 5 that strains grown under sulfite stress for a number of generations gained tolerance to this stressor. It was of interest to investigate whether sulfite tolerance (i.e. adaptation to sulfite stress), is associated with the 'low-ethanol' phenotype in the adaptively evolved variants. To conduct a basic genetic assessment of the 'sulfite tolerance' phenotype, dominance/recessivness of this trait was investigated. The parental strain BY4742, 'low-ethanol' variant B2-c3-1s and heterozygous diploids derived from the latter two (see Section 7.3.4), were grown in YPD-10 supplemented with 30 g/l of sulfite. Inocula for the experiment was prepared as described in Section 2.1.5.2, and incubations were conducted as described in Section 2.1.5.4. The OD_{600} of cultures were measured at 12 hour intervals. Growth of the 'low-ethanol' variant B2-c3-1s and its heterozygous diploids were similar, however, the heterozygotes grew slightly slower and yielded lower biomass (Figure 7.24i). In comparison, growth of the parental strain

Table 7.1 Genes chosen for sequencing and the rationale for this choice. Unless referenced, all information in this table is from *Saccharomyces* Genome Database (SGD) (www.yeastgenome.org).

Alcohol dehydrogenase (*ADH*) genes

In *S. cerevisiae* there are five genes encoding alcohol dehydrogenases (*ADHs*), which are involved in ethanol metabolism. Four of these isoforms, encoded by *ADH1*, *ADH3*, *ADH4* and *ADH5*, are involved in reducing acetaldehyde to ethanol during alcoholic fermentation.

ADH1 This gene encodes cytosolic alcohol dehydrogenase isoenzyme I, which reduces acetaldehyde to ethanol, the last step in the fermentation (Ciriacy 1975). Mutations leading to recreased activity of this gene or its gene product might be expected to result in lowered ethanol production.

ADH3 This gene encodes mitochondrial alcohol dehydrogenase isozyme III, which is involved in the shuttling of mitochondrial NADH to the cytosol under anaerobic conditions and in ethanol production. This gene does not play significant role under aerobic conditions, however under anaerobic conditions mutants for this gene grow noticeably slower than wild type. This may be due to the involvement of Adh3p in an ethanol-acetaldehyde redox shuttle that is involved in maintaining the mitochondrial redox balance during anaerobic growth (Bakker et al. 2000). Decreased activity of this gene or its gene product could have impact on ethanol production.

ADH4 This gene encodes cytoplasmic alcohol dehydrogenase isoenzyme IV, an enzyme involved in ethanol production. There id not much information available on this isooenzyme regarding its metabolic contributions to ethanol production, however some sources state that it is highly expressed in brewing yeast (Dickinson et al. 2003). Also, spontaneous amplifications of this isoenzyme in an *ADH1* null mutant can rescue the wild-type phenotype (Dorsey et al. 1992), which suggests its involvement in ethanol production. Decreased activity in this gene or its gene product could result in lowered ethanol production.

ADH5 This enzyme encodes cytoplasmic alcohol dehydrogenase isoenzyme V; for which the function is currently ambiguous. Triple deletion mutant of *ADH1*Δ, *ADH3*Δ and *ADH5*Δ produced 35% less ethanol than the double deletion *ADH1*Δ, *ADH3*Δ, suggesting that Adh5p is involved in ethanol production (Smith et al. 2004) However, deletion of this isoform in connection with *ADH3*Δ deletion had virtually no effect on ethanol production (Smith et al. 2004). Despite that, and taking into account the unclear function of Adh5p, it was decided to sequence this gene because of its potential, in mutant form, to influence ethanol production.

Table 7.1 *continued*

Pyruvate decarboxylase (*PDC*) genes and their regulator gene *PDC2*

In *S. cerevisiae* there are three genes that encode different isoenzymes of pyruvate decarboxylase: *PDC1, PDC5* and *PDC6*. Pyruvate decarboxylase catalyses degradation of pyruvate to acetaldehyde and carbon dioxide. It has high affinity for pyruvate, thus limiting pyruvate availability from for metabolic fates, such as TCA cycle or gluconogenesis (Pronk et al. 1996).

PDC1 This gene encodes the major pyruvate decarboxylase isozyme I, a key enzyme in alcoholic fermentation (Pronk et al. 1996). Deletion of this isoform results in a 20% reduction of wild type PDC activity (Hohmann and Cederberg 1990). It is highly expressed in active fermenting yeast. Decreased activity in this gene or its gene product might be expected to result in lowered acetaldehyde production, and therefore decrease in ethanol yields.

