Determination of the genetic basis for successful fermentation in high sugar media

by

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Thesis summary

Yeast (*Saccharomyces cerevisiae*) plays a key role in the completion of several fermentations including those used for beverage and bioethanol production. In the wine industry, slow or incomplete alcoholic fermentation is still a challenging problem and often results in increased costs of production and decreased wine quality. One of the reasons for the persistence of this problem could be the trend towards rising sugar concentrations in grape musts. What is already a high sugar concentration fermentation (~200 g L^{-1} or more) has increased by some 20 – 40 g L^{-1} due to climate warming and winemaker pursuit of ripeness. In this project we aim to gain a better understanding of how wine yeast cope in high sugar fermentations (HSF) to help develop strategies for managing these types of grape musts.

With the availability of collections of laboratory yeast including gene deletion and overexpression libraries and the development of techniques used for whole genome analysis, it is now possible to investigate yeast biology under oenological conditions with a systems biology approach. A number of genome-wide studies of yeast have previously been conducted to identify yeast genes involved in sensitivity to individual stresses present during fermentation. However, in reality many of these stresses are often present at the same time, or sequentially throughout the phases of fermentation. This highlights an important gap in current research, that being identification of those genes important for maintenance of fermentation efficiency throughout a complete cycle of fermentation, and in particular an environment which has high initial sugar content such as that found in grapes used to make quality wines. We expected these genes to be related to wine yeast adaption, survival and maintenance of fermentative metabolism.

In this study 93 genes were identified as important for the successful completion of high sugar fermentation as deletants of these resulted in either protracted or incomplete fermentation. We have named this gene set the Fermentation Essential

Genes (FEGs). A gene ontology (GO) analysis of these revealed that vacuolar acidification (VA) is an important biological process required for efficient completion of a high sugar fermentation: 20 of the 93 FEGs annotate to this GO term (vacuolar acidification). Also, this gene set is highly represented in the FEGs since these 20 FEGs represent 77% of all genes annotated to this same GO term. In this study we also report 18 genes (also all FEGs), not previously associated with VA, of which deletants have VA defects. This was achieved through examination of the VA of 93 FEGs using the vacuolar specific probe 6-carboxyfluorescein diacetate (6-CFDA), microscopic and Fluorescence Activated Cell Sorting (FACS) analysis.

It was shown that, nine FEGs were seen to be particular critically to fermentation progression and completion. Their deletion result in the extreme phenotype of arrested or 'stuck' fermentation. Amongst these, featured two genes involved in trehalose biosynthesis. The disaccharide trehalose is an enigmatic compound accumulated in *Saccharomyces* and known to be associated with survival under environmental stress conditions. Deletion of either *TPS1* or *TPS2*, encoding enzymes involved in trehalose biosynthesis, resulted in incomplete fermentation. This phenotype could be reversed by the over-expression of *HXK2* (a paralog of *HXK1* encoding hexokinase isomer 2) in Δtps1 and introduction of the phosphotrehalase gene (TreA), from *Bacillus subtilis*, in Δtps2. *HXK2* over-expression increased the fermentation rate of Δtps1 and the parent BY4743 which actually demonstrated a shorter fermentation duration than the parent having blank plasmid.

To further investigate fermentation of yeast in HSF we sought to examine the fermentation performance of a gene overexpression library, which was constructed in this study by transformation of a Yeast Genomic Tiling Collection into a haploid wine yeast strain; ISOC9dΔleu2. The construction of this library was performed in collaboration with two other PhD students (Mrs Jade Haggerty and Ms Jin Zhang). The clonal identity, degree of plasmid retention and development of methodologies to allow fermentation in high sugar chemically defined grape juice medium (CDGJM) were achieved. However, due to time constraints further evaluation of this library was not possible within the current project.

The collective findings from this project have provided greater insight into the mechanism by which yeast cope with HSF as well as providing direction if not specific gene targets for exploitation in strain improvement programs.

Declaration of Authorship

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge, contains no material previously published or written by another person, except where reference has been made in the text.

Essentially all of the work detailed in Chapter 4 has been submitted for publication.

This thesis may be made available for loan or photocopying.

Trung Dung Nguyen
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Abbreviations

AGRF	Australian Genome Research Facility
ATP	adenosine triphosphate
bp	base pairs of DNA
°C	degrees centigrade
CDGJM	chemically defined grape juice medium
CDGJM+PP	CDGJM enriched with polyphenol extract
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
FACS	Fluorescence Activated Cell Sorting
FAN	free amino acid nitrogen
GO	Gene Ontology
HOG	high osmolarity glycerol
HPLC	high performance liquid chromatography
HSE	heat shock elements
HSF	high sugar fermentation
kb	kilo bases of DNA
LB	Luria-Bertani
LiAc	lithium acetate
M	molar
mL	millilitre
mM	millimolar
mol	mole
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced form
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate reduced form
NOPA	o-Pthaldialdehyde/N-acetyl-L-cysteine
OD	optical density
OE	over-expression
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
RI	refractive index
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
STRE	stress responsive element
TAE	tris acetate EDTA
TCA	tri-carboxylic acid
TE	tris EDTA
Tre6P	trehalose-6-phosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
UDP-Glucose	uridine diphosphate glucose
UTR	untranslated region

URS	upstream regulatory sequence
VA	vacuolar acidification
µL	microliter
µM	micromolar
v/v	volume per volume
w/v	weight per volume
YAN	yeast assimilable nitrogen
YEPD	yeast extract peptone dextrose
X-Gal	5-bromo-4-chloro-indolyl-galactopyranoside

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CHAPTER 1 LITERATURE REVIEW

1.1 Introduction

Yeast (*Saccharomyces cerevisiae*) has been used for fermentation in processes such as baking, brewing and wine making, and more recently bioethanol production. In wine production, yeast is responsible for primary or alcoholic fermentation i.e. the biochemical conversion of fermentable monosaccharides glucose and fructose into ethanol, carbon dioxide, and metabolic compounds. The quality of the final wine product depends on reliable alcoholic fermentation whereby yeast can efficiently catabolise sugar, usually to dryness (Lambrechts and Pretorius 2000, Ribereau-Gayon et al. 2006).

During fermentation, yeast growth and fermentation kinetics are usually affected by factors such as temperature, pH, concentration of sugar, ethanol and nutrients (Alexandre and Charpentier 1998, Bisson and Butzke 2000, Boulton et al. 1996, Cardona et al. 2007, Henschke and Jiranek 1993, Ribereau-Gayon et al. 2006, Santos et al. 2008). Sluggish or stuck fermentation usually results from attenuation of yeast sugar utilisation and restricted biomass formation (Bisson 1999). Consequently, incompletely fermented juice is at risk of microbial spoilage and oxidation in sluggish or stuck fermentation (Bisson 1999), which can result in financial losses. Thus the adaptability of wine yeast to stressful conditions and their ability to effect the completion of alcoholic fermentation are the primary demands of the wine industry when choosing an appropriate strain for winemaking.

The importance of high sugar fermentation (HSF) arises since certain sections of the Australian wine industry have tended to leave grapes on the vine for extended periods of time, in the hope that riper flavour profiles are present in the final wine. Climate change or trends of certain viticultural practices could also lead to increases in the sugar content of grapes. Consequently, the concentration of sugar within grape musts has been found to rise higher than 250 g L^{-1} (Godden and Muhlack 2010). In HSF, yeast needs to grow and undergo fermentation when presented with increased stresses. In these conditions, yeast growth is affected by increased osmotic stress

encountered at earlier stages of fermentation (Jimenez-Marti et al. 2011) and accumulation of ethanol (Yoshikawa et al. 2009) and nutrient deficiency late in fermentation (Ribereau-Gayon et al. 2006). Strain improvement of wine yeast is broadly sought to improve both fermentative efficiency and wine quality. There are several methods used to develop optimised yeast strains. These include classical methods such as clonal selection (which can also be coupled with mutagenesis) or hybridisation between strains. More recently modern techniques have been developed such as adaptive (directed) evolution (Hashimoto et al. 2005, McBryde et al. 2006, Ramirez et al. 1998, Sipiczki 2008) and targeted genetic manipulation (Alper et al. 2006, Cardona et al. 2007, Damore 1992, Dequin 2001, Jimenez-Marti et al. 2009, Parekh et al. 2000). The generation of robust yeast strains better able to cope with the increasing sugar concentrations observed in the wine industry is valuable not only for producing better quality wine with reduced inputs and wastage, but also to simply gain a greater depth of knowledge around the fundamental process of fermentation.

1.2 Yeast growth and fermentation kinetics

Alcoholic fermentation is the anaerobic metabolism of sugar (mainly glucose and fructose) and other nutrient compounds. Products of this process are alcohol, CO_2 and metabolites including glycerol, organic acids, esters, acetaldehydes, etc. This process is mainly carried out by yeast strains which have been selected to complete a particular type of fermentation (Zamora 2009). After inoculation, yeast starts to metabolise sugar and other nutrients for growth and energy accumulation (Figure 1.1). The division or growth of the population of yeast cells is delayed for a short time (termed the lag phase) whilst it adapts to its new environment. After adaptation, the yeast grows quickly and consumes sugar, metabolising it to form alcohol and CO_2 amongst other compounds. During the exponential phase, the population can reach up to $10^7 – 10^8$ cells mL^{-1} in a few days (Ribereau-Gayon et al. 2006, Zamora 2009). As the nutrients are depleted, and deficiency occurs, the yeast stop growing and a stable population is maintained for some days. At the end of the fermentation process, the yeast population decreases due to the lack of nutrients, the effect of ethanol and cell lysis and death.

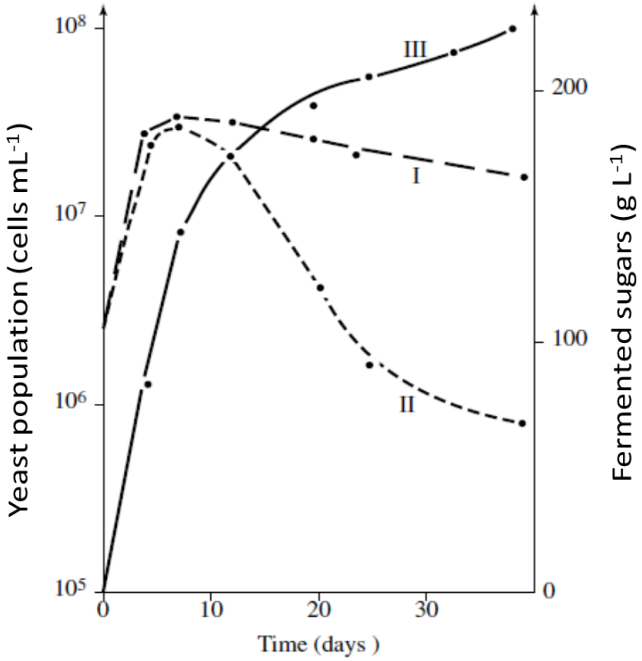

Figure 1.1 Fermentation profile and cycle of yeast growth during wine making (Ribereau-Gayon et al. 2006). Yeast population is depicted in curve I, viable cell number in curve II and fermented sugar in curve III.

1.3 Factors affecting yeast growth and fermentation kinetics during high sugar fermentation

1.3.1 Sugar and osmotic stress

Simple monosaccharide sugars (glucose and fructose) are the main carbon sources for yeast growth and alcoholic fermentation. The sugar content in grape juice for table wine production is usually 16-26%. However, the concentration of sugar may be up to 50% (w/v) for production of dessert wine (Erasmus et al. 2003), which produces the high osmotic stress and has an influence on the yeast strain used to ensure fermentation. In HSF, osmotic stress is present at the time that yeast is inoculated into the must. It has been suggested that during the fermentation in high sugar media, osmotic stress results in a decrease in growth rate and fermentation performance as well as death of yeast cells (Damore 1992, Gibson et al. 2007). High sugar concentrations (between 200 and 300 g L^{-1}) can affect yeast growth by increasing the lag phase, decreasing the cell population and the number of viable cells as well as cell biomass at the end of fermentation (Charoenchai et al. 1998).

1.3.2 Ethanol

Ethanol is a metabolic product of yeast undergoing fermentation. The content of ethanol in wine can reach up to 18% (v/v) (Buescher et al. 2001). The presence of ethanol has a significant effect on yeast growth and activity, especially during the later stages of fermentation when the ethanol reaches its highest concentrations. The presence of ethanol prevents growth and decreases viability, resulting in extension of the fermentation duration (Alexandre and Charpentier 1998, Damore 1992). Ribereau-Gayon et al. (2006) showed that the addition of ethanol decreases the speed of growth initiation and nitrogen assimilation. The inhibition of ethanol on yeast growth can also be enhanced by other factors such as temperature, exposure to oxygen, osmotic pressure and nutrient deficiency (Damore and Stewart 1987). Ethanol not only inhibits cell growth but also represses glucose transport through the modification of the cell membrane (Alexandre et al. 2001, Santos et al. 2008). Consequently the metabolism and energy supply of yeast can be affected (reviewed

in Zhao and Bai 2009). Therefore, it can be inferred that the increase in ethanol leads to a decrease in the rate of both yeast growth and fermentation.

1.3.3 Nutrient deficiencies

There are numerous other nutrients beside carbohydrates which are required for the growth of yeast and successful fermentation kinetics, for instance nitrogen, minerals and vitamins (Ribereau-Gayon et al. 2006). Nitrogen in particular has a significant impact on the metabolic and physiological functions of yeast, and its depletion is one of the main reasons for the slow growth of yeast and decrease in fermentation rate and can result in sluggish or stuck fermentations (Alexandre and Charpentier 1998). Since the high concentration of sugar in HSF both extends the duration of fermentation and increases the nitrogen requirement, nitrogen depletion is more likely in HSF (Alexandre and Charpentier 1998). The nitrogen content in grape juice varies depending on multiple factors, such as grape variety, geographical region, viticultural practices, berry maturation and grape processing (Ribereau-Gayon et al. 2006). The minimum nitrogen concentration required for normal fermentation rate is in the order of 120-140 mg L^{-1} (Alexandre and Charpentier 1998, Jiranek et al. 1995a, Vilanova et al. 2007). The assimilation of nitrogen during fermentation depends on both the activity of nitrogen transport systems and regulation of metabolic systems and other factors such as sugar concentration, temperature and the presence of oxygen in the medium (Henschke and Jiranek 1993, Jiranek et al. 1995a, Ribereau-Gayon et al. 2006). The inactivation of sugar transport results in part from the arrest of protein synthesis, which is linked to nitrogen deficiency, as well as specific inactivation of sugar transporters (Salmon 1989). To avoid the inactivation of sugar transport, it is necessary to maintain a high rate of protein synthesis through nitrogen supplementation (Alexandre and Charpentier 1998).