PDC5 This gene encodes a minor isoform of pyruvate decarboxylase. *PDC5* null mutant do not have reduced pyruvate decarboxylase activity; *PDC1* fully compensates for loss of this isoform (Hohmann and Cederberg 1990). However, in *PDC1* deletion mutants activity of pyruvate decarboxylase is conserved at about 80% of wild type activity, which suggest that *PDC5* compensates for this loss. Because there is no data in the literature on ethanol production in *PDC5*Δ null mutants, it was decided to include this gene in sequencing studies. Decreased activity in this gene or its gene product might result in lowered acetaldehyde production, and therefore decrease in ethanol yields.

PDC6 This gene encodes a minor isoform of pyruvate decarboxylase. Although this enzyme is not expressed during sugar fermentation it plays a significant role when yeast is grown on ethanol. While the function of this isoenzyme in glucose dissimilation is not fully understood, it was decided for completion, to subject this gene to sequencing.

PDC2 This gene encodes a transcription factor required for the synthesis of the glycolytic enzyme pyruvate decarboxylase. Pdc2p, which is DNA binding protein, was shown to be required for high level expression of the *PDC1* and *PDC5* genes (Mojzita and Hohmann 2006). Decreased activity in this protein could result in decreased expression of *PDC* genes and impair ethanol production.

Table 7.1 *continued*

Glycerol-3-phosphate dehydrogenase (*GPD*) genes

Glycerol-3-phosphate dehydrogenases (*GPD*) are the key enzymes of glycerol synthesis. GPD catalyzes conversion of dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate (G-3-P), which is subsequently dephosphorylated to glycerol by glycerol 3-phosphatase. There are two isoenzymes of glycerol-3-phosphate dehydrogenase, encoded: Gpd1p and Gpd2p.

GPD1 This gene encodes NAD-dependent glycerol-3-phosphate dehydrogenase I, which is a key enzyme of glycerol synthesis. Overexpression of this gene in yeast results in higher glycerol production (see, for example, Nevoight and Stahl 1996). Mutations resulting in increased expression of this gene, or increased activity of its gene product, would be expected to result in elevated glycerol production and decreased ethanol production.

GPD2 This gene encodes NAD-dependent glycerol 3-phosphate dehydrogenase II, a paralog of *GPD1*. This isoform play a role in glycerol synthesis under anoxic conditions (Ansell et al. 1997). Overexpression of this gene was shown to result in increased glycerol production and reduced ethanol yields (de Barros Lopes et al. 2000, Eglinton et al. 2002) Increased activity or its gene product would be expected to result in elevated glycerol and lowered ethanol production.

Other candidate genes

TPI1 This gene encodes triose phosphate isomerase, Tpi1p. During fermentative growth on glucose, this enzyme catalyzes the reversible conversion of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. Mutants lacking *TPI1* gene result in greatly impaired ethanol production, with carbon being diverted almost completely to glycerol (Compagno et al. 1996). Mutations resulting in reduced synthesis or activity of this enzyme would be expected to cause reduced ethanol production.

Figure 7.24 Growth profiles under sulfite stress for parental *S. cerevisiae* BY4742, 'low-ethanol' variant B2-c3-1s, two heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)] and [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)], and segregants derived from sporulation of these diploids.

The above strains were incubated under sulfite stress (YPD-10, 30g/l Na_2SO_3, pH 8) (Section 2.1.5.4), and growth was followed by monitoring cultures density (OD_{600}), at 12 hour intervals as described in Section 2.2.1.

i – Growth of BY4742, 'low-ethanol' variant B2-c3-1s and heterozygous diploids [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)] and [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)] under sulfite stress.