Other nutrients considered important for maintenance of efficient fermentation include zinc, magnesium, and oxygen. The limitations of these nutrients often lead to a decrease in fermentation rate (Bauer and Pretorius 2000). Magnesium is important for metabolic and physiological functions involving cell integrity, nucleic acid, protein, polysaccharide and lipid stability (Walker 1994). The deficiency of zinc

results in pervasive changes in expression patterns of genes involved in cellular processes such as zinc homeostasis, cell wall and mitochondrial function and metabolism, causing down-regulation of fermentation efficiency (Lyons et al. 2000, Wu et al. 2008). Industrial fermentations are considered to be anaerobic, but in the early stages of fermentation oxygen can be present (Julien et al. 2000). Oxygen is required for synthesis of a number of cellular compounds, in the context of fermentation the most important being sterols and unsaturated fatty acids, which contribute to the stability of the cell membrane in times of stress (Garre et al. 2010). The dissolved concentration of oxygen in grape juice, called "anaerobic growth factor", depends on grape variety, handling methods, and temperature (Fornairon-Bonnefond et al. 2003). Furthermore, the effect of oxygen is different depending on the composition of the medium, in particular unsaturated fatty acids and sterols (Houtman and Du Plessis 1986).

1.3.4 Environmental factors

Environmental factors such as temperature extremes, SO_2 and toxic chemicals also have significant effects on yeast growth and consequently fermentation. Fermentation speed increases with a rise in temperature within the range of 10 to 35°C. However outside of this range, cell growth and fermentation kinetics are affected by variation in yeast respiration and fermentation intensity (Ribereau-Gayon et al. 2006). This principle is demonstrated in low sugar concentration conditions where the fermentation can go through to completion at temperatures up to 35°C. However, in HSF, the fermentation can become stuck even at 25°C (Cardona et al. 2007, Ribereau-Gayon et al. 2006). Wine yeast are well known to be sensitive to changes in temperature, particularly in the late stage of fermentation, when the level of ethanol is high (Piper 1993). Temperature controlled conditions are nowadays applied in most wine fermentations so that temperatures are maintained between 10-15°C for white and 18-25°C for red wine fermentations. However interestingly, wine fermentation efficiency has been shown to be negatively affected by even a small increase in temperature of 2-3°C. Thus, temperature can still have significant impacts, even in temperature controlled conditions (reviewed in Bauer and Pretorius 2000).

The use of sulphur dioxide (SO_2) in wine making has been considered an appropriate technique to prevent microbial spoilage for some time (Ribereau-Gayon et al. 2006). The principal roles of sulphur dioxide are as an antiseptic, antioxidant and a binder for acetaldehyde and other similar products. However, the high concentration of SO_2 in grape must can affect growth and metabolic activities of wine yeast. Sulphur dioxide inhibits the growth of wild yeast and bacteria whilst selected yeast (*Saccharomyces* ssp.) is typically more tolerant of SO_2. This is very important from the technological point of view as it contributes to the predominance of the selected strains in the fermenting medium. To ensure the successful completion of alcoholic fermentation, the amount of SO_2 added should be regulated according to the pH, temperature, sanitary conditions and other factors (Ribereau-Gayon et al. 2006).

Micro-oxygenation is also widely used under controlled conditions in winemaking. The introduction of oxygen into wine during fermentation can help to maintain yeast cell viability to minimise the risk of stuck fermentation and undesirable production of sulphur compounds. However, the exposure of oxygen must be limited. Either of too much or too little oxygen can lead to adverse effects on wine quality (Fornairon-Bonnefond et al. 2003). Thus the control of oxygenation during wine fermentation may be considered as a method to optimise the fermentation and control yeast lees reactivity towards oxygen (Fornairon-Bonnefond and Salmon 2003).

1.4 Response of yeast to stresses present in high sugar fermentation

A number of yeast "stress response" pathways are induced to resist the impacts of fermentation stresses. Stress tolerance and adaptation of yeast relates closely to the accumulation of trehalose and heat shock proteins in response to osmotic, oxidative, temperature and ethanol stress (Zhao and Bai 2009). Yeast cells also adjust cell membrane biosynthesis according to the particular stress condition(s), which occur during fermentation. The mechanism of response to various stresses involves molecular sensors and transduction pathways which determine the change of mRNA levels for many genes (Cardona et al. 2007, Gasch et al. 2000, Hohmanm and Mager 2003, Zhao and Bai 2009) as generally described in Figure 1.2 (Querol et al. 2003). The mechanisms by which the genes and their products are involved are well

characterised in laboratory, but not industrial strains (Ding et al. 2009, Estruch 2000). Stress tolerance and adaptation of industrial strains may vary and depend on the yeast strains and processes employed.

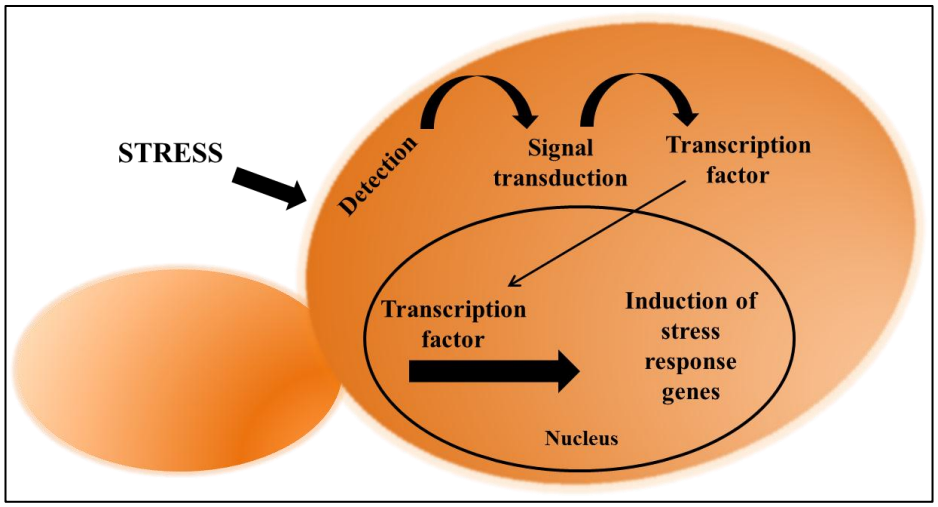

Figure 1.2 Schematic depiction of the molecular and physiological regulation of the yeast cell leading to the stress response. The sensor systems and transduction pathways result in the activation of stress response genes (Querol et al. 2003).

1.4.1 Response to osmotic and environment stresses

Following the inoculation of yeast into grape juice, yeast cells are usually exposed to immediate stresses including hyperosmotic stress. The acidity of must (pH 2.8 – 4.0) can be a further challenge to the yeast upon inoculation. The response of yeast to environmental stresses is indicated by increased expression of genes involved in energy metabolism, protein trafficking, and ion homeostasis (Marks et al. 2008). For instance, a sudden change in environmental conditions can lead to a change in yeast growth, activity of enzymes, metabolic processes and cellular membrane integrity. Yeast will adapt to changes of the external environment by adjusting the internal milieu in order to better grow under the new conditions (Hohmanm and Mager 2003). Generally yeast presents a common response to all environmental stresses. Analysis of the expression of the genes involved in this response reveals that many genes are targets of Msn2p/4p and Yap1p transcription factors, and have already been implicated in a general stress response in yeast (Hohmanm and Mager 2003, Rodrigues-Pousada et al. 2005, Gasch et al. 2000, Cardona et al. 2007). By using DNA microarrays, about 900 genes in *Saccharomyces cerevisiae* were identified to respond to a variety of environmental stresses (Gasch et al. 2000). The genes were divided into two groups based on their expression profile. The first group included nearly 300 genes where expression increased in response to the change of environment, of which approximately 60% are uncharacterised. The genes in this group with known molecular functions are involved in a wide variety of processes, including glycolysis, cell wall modification, DNA damage repair, metabolite transport, mitochondrial function and autophagy. Many genes in this group have also been proposed to offer cellular protection during stress conditions. The second group showed decreased transcription levels as a result of the environment stresses. Most of genes in this group are involved in growth-related processes and aspects of RNA metabolism, whilst others relate to transcript levels (reviewed in Gasch et al. 2000).

Several studies have been carried out to allow a better understanding of ‘stress response’ gene expression during wine fermentation (Cardona et al. 2007, Zuzuarregui and del Olmo 2004, Erasmus et al. 2003, Saito and Posas 2012, Rossignol et al. 2003). Regarding the stress response, some studies have carried out

specific gene modifications and looked at the cellular response. For example, Wojda et al. (2003) demonstrated the over-expression of *FPS1* and *GPD1*, which encode the glycerol transport facilitator and glycerol-3-phosphate dehydrogenase respectively, resulted in an increase of glycerol production and fermentation rate during stationary phase. Another investigation showed that the increased expression of the stress response genes *SPI1* (encoding for a GPI-anchored cell wall protein), *CTT1* (cytosolic catalase T) and *ALD3* (cytoplasmic aldehyde dehydrogenase) were regulated by transcriptional factor Msn2p, which is encoded by *MSN2* (Cardona et al. 2007).

The osmotic stress response of a yeast cell can be defined as occurring in three stages: changes of intracellular milieu contents, operation of primary defences to protect cells and restoration of intracellular milieu contents. Together these responses allow the yeast cells to adapt to changed osmotic conditions. The primary responses to hyperosmotic stress are mostly governed by the high-osmolarity glycerol (HOG) MAP kinase pathway (Saito and Posas 2012, Mager and Siderius 2002, Wojda et al. 2003). Lack of the HOG pathway leads to a decrease in osmotic resistance of the cell and reduced water content (Saito and Posas 2012, Wojda et al. 2003). The HOG pathway together with protein kinase C (PKC) (Gustin et al. 1998) and sterile vegetative growth (SVG) (Lee and Elion 1999) also have a function in controlling cell wall structure. In the yeast cell, the combination of the MAP pathway, PKC and SVG modulates cell wall composition, allowing the cell to cope with high osmotic stress at high temperature (Wojda et al. 2003). Generally, the osmotic stress response of yeast cells is divided into two processes (osmotolerance and osmoadaptation) based on external sensing factors and changes in physiology, biochemistry and other intracellular functions. Osmotolerance is the physiological resistance of the cells to osmotic stresses that is the result of intrinsic factors such as the superior membrane structure, glycerol production, enzymes, vacuolar function and levels of trehalose (Levin 2005). Osmoadaptation represents a highly sensing and responsive system. It activates an acute or chronic response to which cells adjust their normal physiology in order to survive the stress conditions (Wojda et al. 2003, Van Wuytswinkel et al. 2000).

During fermentation, cells are often exposed to rapidly changing temperature which influences the physiology and metabolism of yeast. A number of molecular pathways were identified that involve molecular and physiology response of an organism to temperature changes, commonly known as the heat shock response. The genetic basis of this response is primarily regulated by the heat shock transcription factors encoded by *HSP* genes (Querol et al. 2003). Of these heat shock proteins, Hsp1p is the first transcription factor required for the activation of *HSP* genes which responds not only to heat shock, but also to a variety of other cellular environmental stress conditions (reviewed in Bauer and Pretorius 2000). Abe and Minegishi (2008) screened a yeast deletion library under conditions of high pressure and low temperature to identify deletants that were defective in growth under such conditions. By using micro-scale screening of the deletion library in 96-well culture plates in such conditions, they identified genes involved in major functional classes such as amino acid biosynthesis, microautophagy and functions of the mitochondrion. Of the identified genes, those encoding components of the EGO (**E**scape from rapamycin-induced **G**r**o**wth arrest) complex which is composed of four proteins: Ego1, Ego3, Gtr1 and Gtr2 were substantially responsible for growth by involving the regulation of amino acid uptake.

1.4.2 Response to ethanol stress

In yeast, ethanol tolerance has also been shown to involve the plasma membrane ATPase, energy metabolism, protein trafficking, and ion homeostasis (Alexandre et al. 1994, Ding et al. 2009, Hirasawa et al. 2007, Marks et al. 2008, Teixeira 2009). Interestingly, the alteration of ergosterol and phospholipid biosynthesis has also been shown to affect yeast ethanol stress tolerance (Chi and Arneborg 1999, Zhao and Bai 2009). Sterol synthesis is reduced when the concentration of ethanol increases, which is suggested to lead to a change in general membrane function (Alexandre et al. 1994). Genes that have been shown to be sensitive to ethanol stress are classified into the functional categories of aerobic respiration, cell wall integrity pathway, vacuolar and mitochondrial function. These genes are also required for growth of yeast in such conditions (Marks et al. 2008, Yoshikawa et al. 2009). Stanley et al. (2010) showed that expression of genes associated with mitochondrial and NADH oxidation resulted

in an increase in ethanol tolerance. Similarly, stimulation of glycolysis and other energy-yielding pathways resulted in an increased ethanol stress response (Stanley et al. 2010).

Some studies have been carried out to identify the genes sensitive to ethanol and other stresses during fermentation (Alexandre et al. 2001, Fujita et al. 2006, Yoshikawa et al. 2009). These studies highlight the complexity of the ethanol stress response in yeast, its relationship with other stresses, as well as the importance of the genetic background to the yeast's response to stress. For example, 137 single yeast gene deletion mutants were identified as being sensitive to ethanol (Fujita et al. 2006). Furthermore, Marks and co-workers (2008) showed by genome-wide expression analysis of the wine yeast strain, VIN13, that there were 41 genes important for ethanol stress within the 223 gene dataset designated as Fermentation Stress Response (FSR) genes during alcoholic fermentation in Riesling juice. In addition, Alexandre et al (2001) reported genes involved in trehalose synthesis were up-regulated as a result of ethanol stress, with the authors suggesting this allowed yeast to rapidly adjust trehalose content.

1.4.3 Response to nitrogen deficiencies

Assimilable nitrogen is often limiting in grape juice (Henschke and Jiranek 1993, Gardner et al. 2005, Bell and Henschke 2005) and this is a common cause of fermentation inefficiencies. Nitrogen availability can affect many aspects of yeast metabolism and fermentation (Alexandre and Charpentier 1998, Gardner et al. 2005, Jiranek et al. 1995a, Ribereau-Gayon et al. 2006). Nitrogen deficiency during fermentation is one of the concerns for winemakers because it results in down-regulation of protein synthesis, inhibition of glucose transport and subsequently a reduced fermentation rate and often a sluggish or even stuck fermentation (Lagunas 1993, Lagunas et al. 1982, Salmon 1989, Spoerl et al. 1973). Other problems associated with inadequate nitrogen content of grape must include the formation of undesirable sulphur compounds, in particular hydrogen sulphide (Jiranek et al. 1995b, Spiropoulos and Bisson 2000). However, the utilisation of nitrogen depends not only on the presence of other nutrients in the medium (oxygen, sugar, and

ammonium supplementation (Jiranek et al. 1995a) but also the function of transport and regulation of metabolic systems (reviewed in Ribereau-Gayon et al. 2006). Work within this group has investigated methodology that would allow fermentations to proceed efficiently even when faced with limiting nitrogen. One such study identified genes that when deleted enhanced sugar catabolism, i.e. nitrogen-efficient genes *NGR1* and *GID7* (Gardner et al. 2005). Enhanced sugar catabolism was observed during fermentations using deletion mutants of *NGR1* and/or *GID7*. Furthermore, the final wine product was similar to that of the parental strain, in that there were no changes in the key metabolites such as organic acids, ethanol and glycerol. How these two genes influence fermentation under nitrogen limited conditions is unclear and is the focus of further investigations (Gardner, *pers. comm.*). Ngr1p is a RNA binding protein known to negatively regulate growth rate, possibly through the degradation of Por1p, a mitochondrial porin responsible for maintenance of mitochondrial osmotic stability, mitochondrial membrane permeability, and homeostasis of glutathione in within the mitochondrion and cytosol (Sanchez et al. 2001). Whereas Gid7p, is a subunit of the GID complex involved in the proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase, a key gluconeogenic enzyme, during cellular adaption from a non-fermentable carbon source to glucose (Regelmann et al 2003).

1.4.4 Biological processes related to stress responses of the cell

a) Glycerol production under stress conditions

Glycerol is a poly-hydroxyl alcohol that is produced as a by-product of fermenting sugar to ethanol. Glycerol synthesis in yeast cells serves a number of important physiological functions (Figure 1.3). For instance, an increase of intracellular glycerol production occurs when the yeast is exposed to osmotic shock and other stresses (heat shock and oxidative stress) (Mager and Siderius 2002, Li et al. 2009). Glycerol production by *Saccharomyces cerevisiae* under high osmotic stress results from the enhanced expression of the *GPD1* and *GPD2* genes encoding for glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatase enzymes (Hohmanm and Mager 2003). Glycerol concentration starts to increase mainly in the

initial stage of stress treatment, after which the concentration of glycerol decreases (Li et al. 2009). In the yeast cell glycerol concentration is regulated by the glycerol channel protein (Fps1p) which mediates the efflux of glycerol and is responsible for maintaining cellular glycerol levels. Hohmann and co-workers (2003) also reported that deletion of *FPS1* leads to an imbalance in growth and swelling of the cell under hyperosmotic conditions.

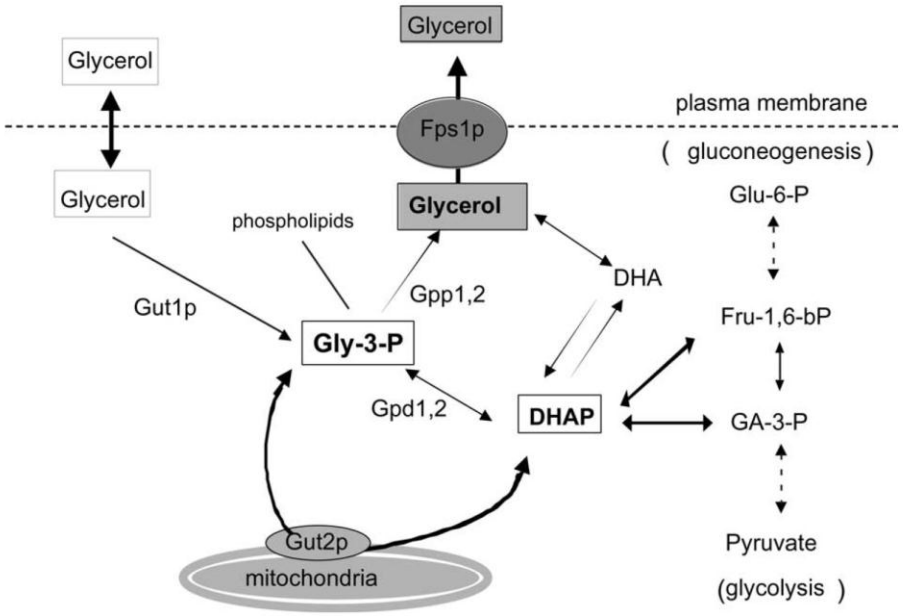

Figure 1.3 Pathway of glycerol synthesis in *Saccharomyces cerevisiae*. Dihydroxyacetone phosphate (DHAP, from glysolysis) is catalysed to glycerol-3-phosphate (Gly-3-P) by Gpd1p and Gpd2p. Gly-3-P is then dephosphorylated to glycerol by Gpp1p and Gpp2p, encoded by *GPD1*, *GPD2*, *GPP1* and *GPP2* respectively. Under high osmotic conditions, GPD1 and GPP2 gene expression is induced and glycerol accumulation is enhanced by an increase in synthesis and decrease in the efflux of glycerol via the Fps1p channel. Glycerol uptake is a diffusion process. Gut1p (glycerol kinase) and Gut2 (mitochondrial glycerol 3-phosphate dehydrogenase) are described as related proteins (Mager and Siderius 2002).

b) Cell wall and plasma membrane integrity

The cell wall is the external layer surrounding the yeast cell. This structure influences cell morphology and protects cells from external environmental conditions by preserving the intracellular osmotic integrity. It is also essential in cell division and bud formation (Levin 2005, Karreman et al. 2005, Scrimale et al. 2009). The cell wall is a layered structure including an electron transparent inner layer and an electron dense outer layer. The inner layer consists of glucan (β-1,3 glucan, β-1,6 glucan), mannoproteins and the N-acetylglucosamine polymer, chitin (Smits et al. 1999, Garcia et al. 2004). The cell wall integrity (CWI) signal pathway consists of a group of cell surface sensors that are coupled to a small G-protein called Rho1p. Rho1p activates a group of individual effectors which regulate processes including β-glucan synthesis, gene expression related to cell wall biogenesis, and actin cytoskeleton organisation (Levin 2005, Smits et al. 1999). The PKC MAP pathway activated by osmotic sensors, such as Sln1p (Wojda et al. 2003) under hyperosmotic stress conditions is considered important in maintenance of cell wall integrity. As a consequence of hyperosmotic stress, an efflux of intracellular water can occur, leading to a decrease in cell volume and changes in cell structure. Recently, osmotic sensors active in the CWI pathway, Msb2p have been identified but their mechanism of action is not well understood (O'Rourke and Herskowitz 2002).

c) Trehalose accumulation

Trehalose is a non-reducing glucose disaccharide that is considered to have an important role in yeast as a protectant in the survival of the cell during environmental stress. Trehalose is synthesised from UDP-glucose and glucose-6-phosphate involving trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase encoded by *TPS1* (Vuorio et al. 1993) and *TPS2* (Devirgilio et al. 1993), respectively. Trehalose can also be degraded through hydrolysis by neutral trehalase encoded by *NTH1* and *NTH2* (Kopp et al. 1993) and/or acid trehalase encoded by *ATH1* (Alizadeh and Klionsky 1996, Destruelle et al. 1995). Trehalose has a role in the preservation of plasma membrane integrity by substituting for water and binding to the phospholipid polar head groups. It also functions to stabilise proteins in yeast cells exposed to heat shock by preventing and repairing protein aggregation, or

facilitating degradation of denatured protein (Mahmud et al. 2009a, Parsell et al. 1993). Under normal growth conditions and during the exponential phase trehalose content is low, but increases in late stationary phase, and similarly under stress conditions. Mahmud et al (2010) proposed that under stress conditions, the accumulation of trehalose was the result of expression of two trehalose synthesis genes, since the content of trehalose was higher in the triple deletants (*Δnth1*, *Δnth2, Δath1*) when compared to the parent strain.

Although the outcome of a yeast's response to several stresses has been extensively studied (Albertyn et al. 1994, Ando et al. 2006, Arguelles 1994, Galafassi et al. 2013, Li et al. 2009, Mager and Siderius 2002, Mahmud et al. 2010, Tofalo et al. 2009, Wojda et al. 2003), the identification of genes which are specifically sensitive to high sugar concentrations, such as that found during wine fermentation has not been studied in great depth. Work to date includes genome-wide screening, which identified 273 deletion mutants whose growth was sensitive to high osmotic pressure on solid media containing 300 g L^{-1} sucrose, of which four genes were specifically sensitive to high sucrose (Ando et al. 2006). Erasmus and colleagues (2003) carried out microarray analysis of genome wide expression in a wine yeast strain under high sugar concentrations (up to 40% w/v) and identified 589 genes sensitive to high sugar stress. Specifically, expression of glycolytic and pentose phosphate pathway genes was shown to be regulated by high sugar stress. Approximately 228 genes identified by Erasmus et al (2003) as being regulated by sugar-induced osmotic stress, still require investigation in terms of their specific function in relation to this condition. In work by Marks and colleagues (2008), 223 fermentative stress response (FSR) genes were identified by genome-wide expression analysis of the wine strain VIN13 during a 15-day fermentation in Riesling juice. Twenty percent of these 223 FSR genes also responded to stresses characterised in the environmental stress response (ESR) and the common environmental response (CER), including changes in temperature, acid, alkali, hydrogen peroxide, salts and sorbitol. Approximately 18% of FSR genes overlap with the response to osmotic stress, increase in ethanol, and oxidative stress (Marks et al. 2008). From the transcriptional data, many of these genes appear to be involved in cellular response and adaptation to multiple stress

conditions, which occur sequentially or consecutively during the extent of a wine fermentation.

In summary, during fermentation, especially under high sugar conditions, yeast is significantly affected by hyperosmotic stress at the beginning of fermentation. Other factors such as ethanol accumulation and nutrient deficiency can impact on yeast growth and fermentation efficiency as fermentation progresses (Cardona et al. 2007). The environmental factors including temperature extremes and the presence of oxygen and oxidative stress also affect fermentation (Marks et al. 2008). Yeast cells are known to have common response elements to some adverse environmental conditions (Gasch et al. 2000), (i.e. *MSN2* and *MSN4* are responsive to high osmolarity (Rep et al. 2000)). The basis of these stress response mechanisms is generally via genetic regulation, in which genes involved in stress tolerance play an important role. Novel insights into the genetic regulation would enable development of strategies for yeast to improve their fermentative behaviour. Several studies have already suggested such strategies, for instance the genetic engineering of genes involved in the stress response for the improvement of yeast stress tolerance during fermentation (reviewed in Zhao and Bai 2009).

1.5 Genetic improvement of wine yeast

1.5.1 Introduction

Saccharomyces cerevisiae is the first eukaryotic organism to have the complete genome sequenced (Goffeau et al. 1996). Also, much of its transcript, protein and metabolite response in a multitude of conditions is known or else is being vigorously studied (Schuller and Casal 2005, Zuzuarregui and del Olmo 2004). The publication of the *S. cerevisiae* genome and development of genetic technologies have led to advances in the fields of yeast molecular genetics, physiology and biology. The construction of specialised commercial strains can possibly be made by heterologous gene expression or altered gene dosage (over-expression or deletion). The most important targets in strain improvement relate to technology and quality of production, such as enhancement of fermentative performance, higher ethanol

tolerance, increase sugar consumption and nitrogen assimilation (reviewed in Schuller and Casal 2005).

New yeast strains can be generated by multiple methods, including hybridization (Sipiczki 2008), genetic engineering (Butzke and Bisson 1996, Marks et al. 2008) and adaptive evolution (Perez-Ortin et al. 2002). However, because of homothallism, poor sporulation and spore viability of yeast, the application of hybridisation can be difficult (Walker et al. 2003). The fermentative performance of yeast may be improved by genetic engineering approaches such as single gene manipulation, global transcription machinery engineering (gTME), evolutionary engineering and genome shuffling (Figure 1.4); many of which have been applied to breed stress resistant strains for ethanol production (reviewed in Zhao and Bai 2009). The completion of the yeast genome project and the ever increasing database of related functions has provided access to information on ~6000 yeast genes and their related protein function, and interactions, thus making future application of recombinant DNA technologies more feasible for industrial wine yeast strain optimisation (reviewed in Giudici et al. 2005).

1.5.2 Genetic manipulation

Genetic manipulation of yeast strains is often used to improve fermentative behaviour, as a logical progression of investigations undertaken to gain a better understanding of the mechanisms involved in stress response during vinification (Cardona et al. 2007). Some authors have shown that genetic modification of yeast could allow improvement of wine production (Cardona et al. 2007, Jimenez-Marti et al. 2009, Pretorius and Bauer 2002, Schuller and Casal 2005). Jimenez-Marti et al. (2009) indicated that the genetic manipulation of genes *HSP26* and *YHR087W* could improve fermentative behaviour in wine yeast. However, the genetics of the stress tolerance of yeast is complex and the manipulation of yeast at the genetic level to facilitate stress tolerance still has many difficulties (Marks et al. 2008).