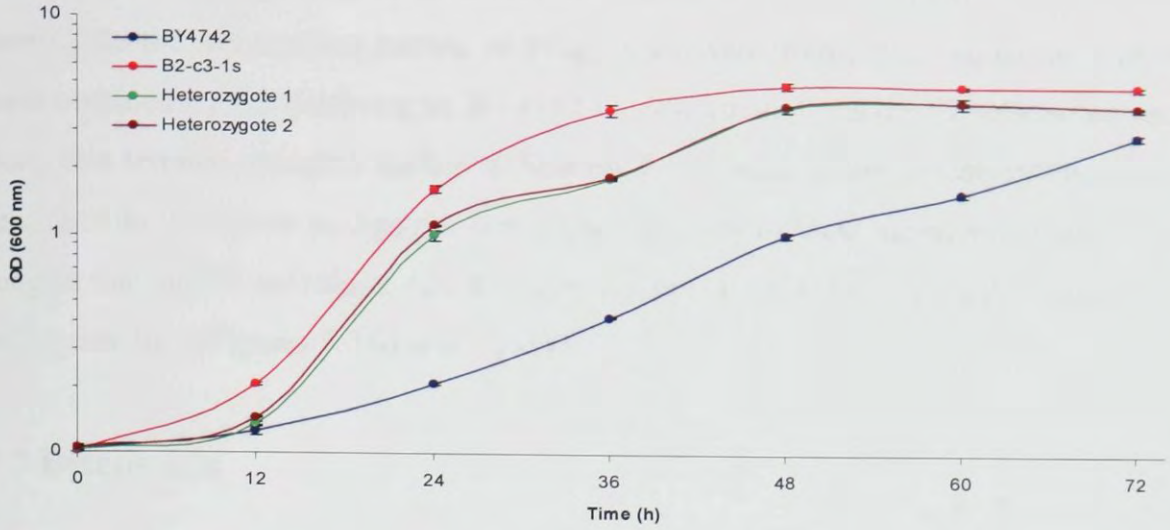
ii – Segregants derived from sporulation of heterozygous diploid [BY4742(*MAT*α) x B2-c3-1s(*MAT*a)] tested for sulfite tolerance. In terms of 'low-ethanol' phenotypes these segregate as follows (see Figure 7.9):

Segregant A1 - intermediate phenotype
Segregant A2 - BY4742-like phenotype
Segregant A3 - intermediate phenotype
Segregant A4 - 'low-ethanol' phenotype

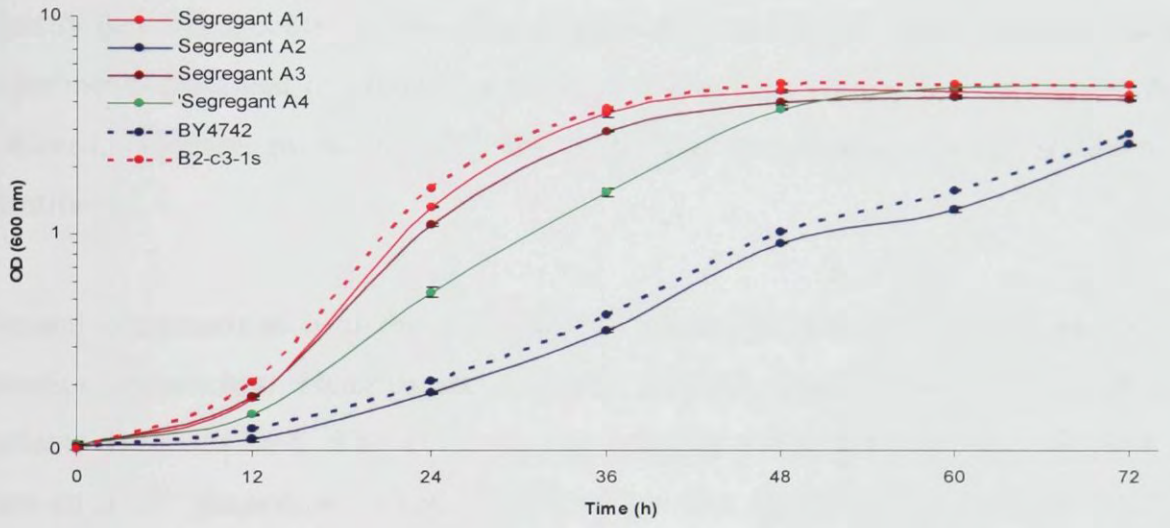
iii – Segregants derived from sporulation of heterozygous diploid [BY4742(*MAT*a) x B2-c3-1s(*MAT*α)] tested for sulfite tolerance. In terms of 'low-ethanol' phenotypes these segregate as follows (see Figure 7.9):

Segregant B1 - intermediate phenotype
Segregant B2 - BY4742-like phenotype
Segregant B3 - intermediate phenotype
Segregant B4 - 'low-ethanol' phenotype