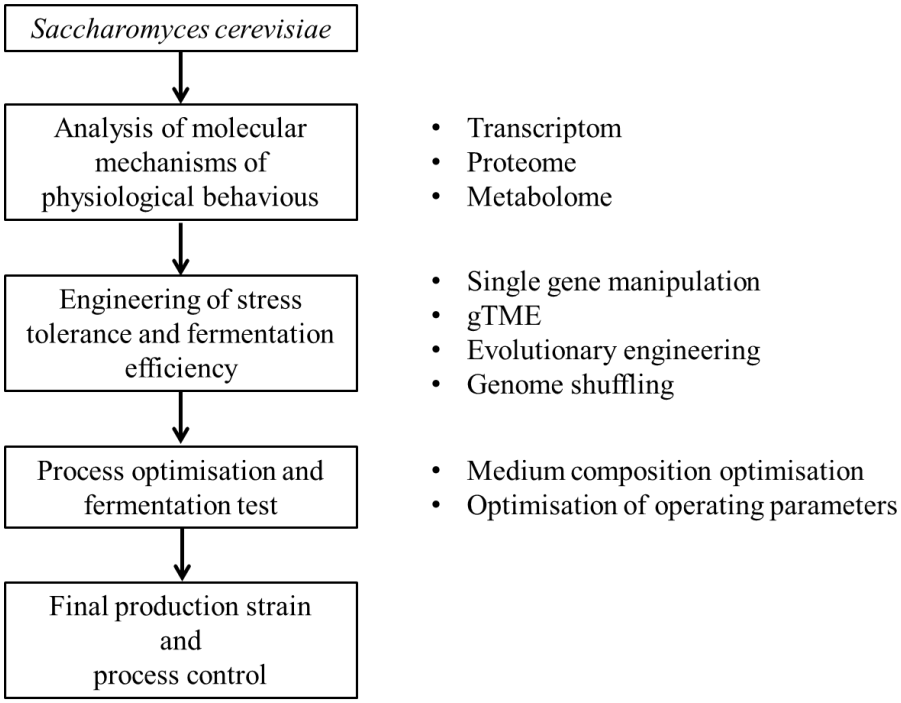

Figure 1.4 Outline for strain improvement and process optimisation of *S. cerevisiae* for superior stress tolerance and fermentation performance (Zhao and Bai, 2009).

Nevertheless, there are studies that have shown that expression and over-expression of a single gene leads to considerable improvement in the stress tolerance of yeast (reviewed in Zhao and Bai, 2009). For instance, ethanol resistance of yeast has been improved by over-expression of tryptophan biosynthesis genes (*TAP1-5*), and tryptophan permease gene (*TAT2*) (Hirasawa et al. 2007), and could be enhanced by disruption of *URA7* and *GAL6* when compared with the parental strain (Yazawa et al. 2007). Screening of deletion mutants of *URA7* and *GAL6* showed an increase of growth in the presence of ethanol and resistance to zymolyase (a cell wall degrading enzyme). The elevated transcription of heat shock factors *HSP1* and *HSP26* in *Δgal6* was suggested to be the mechanism of ethanol resistance, whereas *URA7* participated in membrane phospholipid biosynthesis (reviewed in Zhao and Bai, 2009). Hou et al. (2009) also showed that the modulation of *SPT15* and *SPT3* can improve ethanol production under high gravity conditions by improving the osmotic and ethanol stress resistance of industrial strains. Spt3p interacts with the TAAT-binding protein (TBP) which is encoded by *SPT15*, and is required for the recruitment of TBP to the SAGA-dependent promoters for other genes (Hohmanm and Mager 2003).

1.5.3 Yeast deletion and over-expression libraries

The *Saccharomyces* genome deletion project led to the construction of a near-complete collection of yeast genes as individual deletion clones in both haploid and diploid laboratory yeast genetic backgrounds. The replacement of some 5500 yeast genes with a *KanMX* module allowed the phenotypic analysis of these genes, and so the determination of the genetic function under a range of conditions (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). At the commencement of this PhD research, these libraries were only available in an auxotrophic laboratory yeast background, which meant that the screening medium required adequate supplementation for growth. However, a haploid deletion library is under construction in a wine yeast genetic background (prototrophic) by the Australian Wine Research Institute (Borneman et al. 2008) but is currently not commercially available. Recently, a prototrophic version of the haploid library in laboratory yeast BY4741 has become available (Mulleder et al. 2012). This library

will allow identification of genes involved in nutrition-linked fermentation attributes such as fermentation completion under limited nitrogen conditions.

Despite some limitations, these libraries provide powerful tools as each deletion clone has been functionally characterised to some degree and the gene identified. As such, the libraries are useful in understanding gene function under a wide range of conditions given the ability to analyse the phenotype of each specific gene deletion. Whilst there have been many studies which have screened the libraries for growth sensitivity under single stress parameters related to fermentation (Ando et al. 2006, Fujita et al. 2006, Piggott et al. 2011, Teixeira et al. 2009, van Voorst et al. 2006), the author is unaware of any study which has used the collection of deletants, as separate clones and not a 'pool' to examine which genes are involved in fermentation. This PhD thesis (reported in part in Walker et al. 2013), focused on identification of genes which were responsible for not only growth in high sugar medium but also allowed the timely completion of alcoholic fermentation. Screening of a homozygous diploid deletion library in the defined medium CDGJM+PP (containing polyphenols and 200 g L^{-1} sugar) with sufficient nitrogen to allow for completion of fermentation by the parent, was undertaken. Genes responsible for fermentation were identified as having protracted, and in some cases arrested and incomplete 'stuck' fermentation, when the gene was deleted in the yeast mutant. These genes are referred to as Fermentation Essential Genes (FEGs) in Walker et al. (2013) although the name is interchangeable with High Sugar Fermentation (HSF) genes, as used in this thesis. The FEG or HSF dataset form part of the laboratory yeast 'Fermentome', a term which represents the genes/processes involved when yeast sense and respond to the multiple stresses of the juice environment. These cellular processes enable yeast to grow in grape juice and complete fermentation over the extended period of 4-12 days typical of a wine fermentation.

An alternative approach to the use of deletion libraries to identify genes associated with a specific phenotype is that of over-expression studies, again using a collection of yeast over-expression strains. Whilst there are a number of different over-expression libraries available, not all are suited to fermentation studies. For example,

libraries based on expression driven from the yeast *GAL1* promoter (Lohr et al. 1995) rather than an endogenous promoter (Jones et al. 2008) are not suited to fermentation in glucose media because of transcriptional inhibition. The yeast genomic tiling collection constructed by the Prelich laboratory (Jones et al. 2008) was considered the most suitable commercially available library for systematic gene expression during fermentation under high sugar (glucose and fructose) conditions. The yeast collection consists of ~1500 individual plasmids, each with a genomic fragment (encompassing 3-5 genes), totalling ~97% of the yeast genome as overlapping clones. The library, is based on the episomal plasmid, pGP564, incorporating a function *LEU2* selectable marker to complement leucine auxotrophy in the host yeast, following transformation and growth in media without leucine. CDGJM lacking leucine was chosen as the fermentation medium, allowing for retention of the plasmid to complement the auxotrophic growth requirements of the wine yeast strain. The use of the over-expression library in a wine yeast background allows a comprehensive screening under various fermentative conditions (i.e. high sugar (> 200 g L^{-1}) and variable amounts of nitrogen). As described above, expression of this over-expression library is driven by the endogenous promoter of the cloned genes. Each construct has multiple genes, not all of which are functional (Jones et al. 2008). Micro-scale fermentation was conducted in deep-well plates to allow residual sugar analysis at the time of fermentation completion by the parent strain. A list of potential clonal candidates will then be evaluated in laboratory scale fermentations. Single genes can be annotated to particular GO terms as being required for fermentation identified from clonal identification. And GO analysis will be chosen for re-cloning into pGP564 vector for further assessment.

1.6 Conclusion

In high sugar fermentations where sugar concentrations are elevated, yeast physiology is impacted by the increase of osmotic stress in the early stages of fermentation, and ethanol toxicity and nutrient depletion in the latter phases of fermentation. Consequently, these stress conditions can lower viable cell number, decrease the rate of fermentation and lead to sluggish fermentation and losses in product quality. Therefore, the development of improved or robust wine yeast for

successful fermentation is necessary. The improvement of wine yeast does not only relate to fermentation completion but also improvement of wine quality. Thus the principal targets for yeast strain improvement mainly relate to two broad areas; fermentation performance and final product quality. In wine making, improved fermentation performance relates to biological stress resistance, microbial spoilage control and enhancing sensory quality (Butzke and Bisson 1996, Dequin 2001, Parekh et al. 2000, Barre et al. 1993).

A number of studies on genome wide screening to identify genes sensitive to individual stressors during vinification have been undertaken (Ando et al. 2006, Fujita et al. 2006, Teixeira et al. 2009, 2010, Tucker and Fields 2004, van Voorst et al. 2006, Reiner et al. 2006, Abe and Minegishi 2008, Marks et al. 2008). However, studies on the identification of genes required for completion of HSF are limited. As previously discussed, wine making is a complex process, particularly in HSF, in which several stresses are presented simultaneously or sequentially during fermentation and these impact on yeast growth and fermentation kinetics. The identification of genes involved in HSF will not only be useful in the design and construction of an improved and fermentation robust strain, e.g. through targeting of selection procedures for directed evolution approaches, but also will form the basis of a greater understanding of the molecular mechanisms behind the overall process known as fermentation. Therefore the aims of this research are to:

1. Identify genes required for efficient HSF
2. Analyse the biological processes of this group of identified genes and
3. Relate these biological processes to efficient HSF.

These findings will increase our knowledge in relation to the contribution of microorganisms to fermentation kinetics and the development of strategies for wine yeast strain improvement which could be used to tailor winemaking.

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1 Yeast strains and culture conditions

Yeast strains were cultured at 28°C with shaking at 120 rpm on yeast extract peptone dextrose (YEPD) medium including 20 g L^{-1} glucose, 10 g L^{-1} bacto-yeast extract, 20 g L^{-1} bacto-peptone. Strains were maintained at 4°C on YEPD-agar (by addition of 20 g L^{-1} bacto-agar), or at -80°C as glycerol stocks. The glycerol stocks were prepared by addition of glycerol to an aliquot of overnight culture in YEPD to a final concentration of 15% v/v. Geneticin (G418, from Amresco) was added to YEPD (as required) for culturing yeast deletion strains.

2.2 Bacteria and culture conditions

Escherichia coli was used in this study for the amplification of plasmid DNA. Luria Bertani broth (LB) medium (tryptone 10 g L^{-1}; yeast extract 5 g L^{-1}; sodium chloride 10 g L^{-1}) was used to culture *E. coli* at 37°C overnight with agitation. *E. coli* cultures were maintained at 4°C on LB-agar (by addition of 20 g L^{-1} bacto-agar) or in glycerol stock (as described above). Ampicillin (50-100 mg L^{-1}) or kanamycin (40 mg L^{-1}) was added in LB medium to maintain plasmids during growth (as required).

2.3 Minimal drop-out media

Minimal drop-out media were also used to culture *S. cerevisiae* strains. This medium contained 20 g L^{-1} glucose, 5 g L^{-1} ammonium sulphate, salts, trace minerals, vitamins and drop-out amino acids stock (as listed in Appendix 1). One or two amino acids were omitted or 'dropped-out' (where required) in the amino acid stocks used to prepare the selection medium.

2.4 Chemically Defined Grape Juice Medium (CDGJM)

This medium was described in Henschke and Jiranek (1993) and the components are listed in Appendix 1.

2.5 Laboratory scale fermentation and analysis of key metabolites

2.5.1. Laboratory scale fermentation

Chemical Defined Grape Juice Medium (CDGJM) containing 200 g L^{-1} of equimolar glucose and fructose and 450 mg L^{-1} FAN (Henschke & Jiranek, 1993) was used in fermentation. Supplementation with uracil (150 mg L^{-1}) of the medium was required for the auxotrophic growth requirements of BY4743. Polyphenols were added to the medium as 3 g L^{-1} of polyphenol extract (Cat: Tppr, OenoProd, Sarl) which was dissolved in 5 mL of 100% ethanol and kept 24 hours in the dark, prior to addition to the sterile medium (CDGJM+PP). Yeast strains were grown in YEPD overnight and inoculated into starter CDGJM+PP containing supplementation (McBryde et al. 2006, Henschke and Jiranek 1993), listed in Appendix 1.

2.5.2 Sugar analysis

An accurate measurement of glucose and fructose was conducted by a spectrophotometric enzymatic method, described by the manufacturer, with some modifications by Dr Tommaso Liccioli. In brief, 200 µL of working solution containing 180 µL of NADP/ATP buffer (0.75 M triethanolamine, 10 mM magnesium sulphate, 1.1 mM β-nicotinamide adenine dinucleotide phosphate (NADP), 8 mM adenosine 5'-triphosphate disodium salt (ATP)) and 20 µL of diluted sample were placed into each well of a 96-well plate. Reactions were catalysed by hexokinase/glucose-6-phosphate dehydrogenase (HK/G6P-DH) and phosphoglucose isomerase (PGI). 500 µL of HK (420 U mL^{-1}) + G6P-DH (210 U mL^{-1}) and 40 µL of PGI 1000 U mL^{-1} were separately diluted in 10 mL of MQ water before adding 5 µL into the reaction solutions. Absorbance at 340 nm (A_{340}) was measured with a spectrophotometric plate reader (Tecan M200 Infinite). The reaction preparation was performed using a liquid handling robot (Corbett 3800).

2.5.3 Viable cell counts

The percentage of viable cells present during fermentation was measured by comparison of the number of colonies formed after 2-3 days growth on YEPD from

appropriately diluted cultures (in PBS buffer) to a total of cell count at the same time point, achieved with the use of a haemocytometer.

2.5.4 Plasmid retaining counts

The percentage of plasmid retaining cells during fermentation was determined by comparison of the number of colonies formed after 2-3 days growth on minimal drop-out medium plates from appropriately diluted cultures to the number of colonies on YEPD plates at the same time point.

2.6 Nucleic acid isolation

2.6.1 Preparation of plasmid from E. coli

E. coli cultures (5-10 mL) were grown overnight from a single colony in LB broth with the addition of appropriate antibiotics. Plasmid minipreps were performed using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Cat # A1125), as per manufacturer's instructions.

2.6.2 Genomic DNA isolation from S. cerevisiae

Genomic DNA isolation was adapted from Adams et al. (1997) with some modifications. 10 mL of *S. cerevisiae* cultures were grown overnight from a single colony in YEPD medium and harvested at 2,700 x *g* for 5 min, washed twice with sterile water. Cell pellets were resuspended in 0.2 mL of yeast plasmid buffer containing 2% Triton, 1% SDS, 0.1 M NaCl, 1 mM TRIS and 0.01 mM EDTA. Then 0.2 mL of phenol:chloroform (5:1) and 0.3 g of acid washed glass beads were added. Samples were vortexed for 3 min in 30 second bursts prior to adding 0.2 mL of TE (0.1 mM TRIS, 0.01 mM EDTA, adjusted to pH 8.0) buffer and centrifugation at 20,000 x *g* for 10 min. The aqueous layer was collected into a new tube and 1 mL of ethanol was added. Samples were centrifuged for another 2 min at 20,000 x *g* and the supernatant was discarded. The pellet was resuspended in 0.4 mL of TE buffer and 1 µL of RNAse cocktail enzyme mix (Invitrogen™) was added. Samples were incubated at 37°C for at least 30 min. Genomic DNA was precipitated with 10 µL of

4 M ammonium acetate and 1 mL of absolute ethanol and collected by centrifugation at 20,000 x *g* for 2 min. The supernatant liquid was removed by pipette and the pellet was air-dried and resuspended in deionised water.

2.6.3 Determination of DNA concentration

The concentration of purified DNA preparations was determined by the absorbance of a sample at 260 nm, given that the OD_{260} of a solution of double stranded DNA at 50 µg mL^{-1} is approximately 1. Purity of nucleic acids (from protein) was also estimated by determination of the absorbance at 280 nm, and a comparison of OD_{260}:OD_{280} ratio at the value of 1.8 - 2.0 being deemed adequate.

2.7 Molecular cloning techniques

2.7.1 Polymerase Chain Reaction (PCR)

PCR amplification was performed in 25 - 50 µL reactions typically containing 1 U polymerase enzyme, 1 x polymerase enzyme reaction buffer, 10 nmol primers, 0.5 ng plasmid DNA or 200 ng genomic DNA (as template) and 200 - 400 µM dNTPs. $MgCl_2$ concentration and cycling parameters were optimised for each PCR reaction. Cycling reactions were conducted in a C1000 Thermal Cycler (BIORAD®).

2.7.2 Restriction digestion of DNA

Purified DNA (1 - 10 µg) was incubated with 5 - 50 U of restriction enzyme using buffers recommended by the manufacturer. The final volume of 20 - 50 µL was incubated at 37°C for either 2 - 5 hours or overnight. If desired, digested DNA was cleaned for further use using the Wizard® SV Gel and PCR Clean-Up System (Promega, Cat # A9282) as per manufacturer's instruction.

2.7.3 Ligation of DNA into plasmid

Restriction digested DNA was ligated into appropriately digested and alkaline phosphatase treated vector DNA using T4 DNA ligase (0.1 – 50 Weiss Units) and the appropriate buffer as directed by the manufacturer (New England Biolabs) in a total volume of 10 µL. The molar ratio of insert to vector ends was at 3:1 whenever

possible. In some instances, polyethylene glycol (PEG), $MgCl_2$ and ATP were also added, typically in the concentration of 9.3% (v/v), 3.33 mM and 0.33 mM, respectively.

2.7.4 Preparation of competent E. coli *cells and transformation*

An overnight culture of *E. coli* DH5α from single colony was diluted 1:10 into fresh LB medium and grown to an OD_{600} of 0.4. The cells were chilled on ice and pelleted by centrifugation at 2,190 x *g* for 7 min at 4°C. The pellet was resuspended in 10 mL of cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% glycerol, 10 mM PIPES pH 7.0) and left on ice for 30 minutes. Cells were then washed twice with 10 mL of cold $CaCl_2$ at 4°C for 5 minutes before finally resuspending cells in 2 mL cold $CaCl_2$ solution. 400 µL aliquots were stored at -80°C in 1.5 mL screw capped tubes.

Transformation of the ligation mix into *E. coli* cells was performed using 100 µL of thawed competent cells (kept on ice), 50 µL of *E. coli* transformation buffer (100 µM (pH 7.0), 100 µM Mg Cl_2 and 100 µM $CaCl_2$) and 3 - 10 µL of ligation mix. The transformed cells were left on ice for 30 min and then heat shocked at 42°C for exactly 2 min. Cells were kept at room temperature for 5 min before 1 mL of fresh LB broth was added and cells incubated at 37°C for 1 hr on a rotating drum. Transformed cells were plated on LB agar with the appropriate antibiotic, and incubated overnight at 37°C.

2.7.5 High efficiency yeast transformation using lithium acetate

Competent yeast cells were prepared by inoculation from a single colony on a YEPD plate into YEPD broth and grown overnight. 1 mL of overnight culture was transferred into 100 mL of YEPD and grown for 4-5 hr (to OD_{600} 0.4). Cells were harvested at 2,700 x *g* for 5 min and washed twice in 25 mL of freshly prepared 0.1 M lithium acetate (LiAc) solution. Cells were resuspended in 800 µL of 0.1 M LiAc solution and immediately used in transformation.

Competent cells (100 µL) were pipetted into a microfuge tube, and the cells pelleted. LiAc was removed by micropipette. The transformation mixture consisted of 240 µL

of PEG (50% w/v), 36 µL 1.0 M LiAc solution, 50 µL of carrier DNA (Sigma, cat # D-1626; prepared by boiling a 2 mg mL^{-1} stock in TE buffer for 10 min, and then cooling on ice for 5 min), 2 - 4 µg plasmid DNA in a volume of less than 20 µL. Sterile water was added to give 360 µL total volume. The transformation was then incubated at 30°C for 30 min and followed by heat shock at 42°C for 30 min. Cells were harvested at 3,800 x *g* for 1 min and resuspended in 50 µL of PBS buffer before plating on selected media.

2.7.6 DNA sequencing

DNA sequencing reactions were set up as recommended by Applied Biosystems (ABI): each sequencing reaction contained 9.6 pmol of primer, 50-300 ng of template DNA. The samples were sent to a sequencing service (AGRF, Adelaide, Australia). Sequence analysis was performed using the BLASTN program (Altschul et al. 1997); http://genome-www.stanford.edu/Saccharomyces/; or http://www.ncbi.nlm.nih.gov/blast/.

2.8 Data statistical analysis

The data sets were analysed using data analysis tools (one-way ANOVA and t-test analysis) in GraphPad Prism software version 6.0. Figures were also created using this software.

CHAPTER 3 LABORATORY-SCALE EVALUATION OF FERMENTATION PERFORMANCE OF YEAST HARBOURING SINGLE GENE DELETIONS

3.1 Introduction

A collection of approximately 5000 individual yeast gene deletion clones or deletants were previously screened in this laboratory (Walker et al. 2013). The homozygous diploid deletion clones were screened for fermentation performance in deep 96 well plates containing 0.6 mL of Chemically Defined Grape Juice Medium with polyphenols (CDGJM+PP) and sufficient nutrients for optimal fermentation. 336 gene deletants, which are the subject of this doctorate research, were unable to complete high sugar fermentation after 180 h (with residual sugar being in excess of 2.5 g L^{-1}) whilst growth was not considered greatly different from the parent (having 70% or greater optical density at 600 nm compared with the parent) (Walker et al. 2013). Of these 336 genes, 100 mutants, referred to as the High Sugar Fermentation (HSF) dataset, were identified by direct comparison with published datasets from previous studies which looked at growth response towards typically single stresses relevant to fermentation. For example, hyperosmotic stress (sucrose, glucose, sodium choride or sorbitol), oxidative stress (hydrogen peroxide), and temperature extremes (Abe and Minegishi 2008, Ando et al. 2006, Erasmus et al. 2003, Fujita et al. 2006, Reiner et al. 2006, Teixeira 2009, 2010, Tucker and Fields 2004, van Voorst et al. 2006).

In addition, 81 gene deletants reported to be functionally related to the HSF dataset, but originally excluded from study because of an acceptable fermentation rate or else poor growth (optical density less than 70% of the parent in the micro-scale screening) were retain for re-evaluation. Investigation of these genes by laboratory-scale (100 mL) fermentation of the corresponding yeast deletants, together with bioinformatics analysis of publically accessible database(s) enabled determination of which genes were required for successful completion of alcoholic fermentation in the high sugar medium, CDGJM+PP. Fermentations conducted at this scale were considered more representative as they permitted greater control over parameters that

influence experimental reproducibility and fermentation outcome, e.g. inoculum preparation, maintenance of anaerobiosis and the inclusion of biological triplicates.

3.2 Materials and methods

3.2.1 Yeast strains and culture condition

The yeast strains used in this study were the parent strain *Saccharomyces cerevisiae* BY4743 (*MATa/a his3Δ1/his3Δ1 leu2Δ0 /leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0* (4741/4742)) and a collection of its derived diploid deletion strains developed by the Yeast Genome Deletion Project (Winzeler et al. 1999), which is available from Open Biosystems. The homozygous (*Δorf::KanMX*) gene deletions were in BY4743. All strains were cultured on YEPD as described in Section 2.1. Deletion strains were confirmed by PCR using primer A corresponding to the gene deleted (as listed in the Yeast Deletion Web Page (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html and Appendix 2, Table 2.1) and primer KanB which is an internal primer for the *KanMX* cassette.

3.2.2 Laboratory scale fermentation and monitoring of key metabolites

Chemical Defined Grape Juice Medium (Henschke & Jiranek, 1993) with polyphenols (CDGJM+PP) containing 200 g L^{-1} of equimolar glucose and fructose and 450 mg L^{-1} FAN and polyphenol extract was used in fermentation (see Section 2.5.1). Polyphenols were added to mimic a red wine fermentation wherein varying amounts of these known growth inhibitors (and hence an additional source of stress) are present (Xia et al. 2010). The parent strain BY4743 and a collection of its derived diploid deletants were grown in YEPD overnight and inoculated in starter CDGJM+PP containing supplementation (McBryde et al. 2006, Henschke and Jiranek 1993), as listed in Appendix 1. The starter cultures were grown in 250 mL Erlenmeyer flasks loosely fitted with a screw cap lid and incubated at 28°C overnight with shaking at 120 rpm. Fermentations were performed in 100 mL of CDGJM+PP in 250 mL Erlenmeyer flasks fitted with air-locks to allow release of CO_2. Samples were extracted using a 1 mL syringe via a septum-sealed port on the side of flask. Starter culture was transferred to 5 x 10^6 cells mL^{-1} into fermentation flasks. The

fermentations were conducted in triplicate at 28ºC with shaking at 120 rpm. Fermentation progress was monitored regularly by approximating residual sugars via refractive index (RI) measurement of sample supernatants. The samples were regularly collected, clarified by centrifugation and stored at -20°C prior to sugar analysis by a spectrophotometric enzymatic method (Walker et al. 2003) (Section 2.5.2). Growth was estimated by optical density at 600 nm (OD_{600}) of the culture sample. Fermentation data was presented as an average of triplicate determinations with the standard deviation included. Fermentations were considered 'dry' (complete) when the total residual sugar was less than 2.5 g L^{-1} as determined by using Clinitest™ tablets (Cat: 2107, Bayer). The time taken for individual deletion mutants to complete fermentation was expressed as relative fermentation duration compared to the parent strain BY4743.

3.2.3 Classification and numerical enrichment of identified genes annotated to Gene Ontology (GO) terms using computational software tools

The datasets were analysed using GOToolBox; specifically GO-Stats (http://genome.crg.es/GOToolBox/) (Martin et al. 2004) and GO Finder Version 0.83 software (http://www.yeastgenome.org/cgibin/GO/goTermFinder.pl) (Ashburner et al. 2000), which allowed the hierarchical clustering (and over-representation/enrichment or under-representation/depletion) of genes based on shared Gene Ontology (GO) terms. Both computational tools use a hypergeometric distribution with Multiple Hypothesis (Bonferroni) Correction (and additional False Discovery Rate; GO Finder) to calculate *p*-values. The clustering of genes based on their shared gene annotations was also determined using GO-Proxy (http://genome.crg.es/GOToolBox/) (Martin et al. 2004) and SGD Gene Ontology Slim Mapper (http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl) (Ashburner et al. 2000). The yeast genetic interaction networks between genes annotated to a particular GO term were visualised using the DRYGIN database (http://www.drygin.ccbr.utoronto.ca/) (Koh et al. 2010).

3.3 Results

3.3.1 Screening of 181 gene deletants in laboratory-scale fermentation

The 181 homozygous diploid gene deletants (identified in an initial micro-scale fermentation were compared to the parent strain BY4743 in triplicate 100 mL fermentations. Fermentations were conducted in Chemical Defined Grape Juice Medium containing 3 g L^{-1} grape skin polyphenols (CDGJM+PP) as described in Section 2.5.1. Figure 3.1 is representative of the results derived from the 100 mL scale fermentations performed in this study. Deletants △doa4 and △rav1 are example of strains with delayed or arrested fermentation thereby making them targets of further investigation. The relative fermentation duration of all 181 deletants was determined as a percentage of the time taken to finish fermentation when compared with the parent (Figure 3.2). Mutants which were shown to require an extra 20% or more time to complete fermentation were considered to exhibit protracted fermentation. From this, 88 deletants were shown not to be affected in fermentation, instead completing fermentation in a similar time to the parent. However, 84 gene deletants were classified as having protracted fermentation whilst another nine deletants were unable to complete fermentation at all. Fermentations performed by these 9 mutants actually arrested alcoholic fermentation and are thus referred to as 'stuck' deletants (Figure 3.2). Together, these 93 genes are hereafter referred to as Fermentation Essential Genes or FEGs (Walker et al. 2013), as their deletion results in fermentation dysfunction in the corresponding yeast mutants. The fermentation profiles of the 93 gene deletants are reported in Appendix Figure 2.1.