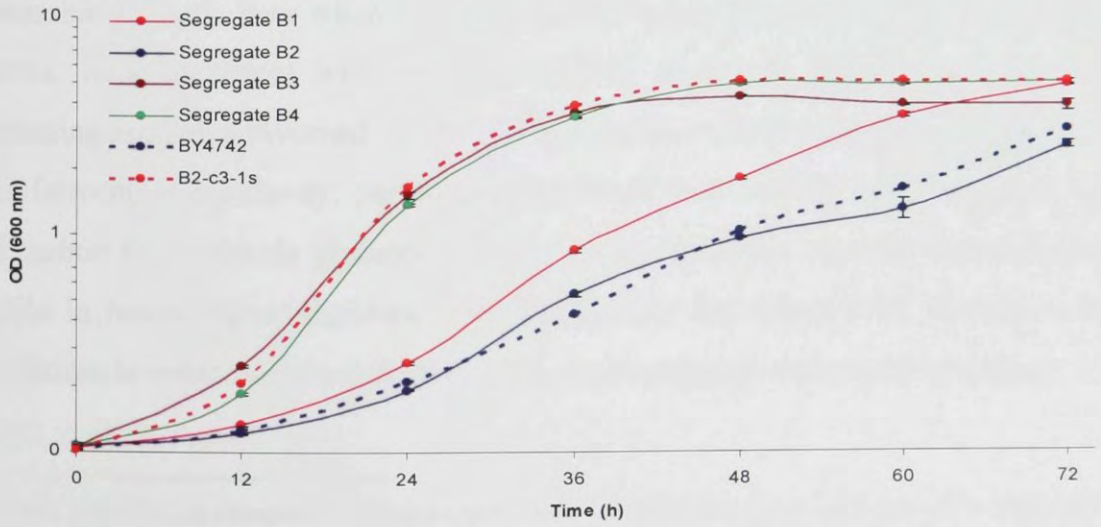
7.24i



7.24ii



7.24iii



BY4742 was clearly slower compared to the latter two. This suggests that the mutation(s) conferring sulfite tolerance in 'low-ethanol' variant B2-c3-1s are partially dominant. To investigate the segregation pattern of progeny derived from heterozygous diploids that were obtained by backcrossing of BY4742 to 'low-ethanol' variant B2-c3-1s, the spores from two tetrads, assessed earlier in Section 7.3.4, were grown under sulfite stress as described in the above paragraph. The growth profiles of these segregants varied, which suggest that sulfite resistance, like the 'low-ethanol' phenotype, is probably conferred by multigenic loci (Figures 7.24ii and 7.24iii).

7.7 Discussion

The main objectives of the work described in this chapter were to characterize and identify genes that confer a 'low-ethanol' phenotype in isolates from adaptive evolution experiments described in Chapters 4 and 5. Some of these objectives were successfully achieved, however, mutations in genes conferring 'low-ethanol' phenotypes were not identified.

General characterization of the 'low-ethanol' phenotype was performed using classical genetics approaches. Heterozygous diploids derived from crosses of 'low-ethanol' variants B2-c3-1s and B2/1-c4-2s to the parental strain BY4742, entirely lost the 'low-ethanol' phenotype when compared to the homozygous diploids of both 'low-ethanol' variants[4]. This result shows that 'low-ethanol' conferring genes are recessive in both 'low-ethanol' variants. While this does not assist in identification of genes, it is consistent with the 'low-ethanol' mutations being associated with genes encoding enzymes involved in central metabolism, which impact on metabolic fluxes in the fermentative pathway; partial impairment of their activity might result in redirection of carbon flux towards glycerol production. The presence of a non-mutated copy of the allele in heterozygous diploids, encoding a fully functional form of protein should be sufficient to overcome the defective gene, thus rendering the mutant resessive.

[4] It was important to compare the heterozygous diploids to the homozygous forms of 'low-ethanol' variants, because ploidy was shown to have a significant effect on the phenotypes of both parental strains, and the 'low-ethanol' variants.

Spores from tetrads derived from heterozygous products of backcross experiment were analyzed to estimate the number of loci (mutations) involved in conferring 'low-ethanol' phenotypes of the variants. Two tetrads derived from 'low-ethanol' variant B2-c3-1s backcrosses had complex segregation pattern suggesting involvement of more that two loci. More detailed characterization of the same variant supported this; the segregation patterns across 10 tetrads were highly diverse indicating involvement of multiple mutations shaping this 'low-ethanol' phenotype. To confirm the multigenic nature of the 'low-ethanol' phenotype of B2-c3-1s, the segregation pattern was assessed for tetrads derived from a cross of two intermediates, A1 and A3, that were derived from a heterozygous diploid. Hypothetically, if more than two genes were involved in conferring the 'low-ethanol' phenotype of B2-c3-1s, segregation of tetrads derived from [A1 x A3] diploids should be more complex than would be expected for trait conferred by 2 genes, which is typically 1:1:2 (parental : mutant : intermediate) (see figure 7.9). The segregation patterns of two tetrads were more complex than this, and impossible to classify (see Figure 7.15). These results support the argument that more than 2 loci shape the 'low-ethanol' phenotype of B2-c3-1s variant.