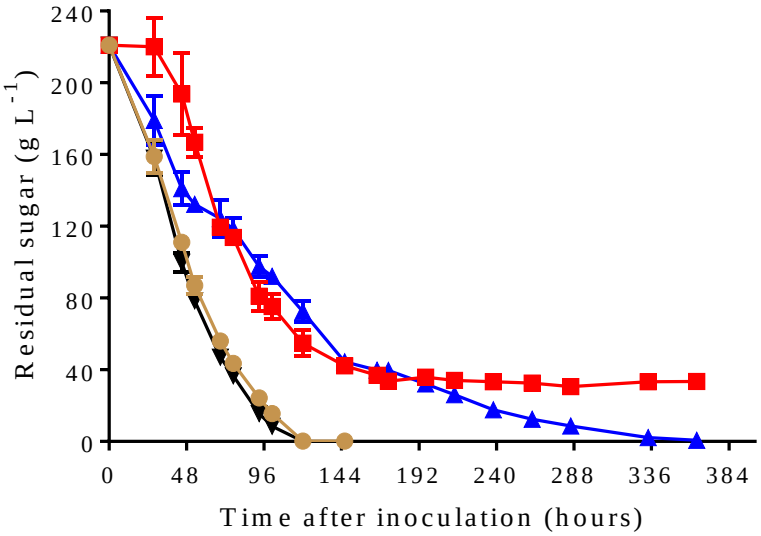

Figure 3.1 Comparative fermentation with the parent strain BY4743 (▼) and gene deletants Δdoa4 (■), Δrav1 (▲) and Δkes1 (●) in high sugar media. Fermentations were conducted in high sugar media (CDGJM+PP, 450 mg FAN L^{-1}, 200 g L^{-1} total sugar (equimolar glucose + fructose). Genes *DOA4* and *RAV1* appear to be required for high sugar fermentation since their corresponding deletants resulted in protracted (Δrav1) or stuck (Δdoa4) fermentation, whilst *KES1* does not appear to be essential as the *kes1*Δ deletant has similar fermentation kinetics to that of the parent.

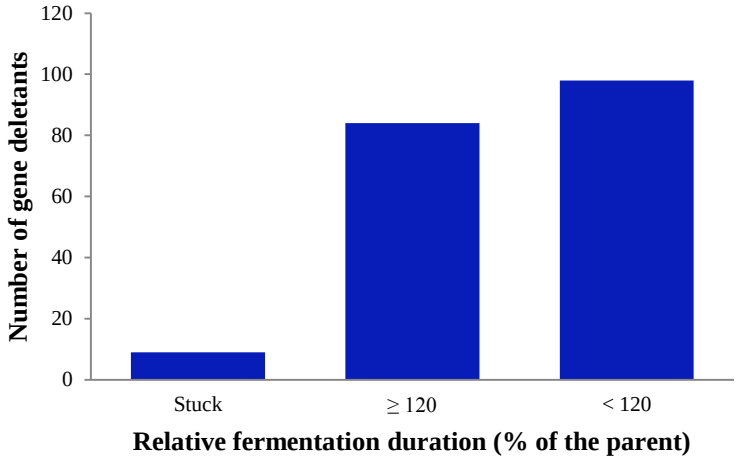

Figure 3.2 Categorisation of 181 gene deletants based on their relative fermentation duration. 100 mL fermentations were conducted in triplicate for each deletant and the parent using shake flasks fitted with air-locks as described in Materials and Methods. Fermentation progress was monitored by enzymatic determination of residual sugar (Walker et al. 2003). The relative fermentation duration was calculated as a percentage of the parent. In the case of those deletants with arrested or 'stuck' alcoholic fermentation, the duration was defined as the point when there was no longer a change in measured residual sugar.

3.3.2 Gene ontology analysis of the FEG dataset

In order to identify related functions of genes of the FEG dataset and to look for functional relationships to fermentation performance, computational software tools were used to both classify and look for enrichment of Gene Ontology (GO) terms. The description and annotation of the 93 genes of the FEG dataset, together with the relative fermentation duration of corresponding mutants is shown in Table 3.1 and Table 3.2. The annotation and enrichment of individual genes within the FEG dataset to particular GO terms, provides valuable insight into the potential cellular mechanisms by which yeast are able to respond and adapt to a grape must environment. These mechanisms allow yeast to succeed under such harsh conditions and complete fermentation. Several processes with significant enrichment of genes (annotated to the corresponding GO term) within the FEG dataset ($p < 0.01$) in reference to the genome, were chosen as likely to be important for efficient high sugar fermentation. Figure 3.3 is representative of the following cellular processes identified as having significant enrichment, namely, membrane invagination, autophagy; cellular (ion) homeostasis and vacuolar acidification.

Table 3.1 Annotation of 93 genes of the FEG dataset with cellular processes using GO Slim mapper software in the *Saccharomyces* Genome Database.

GO ID	GO term	Gene name
6873	Cellular ion homeostasis	*VMA2, VMA1, RAV2, NHX1, VMA3, VMA8, VMA7, DBF2, VMA16, VMA10, VMA22, TRK1, RAV1, PTK2, VMA5, VPH2, OCT1, MEH1, SSQ1, VMA6, HRK1, VPH1, VMA4, VMA11, VMA13*
19725	Cellular homeostasis	*VMA2, GPR1, VMA1, DOA4, RAV2, NHX1, VMA3, VMA8, GPA2, VMA7, DBF2, VMA16, VMA10, VMA22, TRK1, RAV1, PTK2, VMA5, VPH2, OCT1, MEH1, SSQ1, VMA6, ASC1, HRK1, VPH1, VMA4, VMA11, VMA13*
50801	Ion homeostasis	*VMA2, VMA1, RAV2, NHX1, VMA3, VMA8, VMA7, DBF2, VMA16, VMA10, VMA22, TRK1, RAV1, PTK2, VMA5, VPH2, OCT1, MEH1, SSQ1, VMA6, HRK1, VPH1, VMA4, VMA11, VMA13*
6885	Regulation of pH	*VMA2, VMA1, RAV2, NHX1, VMA3, VMA8, VMA7, DBF2, VMA16, VMA10, VMA22, RAV1, VMA5, VPH2, MEH1, VMA6, VPH1, VMA4, VMA11, VMA13*
51452	Intracellular pH reduction	*VMA2, VMA1, RAV2, NHX1, VMA3, VMA8, VMA7, DBF2, VMA16, VMA10, VMA22, RAV1, VMA5, VPH2, MEH1, VMA6, VPH1, VMA4, VMA11, VMA13*
7035	Vacuolar acidification	*VMA2, VMA1, RAV2, NHX1, VMA3, VMA8, VMA7, DBF2, VMA16, VMA10, VMA22, RAV1, VMA5, VPH2, MEH1, VMA6, VPH1, VMA4, VMA11, VMA13*

6366	Transcription from RNA polymerase II promoter	*CCR4, PAF1, SNF5, DST1, SPT4, OPI1, ZAP1, NUP133, HOG1, SAP30, MKS1, SIN3, HFI1*
6360	Transcription from RNA polymerase I promoter	*PAF1, RXT3, DST1, SPT4, SAP30, SIN3*
7154	Cell communication	*SLM4, CCZ1, SNF5, GPR1, VAC8, GPA2, OPI1, ATG7, ZAP1, PBS2, HOG1, ASC1, MKS1, VAM3, TCO89, PLC1*
23052	Signaling	*SLM4, GPR1, GPA2, OPI1, PBS2, PEX1, HOG1, ASC1, MKS1, TCO89, PLC1*
6605	Protein targeting	*ATG7, CCZ1, NUP133, NUP188, OCT1, PBS2, PEX1, SNX4, VAC8, VMA3*
10324	Membrane invagination	*SLM4, VPS41, VAC8, ATG7, SNX4, MEH1, VAM3, CIS1 DOA4, VMA3, CCZ1*
61025	Membrane fusion	*CCZ1, VAC8, VAM3, VPS41*
34727	Piecemeal microautophagy of nucleus	*VPS41, VAC8, ATG7, SNX4, VAM3*
6914	Autophagy	*SLM4, CCZ1, VPS41, VAC8, ATG7, SNX4, MEH1, VAM3*
16237	Microautophagy	*ATG7, CIS1, SLM4, SNX4, VAC8, VAM3, VPS41, CCZ1*
42594	Response to starvation	*CCZ1, VAC8, ATG7, ZAP1, VAM3*
7033	Vacuole organization	*TPM1, VAC8, VAM3, VPS41, VMA3*
70072	Vacuolar proton-transporting V-type ATPase complex assembly	*VMA22, VPH2, PKR1*

70271	Protein complex biogenesis	*PKR1, RAV1, RAV2, RBL2, VPH1, VPH2, VPS41, DST1*
6970	Response to osmotic stress	*HOG1, PBS2, TCO89*
34605	Response to heat	*HOG1, PBS2, TCO89, NUP133, NUP188, SAP30, SINE3*
6974	Response to DNA damage stimulus	*DCC1, SIN3, SNF5, SPT4, XRS2, YDR433W*
9311	Oligosaccharide metabolic process	*TPS1, SNF5, TPS2*
5975	Carbohydrate metabolic process	*HXK1, TCO89, TKL1, TPS1, TPS2*
6811	Ion transport	*NHX1, VMA3, VPH1*
45333	Cellular respiration	*MCT1*
6520	Cellular amino acid metabolic process	*MSW1*
55086	Nucleobase, nucleoside and nucleotide metabolic process	*ADO1, GPA2, NPT1, TKL1*
51186	Cofactor metabolic process	*NPT1, TKL1, SSQ1*
6629	Lipid metabolic process	*ERG6, MCT1, OPI1, PLC1, RML2*
6457	Protein folding	*GSF2, RBL2, ZUO1*
278	Mitotic cell cycle	*CCR4, CNM67, DCC1, SIN3, SPC72, TOM1, VHS1*
7010	Cytoskeleton organization	*CNM67, PBS2, RBL2, SPC72, TPM1*

910	Cytokinesis	*CYK3, BUD31*
6401	RNA catabolic process	*CCR4, LSM1, PAT1*
6397	mRNA processing	*BUD31, CCR4, DST1, SNT309*
6353	Transcription termination, DNA-dependent	*RTT103, DST1*
6417	Regulation of translation	*ASC1, PAT1, ZUO1*
6413	Translational initiation	*BUD27, PAT1*
51052	Regulation of DNA metabolic process	*DIA2, CCR4, DOA4, SIN3*
other	Other	*PUG1, YGR064W*
8150	Biological process unknown	*DUF1, IES6, YCL007C, YFL012W, YLL007C*

Table 3.2 Description and relative fermentation duration of 93 identified genes.

Gene name	Systematic name	Relative fermentative duration (%)	Description
ADO1	*YJR105W*	*182*	Adenosine kinase, required for the utilization of S-adenosylmethionine (AdoMet); may be involved in recycling adenosine produced through the methyl cycle
ASC1	*YMR116C*	*117*	G-protein beta subunit and guanine nucleotide dissociation inhibitor for Gpa2p; ortholog of RACK1 that inhibits translation; core component of the small (40S) ribosomal subunit
ATG7	*YHR171W*	*152*	Autophagy-related protein and dual specificity member of the E1 family of ubiquitin-activating enzymes; mediates the conjugation of Atg12p with Atg5p and Atg8p with phosphatidylethanolamine, required steps in autophagosome formation
BUD27	*YFL023W*	*134*	Unconventional prefoldin protein involved in translation initiation; mutants have inappropriate expression of nutrient sensitive genes due to translational derepression of Gcn4p transcription factor; diploid mutants show random budding
BUD31	*YCR063W*	*234*	Component of the SF3b subcomplex of the U2 snRNP; diploid mutants display a random budding pattern instead of the wild-type bipolar pattern
CCR4	*YAL021C*	*151*	Component of the CCR4-NOT transcriptional complex, which is involved in regulation of gene expression; component of the major cytoplasmic deadenylase, which is involved in mRNA poly(A) tail shortening
CCZ1	*YBR131W*	*172*	Protein involved in vacuolar assembly, essential for autophagy and the cytoplasm-to-vacuole pathway
CIS1	*YDR022C*	*132*	Autophagy-specific protein required for autophagosome formation; may form a complex with Atg17p and Atg29p that localizes other proteins to the pre-autophagosomal structure; high-copy suppressor of CIK1 deletion
CNM67	*YNL225C*	*121*	Component of the spindle pole body outer plaque; required for spindle orientation and mitotic nuclear migration
CYK3	*YDL117W*	*152*	SH3-domain protein located in the mother-bud neck and the cytokinetic actin ring; mutant

			phenotype and genetic interactions suggest a role in cytokinesis
DBF2	*YGR092W*	*145*	Ser/Thr kinase involved in transcription and stress response; functions as part of a network of genes in exit from mitosis
DCC1	*YCL016C*	*140*	Subunit of a complex with Ctf8p and Ctf18p that shares some components with Replication Factor C, required for sister chromatid cohesion and telomere length maintenance
DIA2	*YOR080W*	*115*	Origin-binding F-box protein that forms an SCF ubiquitin ligase complex with Skp1p and Cdc53p; plays a role in DNA replication, involved in invasive and pseudohyphal growth
DOA4	*YDR069C*	*STUCK**	Ubiquitin isopeptidase, required for recycling ubiquitin from proteasome-bound ubiquitinated intermediates, acts at the late endosome/prevacuolar compartment to recover ubiquitin from ubiquitinated membrane proteins en route to the vacuole
DST1	*YGL043W*	*124*	General transcription elongation factor TFIIS, enables RNA polymerase II to read through blocks to elongation by stimulating cleavage of nascent transcripts stalled at transcription arrest sites
DUF1	*YOL087C*	*138*	Putative protein of unknown function
ERG6	*YML008C*	*185*	Delta(24)-sterol C-methyltransferase, converts zymosterol to fecosterol in the ergosterol biosynthetic pathway by methylating position C-24; localized to both lipid particles and mitochondrial outer membrane
GPA2	*YER020W*	*133*	Nucleotide binding alpha subunit of the heterotrimeric G protein that interacts with the receptor Gpr1p, has signaling role in response to nutrients; green fluorescent protein (GFP)-fusion protein localizes to the cell periphery
GPR1	*YDL035C*	*123*	Plasma membrane G protein coupled receptor (GPCR) that interacts with the heterotrimeric G protein alpha subunit, Gpa2p, and with Plc1p; sensor that integrates nutritional signals with the modulation of cell fate via PKA and cAMP synthesis
GSF2	*YML048W*	*136*	ER localized integral membrane protein that may promote secretion of certain hexose transporters, including Gal2p; involved in glucose-dependent repression
HFI1	*YPL254W*	*229*	Adaptor protein required for structural integrity of the SAGA complex, a histone acetyltransferase-coactivator complex that is involved in global regulation of gene expression through acetylation and transcription functions