The segregation pattern of 'low-ethanol' genes from variant B2/1-c4-2s was also assessed. This variant, however, displayed even more complex segregation pattern than B2-c3-1s (see Figure 7.10). This might be explained by the fact that B2/1-c4-2s was derived from B2-c3-1s from the second round of adaptive evolution. Thus, it is not unreasonable to speculate that this variant harbors the mutations of B2-c3-1s, plus additional mutations acquired during the second round of adaptive evolution.

With basic genetic characterization of the 'low-ethanol' phenotype in place, it was of particular interest to identify genes conferring the 'low-ethanol' phenotype and Comparative Genomic Hybridization (CGH) was the method chosen for this. This approach has the potential to identify mutations down to single nucleotide polymorphism resolution across the entire genome (Gresham et al. 2006). Unfortunately, however, the method did not work here; it was not possible to distinguish real mutations from noise in the data sets obtained. This was most likely due to problems associated with the processing of the Affymetrix tiling arrays and may result from improper DNA isolation,

probe preparation, or processing of the array. However, a clear-cut answer which explains the ambiguous results obtained from the CGH arrays is not possible. To improve this method for future work, some alternative protocols will need to be introduced. Firstly, the array work could be performed under conditions which allow greater control; as these arrays were processed by a secondary party, there was very little which could be controlled by direct intervention. Alternatively, an array platform from a different manufacturer (e.g. Nimblegen, Agilent) could be trialed. However, the most direct method to provide the complete picture of the mutations present within the entire genome of each mutant is whole genome sequencing. Unfortunetaly, this could not be applied in this project due to the high costs involved. While CGH did not provide the means to identify 'low-ethanol' mutation it is nonetheless technology that, with future refinement, should be suitable for this analysis.

An alternative approach that might have led to identifying mutations that confer 'low-ethanol' phenotype of variants was to choose and sequence a group of candidate genes that might, if mutated, confer a 'low-ethanol' phenotype. Potential genes (see Table 7.1) were chosen based on their roles in metabolism as described in the literature and at (www.yeastgenome.org). Of particular interest were genes of the fermentative pathway, including alcohol dehydrogenases (*ADHs*), and pyruvate decarboxylases (*PDCs*), mutations in which may have led to impaired ethanol production and increases in glycerol yields; the genes targeted and the rationale for their inclusion is given in Table 7.1. Unfortunately, none of the sequenced loci revealed differences between the 'low-ethanol' variants and their parental strains for any of B2-c3-1s, B2/1-c4-1s or A3-c2-1s. Approximately 350 bp regions of up-stream and 200 down-stream of each gene were also sequenced. However, there were no differences within these regions when variants were compared with parental strains. It is possible that mutations further up-stream than 350 bp from ORF might have an effect on the expression regulation of particular genes, however, limited time did not permit further investigation of this. There are, of course, many other genes that might have been candidates for sequencing, including other genes associated with central metabolism and genes involved in regulation of their expression or activity.

Interestingly, segregation of sulfite tolerance and 'low-ethanol' phenotypes from heterozygous diploids derived from B2-c3-1s suggests that these two traits are not genetically linked. Evidence for this comes from comparative analysis of 'low-ethanol' and 'sulfite tolerance' segregation patterns of spores derived from heterozygous diploids BY4742 x B2-c3-1s. Segregant A4, for example, has a 'low-ethanol' phenotype (Figure 7.9), but its sulfite tolerance is impaired compared to the 'low-ethanol' variant B2-c3-1s (Figure 24ii). Other segregants from the same tetrad, A1 and A3, have intermediate 'low-ethanol' phenotypes and high sulfite tolerance. Segregants B1 and B3 from the second tetrad, although possessing similar intermediate 'low-ethanol' phenotypes (Figure 7.9), have clearly different levels of sulfite tolerance; segregant B1 is not sulfite tolerant while B3 retains high sulfite tolerance (Figure 24iii). Consistent with this, while the sulfite tolerant phenotype is partially dominant, the 'low-ethanol' phenotype is recessive, suggesting the involvement of different loci for these traits.