HOG1	*YLR113W*	*209*	Mitogen-activated protein kinase involved in osmoregulation via three independent osmosensors; mediates the recruitment and activation of RNA Pol II at Hot1p-dependent promoters; localization regulated by Ptp2p and Ptp3p
HRK1	*YOR267C*	*289*	Protein kinase implicated in activation of the plasma membrane $H(^{+})$-ATPase Pma1p in response to glucose metabolism; plays a role in ion homeostasis
HXK1	*YFR053C*	*142*	Hexokinase isoenzyme 1, a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism; expression is highest during growth on non-glucose carbon sources; glucose-induced repression involves the hexokinase Hxk2p
IES6	*YEL044W*	*124*	Protein that associates with the INO80 chromatin remodeling complex under low-salt conditions; human ortholog INO80C is a member of the human INO80 complex; implicated in DNA repair based on genetic interactions with RAD52 epistasis genes
LSM1	*YJL124C*	*129*	Lsm (Like Sm) protein; forms heteroheptameric complex (with Lsm2p, Lsm3p, Lsm4p, Lsm5p, Lsm6p, and Lsm7p) involved in degradation of cytoplasmic mRNAs
MCT1	*YOR221C*	*117*	Predicted malonyl-CoA:ACP transferase, putative component of a type-II mitochondrial fatty acid synthase that produces intermediates for phospholipid remodeling
MEH1	*YKR007W*	*165*	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; loss results in a defect in vacuolar acidification
MKS1	*YNL076W*	*160*	Pleiotropic negative transcriptional regulator involved in Ras-CAMP and lysine biosynthetic pathways and nitrogen regulation; involved in retrograde (RTG) mitochondria-to-nucleus signaling
MSW1	*YDR268W*	*152*	Mitochondrial tryptophanyl-tRNA synthetase
NHX1	*YDR456W*	*175*	Endosomal Na^{+}/H^{+} exchanger, required for intracellular sequestration of Na^{+}; required for osmotolerance to acute hypertonic shock
NPT1	*YOR209C*	*STUCK**	Nicotinate phosphoribosyltransferase, acts in the salvage pathway of NAD^{+} biosynthesis; required for silencing at rDNA and telomeres and has a role in silencing at mating-type loci; localized to the nucleus
NUP133	*YKR082W*	*124*	Subunit of the Nup84p subcomplex of the nuclear pore complex (NPC), localizes to both sides of the NPC, required to establish a normal nucleocytoplasmic concentration gradient of the

			GTPase Gsp1p
NUP188	*YML103C*	*151*	Subunit of the nuclear pore complex (NPC), involved in the structural organization of the complex and of the nuclear envelope, also involved in nuclear envelope permeability, interacts with Pom152p and Nic96p
OCT1	*YKL134C*	*136*	Mitochondrial intermediate peptidase, cleaves destabilizing N-terminal residues of a subset of proteins upon import, after their cleavage by mitochondrial processing peptidase (Mas1p-Mas2p); may contribute to mitochondrial iron homeostasis
OPI1	*YHL020C*	*168*	Transcriptional regulator of a variety of genes; phosphorylation by protein kinase A stimulates Opi1p function in negative regulation of phospholipid biosynthetic genes; involved in telomere maintenance
PAF1	*YBR279W*	*127*	Component of the Paf1p complex that binds to and modulates the activity of RNA polymerases I and II; required for expression of a subset of genes, including cell cycle-regulated genes; homolog of human PD2/hPAF1
PAT1	*YCR077C*	*157*	Topoisomerase II-associated deadenylation-dependent mRNA-decapping factor; also required for faithful chromosome transmission, maintenance of rDNA locus stability, and protection of mRNA 3'-UTRs from trimming; functionally linked to Pab1p
PBS2	*YJL128C*	*227*	MAP kinase kinase that plays a pivotal role in the osmosensing signal-transduction pathway, activated under severe osmotic stress; plays a role in regulating Ty1 transposition
PEX1	*YKL197C*	*117*	AAA-peroxin that heterodimerizes with AAA-peroxin Pex6p and participates in the recycling of peroxisomal signal receptor Pex5p from the peroxisomal membrane to the cystosol; induced by oleic acid and upregulated during anaerobiosis
PKR1	*YMR123W*	*118*	V-ATPase assembly factor, functions with other V-ATPase assembly factors in the ER to efficiently assemble the V-ATPase membrane sector
PLC1	*YPL268W*	*STUCK**	Phospholipase C, hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP2) to generate the signaling molecules inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG); involved in regulating many cellular processes
PTK2	*YJR059W*	*STUCK**	Putative serine/threonine protein kinase involved in regulation of ion transport across plasma membrane; enhances spermine uptake

PUG1	*YER185W*	*149*	Plasma membrane protein with roles in the uptake of protoprophyrin IX and the efflux of heme; expression is induced under both low-heme and low-oxygen conditions; member of the fungal lipid-translocating exporter (LTE) family of proteins
RAV1	*YJR033C*	*249*	Subunit of the RAVE complex (Rav1p, Rav2p, Skp1p), which promotes assembly of the V-ATPase holoenzyme; required for transport between the early and late endosome/PVC and for localization of TGN membrane proteins; potential Cdc28p substrate
RAV2	*YDR202C*	*178*	Subunit of RAVE (Rav1p, Rav2p, Skp1p), a complex that associates with the V1 domain of the vacuolar membrane (H^+)-ATPase (V-ATPase) and promotes assembly and reassembly of the holoenzyme
RBL2	*YOR265W*	*202*	Protein involved in microtubule morphogenesis, required for protection from excess free beta-tubulin; proposed to be involved the folding of beta-tubulin; similar to mouse beta-tubulin cofactor A
RML2	*YEL050C*	*152*	Mitochondrial ribosomal protein of the large subunit, has similarity to E. coli L2 ribosomal protein; fat21 mutant allele causes inability to utilize oleate and may interfere with activity of the Adr1p transcription factor
RTT103	*YDR289C*	*126*	Protein that interacts with exonuclease Rat1p and Rai1p and plays a role in transcription termination by RNA polymerase II, has an RPR domain (carboxy-terminal domain interacting domain); also involved in regulation of Ty1 transposition
RXT3	*YDL076C*	*183*	Subunit of the RPD3L complex; involved in histone deacetylation
SAP30	*YMR263W*	*162*	Subunit of a histone deacetylase complex, along with Rpd3p and Sin3p, that is involved in silencing at telomeres, rDNA, and silent mating-type loci; involved in telomere maintenance
SIN3	*YOL004W*	*STUCK**	Component of the Sin3p-Rpd3p histone deacetylase complex, involved in transcriptional repression and activation of diverse processes, including mating-type switching and meiosis; involved in the maintenance of chromosomal integrity
SLM4	*YBR077C*	*165*	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; gene exhibits synthetic genetic interaction with MSS4
SNF5	*YBR289W*	*255*	Subunit of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation; functions interdependently in transcriptional activation with Snf2p and Snf6p

SNT309	*YPR101W*	*132*	Member of the NineTeen Complex (NTC) that contains Prp19p and stabilizes U6 snRNA in catalytic forms of the spliceosome containing U2, U5, and U6 snRNAs; interacts physically and genetically with Prp19p
SNX4	*YJL036W*	*124*	Sorting nexin, involved in retrieval of late-Golgi SNAREs from post-Golgi endosomes to the trans-Golgi network and in cytoplasm to vacuole transport; contains a PX phosphoinositide-binding domain; forms complexes with Snx41p and with Atg20p
SPC72	*YAL047C*	*172*	Component of the cytoplasmic Tub4p (gamma-tubulin) complex, binds spindle pole bodies and links them to microtubules; has roles in astral microtubule formation and stabilization
SPT4	*YGR063C*	*169*	Protein involved in the regulating Pol I and Pol II transcription, pre-mRNA processing, kinetochore function, and gene silencing; forms a complex with Spt5p
SSQ1	*YLR369W*	*STUCK**	Mitochondrial hsp70-type molecular chaperone, required for assembly of iron/sulfur clusters into proteins at a step after cluster synthesis, and for maturation of Yfh1p, which is a homolog of human frataxin implicated in Friedreich's ataxia
TCO89	*YPL180W*	*143*	Subunit of TORC1 (Tor1p or Tor2p-Kog1p-Lst8p-Tco89p), a complex that regulates growth in response to nutrient availability; cooperates with Ssd1p in the maintenance of cellular integrity; deletion strains are hypersensitive to rapamycin
TFP1	*YDL185W*	*286*	VMA1; Subunit A of the eight-subunit V1 peripheral membrane domain of the vacuolar H^+-ATPase; protein precursor undergoes self-catalyzed splicing to yield the extein Tfp1p and the intein Vde (PI-SceI), which is a site-specific endonuclease
TKL1	*YPR074C*	*123*	Transketolase, similar to Tkl2p; catalyzes conversion of xylulose-5-phosphate and ribose-5-phosphate to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate in the pentose phosphate pathway; needed for synthesis of aromatic amino acids
TOM1	*YDR457W*	*152*	E3 ubiquitin ligase of the hect-domain class; has a role in mRNA export from the nucleus and may regulate transcriptional coactivators; involved in degradation of excess histones
TPM1	*YNL079C*	*117*	Major isoform of tropomyosin; binds to and stabilizes actin cables and filaments, which direct polarized cell growth and the distribution of several organelles; acetylated by the NatB complex and acetylated form binds actin most efficiently
TPS1	*YBR126C*	*STUCK**	Synthase subunit of trehalose-6-phosphate synthase/phosphatase complex, which synthesizes the storage carbohydrate trehalose; also found in a monomeric form; expression is induced by

			the stress response and repressed by the Ras-cAMP pathway
TPS2	*YDR074W*	*STUCK**	Phosphatase subunit of the trehalose-6-phosphate synthase/phosphatase complex, which synthesizes the storage carbohydrate trehalose; expression is induced by stress conditions and repressed by the Ras-cAMP pathway
TRK1	*YJL129C*	*139*	Component of the Trk1p-Trk2p potassium transport system; 180 kDa high affinity potassium transporter; phosphorylated in vivo and interacts physically with the phosphatase Ppz1p
VAC8	*YEL013W*	*152*	Phosphorylated and palmitoylated vacuolar membrane protein that interacts with Atg13p, required for the cytoplasm-to-vacuole targeting (Cvt) pathway; interacts with Nvj1p to form nucleus-vacuole junctions
VAM3	*YOR106W*	*178*	Syntaxin-related protein required for vacuolar assembly; functions with Vam7p in vacuolar protein trafficking; multispecificity vacuolar t-SNARE that mediates docking/fusion of multiple distinct late transport intermediates with the vacuole
VHS1	*YDR247W*	*116*	Cytoplasmic serine/threonine protein kinase; identified as a high-copy suppressor of the synthetic lethality of a sis2 sit4 double mutant, suggesting a role in G1/S phase progression; homolog of Sks1p
VMA10	*YHR039C-A*	*270*	Subunit G of the eight-subunit V1 peripheral membrane domain of the vacuolar H^+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; involved in vacuolar acidification
VMA11	*YPL234C*	*190*	Vacuolar ATPase V0 domain subunit c', involved in proton transport activity; hydrophobic integral membrane protein (proteolipid) containing four transmembrane segments
VMA13	*YPR036W*	*245*	Subunit H of the eight-subunit V1 peripheral membrane domain of the vacuolar H^+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; serves as an activator or a structural stabilizer of the V-ATPase
VMA16	*YHR026W*	*165*	PPA1; Subunit c" of the vacuolar ATPase, which functions in acidification of the vacuole; one of three proteolipid subunits of the V0 domain
VMA2	*YBR127C*	*199*	Subunit B of the eight-subunit V1 peripheral membrane domain of the vacuolar H^+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system

VMA22	*YHR060W*	*160*	Peripheral membrane protein that is required for vacuolar H^+-ATPase (V-ATPase) function, although not an actual component of the V-ATPase complex; functions in the assembly of the V-ATPase; localized to the yeast endoplasmic reticulum (ER)
VMA3	*YEL027W*	*242*	Proteolipid subunit of the vacuolar H^+-ATPase V0 sector (subunit c; dicyclohexylcarbodiimide binding subunit); required for vacuolar acidification and important for copper and iron metal ion homeostasis
VMA4	*YOR332W*	*230*	Subunit E of the eight-subunit V1 peripheral membrane domain of the vacuolar H^+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; required for the V1 domain to assemble onto the vacuolar membrane
VMA5	*YKL080W*	*274*	Subunit C of the eight-subunit V1 peripheral membrane domain of vacuolar H^+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; required for the V1 domain to assemble onto the vacuolar membrane
VMA6	*YLR447C*	*210*	Subunit d of the five-subunit V0 integral membrane domain of vacuolar H^+-ATPase (V-ATPase), an electrogenic proton pump found in the endomembrane system; stabilizes V0 subunits; required for V1 domain assembly on the vacuolar membrane
VMA7	*YGR020C*	*185*	Subunit F of the eight-subunit V1 peripheral membrane domain of vacuolar H^+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; required for the V1 domain to assemble onto the vacuolar membrane
VMA8	*YEL051W*	*165*	Subunit D of the eight-subunit V1 peripheral membrane domain of the vacuolar H^+-ATPase (V-ATPase), an electrogenic proton pump found throughout the endomembrane system; plays a role in the coupling of proton transport and ATP hydrolysis
VPH1	*YOR270C*	*178*	Subunit a of vacuolar-ATPase V0 domain, one of two isoforms (Vph1p and Stv1p); Vph1p is located in V-ATPase complexes of the vacuole while Stv1p is located in V-ATPase complexes of the Golgi and endosomes
VPH2	*YKL119C*	*148*	Integral membrane protein required for vacuolar H^+-ATPase (V-ATPase) function, although not an actual component of the V-ATPase complex; functions in the assembly of the V-ATPase; localized to the endoplasmic reticulum (ER)
VPS41	*YDR080W*	*171*	Vacuolar membrane protein that is a subunit of the homotypic vacuole fusion and vacuole protein sorting (HOPS) complex; essential for membrane docking and fusion at the Golgi-to-

			endosome and endosome-to-vacuole stages of protein transport
XRS2	*YDR369C*	*149*	Protein required for DNA repair; component of the Mre11 complex, which is involved in double strand breaks, meiotic recombination, telomere maintenance, and checkpoint signaling
YCL007C	*YCL007C*	*170*	Dubious ORF unlikely to encode a protein
YDR433W	*YDR433W*	*149*	Dubious open reading frame unlikely to encode a functional protein
YFL012W	*YFL012W*	*133*	Putative protein of unknown function; transcribed during sporulation; null mutant exhibits increased resistance to rapamycin
YGR064W	*YGR064W*	*153*	Dubious open reading frame unlikely to encode a protein
YLL007C	*YLL007C*	*168*	Putative protein of unknown function
ZAP1	*YJL056C*	*STUCK**	Zinc-regulated transcription factor; binds to zinc-responsive promoters to induce transcription of certain genes in presence of zinc, represses other genes in low zinc; regulates its own transcription; contains seven zinc-finger domains
ZUO1	*YGR285C*	*151*	Cytosolic ribosome-associated chaperone that acts, together with Ssz1p and the Ssb proteins, as a chaperone for nascent polypeptide chains; contains a DnaJ domain and functions as a J-protein partner for Ssb1p and Ssb2p