7.8 Conclusions

- Mutations conferring the 'low-ethanol' phenotypes of both variants are recessive.
- The 'low-ethanol' phenotype of variant B2-c3-1s multigenic, with at least 3 loci involved. The 'low-ethanol' phenotype of B2/1-c4-2s variant is also multigenic and probably involves more loci than was the case for B2-c3-1s.
- Mutations conferring sulfite tolerance are partially dominant.
- Mutations conferring the 'low-ethanol' phenotype of B2-c3-1s variant may segregate independently from those conferring sulfite tolerance.
- Mutations conferring the 'low-ethanol' phenotypes of B2-c3-1s, B2/1-c4-2s and A3-c2-1s do not reside in any of the ORFs listed in Table 7.1

CHAPTER 8

CONCLUDING REMARKS AND FUTURE STUDIES

The main objective of work described in this thesis was to develop a non-genetic engineering approach for generating strains of *S. cerevisiae* that produce less ethanol than the parents they were derived from. Adaptive evolution was used with sub-lethal concentrations of sodium sulfite (Na_2SO_3) providing the selection pressure. The rationale for this was that sulfite is an acetaldehyde sequestering agent, and therefore re-direct metabolism away from ethanol production. It was hypothesized that, under such conditions, natural selection would favor mutations that direct carbon metabolism to glycerol production, as this would enable regeneration of NAD^+, which is usually achieved during the NADH-driven reduction of acetaldehyde to ethanol. The action of sulfite as an acetaldehyde trapping agent was also expected to reduce acetic acid production; acetaldehyde is a substrate for acetic acid production. In a wine context such an outcome would be regarded as highly desirable.

Empirical data from this thesis provides evidence that adaptive evolution with sulfite as a selection pressure indeed drives the creation of genetically stable variants, that produce lowered ethanol yields than their parents. All 'low-ethanol' variants isolated in this work also displayed 'hi-glycerol' phenotypes, with variant A3-c2-1s almost doubling its glycerol production under anaerobic conditions, compared to its parent AWRI1628. The magnitude of metabolic redirection away from ethanol production, however, was only modest; the greatest decrease being of 2.9 g/l for variant A3-c2-1s, grown on 100g/l of glucose. This reduction represents only a 0.36% (v/v) decreases in ethanol production; decreases of greater than 1 - 2% (v/v) are being sought by the wine industry.

One disadvantage of adaptive evolution in sulfite appeared to be the unexpected increase in acetic acid yields, which, under anaerobic conditions, was 73% greater for A3-c2-1s variant, compared to its parent strain. The reason for this is uncertain, but it might be related to respiratory contributions to NAD^+ regeneration. It was argued in Chapter 6 that

some phenotypic traits of the 'low-ethanol' variants suggest that adaptive evolution in aerobic conditions might have contributed to creation of adaptations resulting in energetically more favorable utilization of respiratory metabolism for NAD^+ regeneration. Under anaerobic conditions, when these potential contributions are 'switched off', glycerol synthesis is absolutely required to compensate for waning NAD^+. This might subsequently result in increased demand for NADH, and acetic acid production might have been used by cells to meet this demand. As most wine fermentations are essentially anaerobic, this might be problematic from an industry perspective.

Nevertheless, demonstrating in principle that adaptive evolution using sulfite as a selection pressure can generate yeast strains that produce reduced levels of ethanol opens the possibility of using this non-GM approach to produce 'low-ethanol' wine yeast strains. The following suggestions are offered as a way of improving on what has been achieved:

- **Adaptive evolution in bioreactors** - Adaptive evolution experiments for this thesis were conducted using serial-batch transfer. This method does not allow ongoing control of proliferating cultures; it is particularly difficult to determine when an evolving population has reached optimal adaptation to a selection pressure, and thus one is not able to push the selection pressure to its optimum level. Use of bioreactors offers a promising solution to this problem, enabling continuously increasing selection pressure on evolving populations. Preliminary experiments at The Australian Wine Research Institute indicate that adaptive evolution under sulfite stress conducted in bioreactors result in the generation of 'low-ethanol' variants a great deal more rapidly than was the case for this thesis, and with greater reduction on ethanol yields (unpublished data).

- **Adaptive evolution under anaerobic conditions** – As discussed above the action of sulfite on yeast cells leads to disruption of the NAD^+/NADH redox balance; cytoplasmic NAD^+ regeneration from ethanol production is compromised, leading

to an induction of glycerol production. However, adaptive evolution under aerobic conditions, as was the case for work described in Chapters 4 and 5, might favor adaptations that utilize aerobic metabolism to generate NAD^+; mitochondrial activity has been reported to contribute to the cytoplasmic pool of NAD^+ (see Section 1.3.3.2). Using anaerobic conditions during adaptive evolution in sulfite should negate this, leaving glycerol production as the only means of regenerating NAD^+.

- **Increasing genetic variation to provide more raw material for adaptive evolution** - As previously discussed in Section 4.3, adaptive evolution requires genetic variation; the rate of evolution is limited by the amount of variation in a population. Thus anything that can be done to increase variation should increase the rate of evolutionary change. With this in mind, it would be beneficial to mutagenise founder populations, as discussed in Chambers et al. 2007.

  Sexual reproduction also increases variation. 'Low-ethanol' phenotypes generated in work described in this thesis were multigenic, and it is possible that different 'low-ethanol' variants acquire different 'low-ethanol' mutations. Crosses of different 'low-ethanol' variants may therefore result in the 'accumulation' of 'low-ethanol' conferring mutations, enabling the adaptive evolution of variants with improved 'low-ethanol' phenotypes. Complicating factors that might impede this approach are, firstly, that 'low-ethanol' mutations are recessive, and, secondly, ongoing rounds of selection in sulfite are likely to lead to the generation of increased sulfite tolerance but, as shown in this thesis (Section 7.6), this is not necessarily linked to the 'low-ethanol' phenotype. To get around this issue, selection should be performed only on haploids (from sporulated progeny), and careful screening for the desired phenotype should be performed at regular intervals to identify the best candidates for subsequent rounds of mating and selection.

- **Selection pressures other than sulfite** - Osmotic stress was trialed as an alternative selection pressures to create a 'low-ethanol' phenotype using adaptive evolution approach. However, empirical data did not give clear-cut answer on whether this selection pressure favors the creation of 'low-ethanol' variants. Time constrains did not permit further exploration of this, but the strategy undoubtedly deserves further and more detailed investigation.

  Use of pyruvate decarboxylase inhibitors, discussed in more detail in Section 6.3.3, in combination with sulfite might create a more intense selection than sulfite alone, targeting two different points of metabolism; acetaldehyde production from pyruvate and ethanol production from acetaldehyde.

- **Screening more isolates** - Larger scale screenings for 'low-ethanol' variants from evolving populations would increase the chance of isolating variants with more exaggerated 'low-ethanol' phenotypes; i.e. the larger the sample, the greater the chance of finding rare phenotypes. This could be achieved if high-throughput screening method could be adopted, using, for example, microtitre plates rather than flasks.

Appendix A

SOLUTIONS AND BUFFERS

TE buffer

10mM Tris.HCl (pH 8.0)
1mM EDTA (pH 8.0)
Sterilized by autoclaving

0.5M EDTA (pH 8.0)

Dissolve 186.1 g of disodium ethylenediamine tetra acetate.$2H_20$, (EDTA), in 800ml H_20 and adjust pH to 8.0 with 5m NaOH. Adjust final volume of the solution to 1000ml with H_2O and autoclave.

1M Tris HCl

Dissolve 121.14 g Tris.HCl in 800ml H_2O and adjust pH to 7.5 or 8.0 with concentrated HCl. Adjust total volume to 1000ml, sterilize by autoclaving.

10X TBE Buffer (Tris-Borate-EDTA)

0.89 M Tris-base,
0.89 M Boric Acid,
20 mM EDTA (disodium) (pH 8.0),

The pH is approximately 8.3 and requires no adjustment.

Breaking Buffer (for isolation of genomic DNA)

2% (v/v) Triton-X 100
1% (v/v) SDS
100 mM NaCl
10 mM Tris.HCl (pH 8.0)
1 mM EDTA (pH 8.0)

Sterilize by autoclaving.

6X Ficoll (DNA loading buffer)

15% (w/v) Ficoll 400
0.25% Bromophenol Blue
1X TBE

Phenyl: Chloroform: Isoamyl Alcohol (25:24:1, v/v)

Phenol: Chloroform: Isoamyl Alcohol (25:24:1, v/v) is prepared from highly pure chloroform, isoamyl alcohol, and phenol saturated with 0.1 M Tris-HCl buffer (pH 8). For every 25 ml of buffered phenol, add 24 ml chloroform and 1 ml IAA. Store at 4°C.

RNase A

Dissolve pancreatic RNase A at a concentration of 10 mg/ml in 10mM Tris HCl (pH 7.5) and 15mM NaCl in sterile tube. Heat at 100°C for 15 minutes and cool slowly to room temperature. Aliquot to sterile tubes and store at -20°C.

Ethidium bromide (stock solution 10mg/ml)

Dissolve 1g Ethidium bromide in 100ml H_2O and stir on magnetic stirrer for several hours until dye is totally dissolved. Wrap storage container with aluminium foil or keep in dark place at room temperature.

1M Sorbitol

Dissolve 18.2 g $C_6H_{14}O_6$ in 100ml H_2O
Sterilize by autoclawing

5M NaOH

Dissolve 20g NaOH in 100ml H_2O
Sterilize by autoclaving

1M Lithium acetate

Dissolve 6.6 g $C_2H_3LiO_2$ in 100 ml H_2O
Sterilize by autoclaving

CloNAT (stock solution)

Dissolve CloNAT at concentration of 25mg/ml in H_2O. Filter sterilize through 0.22µm membrane filter into sterile container. Aliquot into sterile micro-centrifuge tubes and store at -20°C.

APPENDIX B

Primers used for gene sequencing:

ADH1

Forward primer: 5'-**TTTTTTCTTTTCTCTCTCCCCCGT**-3'
Reverse primer: 5'-**GCCGGTAGAGGTGTGGTCAATAAG**-3'

Sequencing primers: 5'-**GGAATACAAAGATATTCC**-3'
5'-**CGGTCACTGGGTTGCTAT**-3'
5'-**AATCAAGGTTGTCGGCTT**-3'

ADH3

Forward primer: 5'- **CAAGTGATCATTCGCTCGTTACTAC** -3'
Reverse primer: 5'- **GGTGATAATGTCTCTCAAACGTTC** -3'

Sequencing primers: 5'-CAACGTTAAATATTCTGG -3'
5'-ATATGGTTTCTGACATTC -3'

ADH4

Forward primer: 5'- **TCAAGAAGTCCCTTAGTCTTTGTG** -3'
Reverse primer: 5'- **TTTTCAACAAACGTACCGGTCTC** -3'

Sequencing primers: 5'- ATCAAACAGGAAAAAAGG -3'
5'- ATCGGAGACTATGAAGGT -3'
5'- AGGCCAACATGCAATGTC -3'

ADH5

Forward primer: 5'- **AAGTCCCGCGAAAAATGACTGAT** -3'
Reverse primer: 5'- **TGTATAGGTTGTTCTTGTAAGGCA** -3'

Sequencing primers: 5'- GCACGGTGATTGGCCATT -3'
5'- CTATAATAAAGGCCACTA -3'

PDC2

Forward primer: 5'- **CGCCTTCTTGATGAGCTATATGTC** -3'
Reverse primer: 5'- **AATCCAGTTTCCAGTGATCGGTAC** -3'

Sequencing primers: 5'- TGAATTGTAAAGAGCATG -3'
5'- GTGTCACAATCAATGTTG -3'
5'- AGAAAAACGAAGTCTTAG -3'
5'- TAATACAATGAAACAGCC -3'
5'- GGTGCTGTTTCCGGTATG-3'

PDC5

Forward primer: 5'- **TGCATACTTTATGCGTTTATGCG** -3'
Reverse primer: 5'- **AACCTGGAAGACAGGACAGAAAA** -3'

Sequencing primers: 5'- TGCTGATGGTTACGCTCG -3'
5'- TGCTGATGGTTACGCTCG -3'
5'- TTTTATTCATTGGTGACG -3'

PDC6

Forward primer: 5'- **CCCACACCAAAGGATGTTTTTTT** -3'
Reverse primer: 5'- **TCCCAATTGGTAAATATTCAACAAG** -3'

Sequencing primers: 5'- CGATGGTTACGCACGCAT -3'
5'- AGGTAAAGGGTCAATAGA -3'
5'- AGTCATCTTATTCATAGG -3'

PDC1

Forward primer: 5'- **CAATCAATTGAGGATTTTATGCA** -3'
Reverse primer: 5'- **TCGTTTAAGAGAAATTCTCCAAA** -3'

Sequencing primers: 5'- ACCGTTTTCGGTTTGCCA -3'
5'- TGAACGTCCCAGCTAAGT -3'
5'- TTCGTTTTGCAAAAGTTG -3'
5'- CACGGTCCAAAGGCTCAA -3'

GPD1

Forward primer: 5'- **ACAAATCAAACACCCACACCC** -3'
Reverse primer: 5'- **GCCTCTGAATGAGTGGTGTTG** -3'

Sequencing primers: 5'- CTTCTGTTTCTTTGAAGG -3'
5'- GGTGCTAACATTGCCACC -3'
5'- CGCTCAAGGTTTAATTAC -3'

GPD2

Forward primer: 5'- **CGATGGCTCTGCCATTGTTAT** -3'
Reverse primer: 5'- **CAGGGTGTCGTATCTTTCATT** -3'

Sequencing primers: 5'- TCAAGATCTACTTTCCTA -3'
5'- TAAGGGCCATCTCGTGTC -3'
5'- CGGTAGAAACGTCAAGGT -3'

TPI1

Forward primer: 5'- **CCCGTTCTAAGACTTTTCAGC** -3'
Reverse primer: 5'- **AAGCGCCTTGCTTTTTGTTG** -3'

Sequencing primers: 5'- CCATTAAGGAAATTGTTG -3'
5'- GGCCATTGGTACCGGTTT -3'

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