** the fermentation was incomplete*

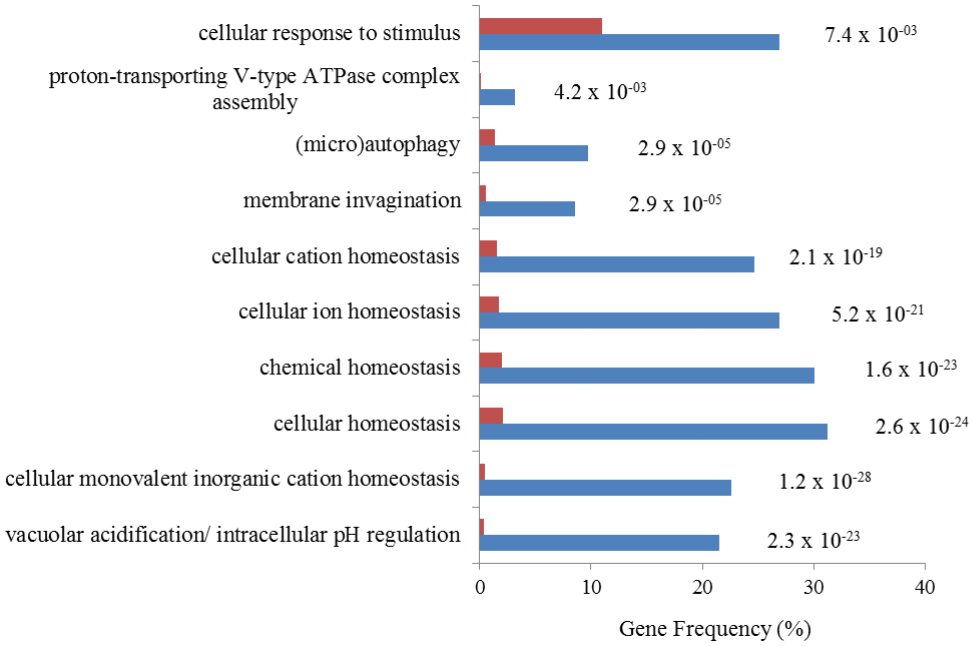

Figure 3.3 The frequency of genes in the genome (red bars) and FEG dataset (blue bars) annotated to each molecular biological process. The number on the side of each bar indicates the enrichment value based on Gene Ontology analysis using GO Finder software (Ashburner et al. 2000).

3.3.3 Fermentation profiles of nine stuck gene deletants

The fermentation of nine of the 93 FEG deletants resulted was arrested. The growth and fermentation profile of these nine yeast deletants was determined in an independent experiment (Figure 3.4). Fermentation arrest was noted after an extended fermentation for the three deletants Δssq1, Δnpt1 and Δplc1, as shown in Table 3.3, where the residual sugar was 60.2 ± 0.8 g L^{-1}, 18.5 ± 7.8 g L^{-1} and 60.1 ± 5.3 g L^{-1}, respectively. Growth was observed not to be markedly different within the first 24 hours of incubation between all nine mutants and parent (Figure 3.4). However, all mutants with the exception of Δptk2 and Δtps1 only grew to a final optical density at 600 nm of less than 70% of the parent. Whilst five mutants had similar growth behaviour, the timing of fermentation cessation differed, with Δdoa4, Δtps1 and Δtps2 having similar fermentation profiles, which was not as protracted as observed with Δsin3 and Δzap1. Interestingly, four vma mutants (Δvma4, Δvma6, Δvma11 and Δvma22) defective in vacuolar ATPase had similar growth deficiency to the 'stuck' mutants, but were able to catabolise sugar albeit more slowly than the parent BY4743 (Walker, *pers. comm*). These findings would indicate that final biomass (as indicated by maximal OD_{600}) does not necessarily determine whether yeast are able to catabolise sugar albeit in an extended time frame (protracted) or fail to catabolise all of the sugar (stuck fermentation). Rather, the final outcome is likely dependent on the interplay of the gene and its encoded protein, in the myriad of cellular processes required for adaptation to such harsh environments.

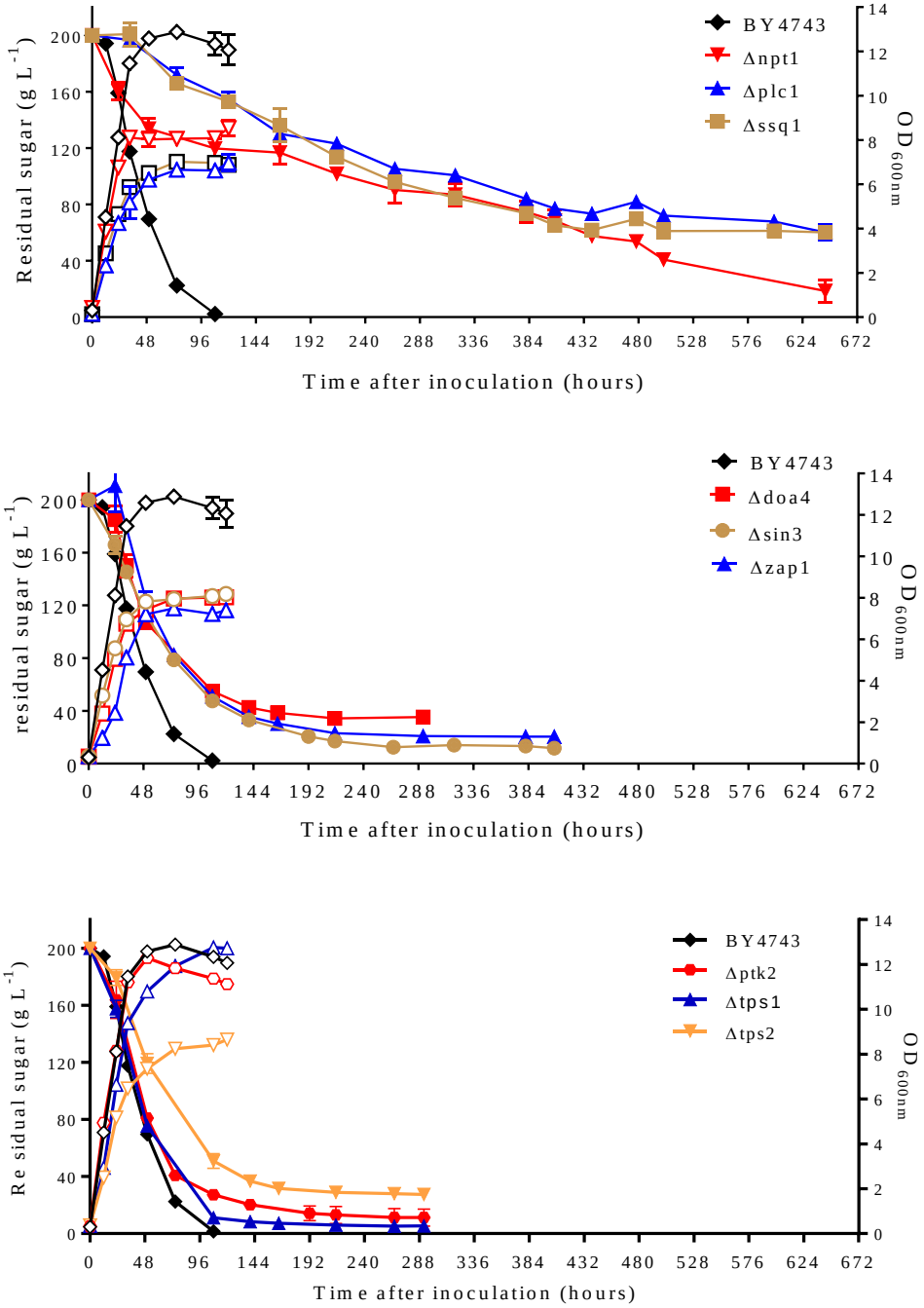

Figure 3.4 Fermentation and growth profiles of the parent (BY4743) and nine yeast deletants which result in 'stuck' fermentation. Triplicate (100 mL) fermentations were conducted in CDGJM+PP medium (450 mg FAN L^{-1}, 200 g L^{-1} total sugar) according to Material and Methods. Growth was monitored by optical density (OD_{600}) using 1:10 diluted culture samples (open symbols). Fermentation progress was monitored by determination of residual sugar (solid symbols). The standard deviations are shown as error bars (typically no visible beyond the symbol).

Table 3.3 Fermentation duration and residual sugar of the 9 'stuck' deletants conducting in the CDGJM containing 200 g L^{-1} sugars.

Gene name	Systematic name	Measured fermentation duration until arrest hours (residual sugar)	Time of completion for parent BY4743 (hours)	Cellular process
DOA4	*YDR069C*	292 (35.3 ± 2.8 g L^{-1})	108	Ubiquitin recycling
SIN3	*YOL004W*	406.5 (11.7 ± 2.2 g L^{-1})	108	Transcription from RNA polymerase II promoter
PTK2	*YJR059W*	292 (12.0 ± 3.3 g L^{-1})	108	Ion homeostasis
ZAP1	*YJL056C*	406.5 (20.4 ± 1.9 g L^{-1})	108	Transcription from RNA polymerase II promoter
PLC1	*YPL268W*	644 (60.1 ± 5.3 g L^{-1})	108	Signalling
SSQ1	*YLR369W*	644 (60.2 ± 0.8 g L^{-1})	108	Ion homeostasis
NPT1	*YOR209C*	644 (18.5 ± 7.8 g L^{-1})	108	NAD recycling
TPS1	*YBR126C*	292 (5.4 ± 0.2 g L^{-1})	108	Trehalose synthesis
TPS2	*YDR074W*	292 (27.3 ± 2.0 g L^{-1})	108	Trehalose synthesis

3.4 Discussion

3.4.1 Vacuolar acidification regulated by the V-ATPase complex is important in fermentation

Gene Ontology analysis of the 93 FEG genes showed that a large number (20 of the 26 genes present in the genome) were annotated to vacuolar acidification. Most of these genes were involved in function and assembly of the V-ATPase complex, the major proton pump involved in vacuolar acidification (Kane 2006). Other researchers have shown vacuolar acidification to play a crucial role in regulating other cellular processes such as pH regulation, ion (calcium, iron, zinc) homeostasis, amino acid storage and recycling and membrane trafficking (Corbacho et al. 2012, Klionsky et al. 1990, Makrantoni et al. 2007, Nass and Rao 1999, Plant et al. 1999, Ungermann et al. 1999, Yamashiro et al. 1990). The present study indicates that vacuolar acidification maybe an important adaptive mechanism when yeast undergo fermentation in the presence of high concentrations of sugar. Mutants defective in genes annotated to this GO term were shown to have protracted fermentation. 13 genes of the 20 identified vacuolar acidification genes encode for all the components of V-ATPase whose major role is to maintain the acidity of the vacuole (Table 3.4). The internal pH of the vacuole is reported to normally be around 5.27 and maintenance is necessary for glucose metabolism, cytosolic pH homeostasis (Brett et al. 2011) and the stable localisation of Pmp1p in the plasma membrane (Martinez-Munoz and Kane 2008). In terms of cell growth, whilst V-ATPase does not regulate cell growth, vma mutants which lack genes encoding individual V-ATPase subunits, exhibited growth sensitivity in highly acidic (pH 3) and neutral (pH 7) or alkaline conditions (Cyert and Philpott 2013). Furthermore, Kane (2006) showed that extracellular calcium was inhibitory to growth, and vma mutants were unable to grow on non-fermentable carbohydrates. Other genes involved in the assembly of the V-ATPase complex such as those encoding the assembly factors Vma22p, Pkr1p as well as the RAVE complex (Rav1p and Rav2p) were also included in the FEG data set. The RAVE complex is required for the reversible assembly and disassembly of the V-ATPase complex in response to glucose deprivation (Kane 2006).

Table 3.4 Genes encoding the 13 subunits of the V-ATPase complex and the fermentation performance (as relative fermentation duration) of the corresponding yeast deletants

Yeast gene	Domain	Subunit	Relative fermentation duration (%)
VMA1 (*TFP1*)	V1	A	286
VMA2		B	199
VMA5		C	274
VMA8		D	165
VMA4		E	230
VMA7		F	185
VMA10		G	269
VMA13		H	245
*VPH1**	V0	a	177
VMA6		d	210
VMA3 (*CUP5*)		c	241
VMA11 (*TFP3*)		c'	190
VMA16 (*PPA1*)		c"	165

**VPH1* encodes vacuolar ATPase subunit a, whilst *STV1* encodes the equivalent isomer of subunit a, Stv1p in the golgi and endosome.

3.4.2 Other cellular mechanisms have a role in fermentation

Interestingly, of the nine deletants which resulted in stuck fermentation, two were related to trehalose synthesis. TPS1 encodes the trehalose-6-phosphate synthase (TPS), which catalyses the formation of alpha, alpha-trehalose 6-phosphate from glucose 6-phosphate and UDP-glucose. TPS2 encodes the trehalose-6-phosphate phosphatase (TPP) catalysing the conversion of alpha, alpha trehalose-6-phosphate in water to trehalose and phosphate (Francois and Parrou 2001, Thevelein and Hohmann 1995). Trehalose is a non-reducing carbohydrate which is known to be accumulated by the cell in the stationary phase of fermentation (Gibson et al. 2008). The accumulation of trehalose is involved in cellular protection and adaptation to various stresses present during fermentation (Alexandre et al. 1998). The trehalose synthetic genes TPS1 and TPS2 were previously identified in various studies, with Δtps1 being sensitive to ethanol (Ando et al. 2006, Teixeira 2009, van Voorst et al. 2006) and oxidative stress (Tucker and Fields 2004) and Δtps2 to ethanol, hyper-osmolarity (Auesukaree et al. 2009), oxidative stress (Tucker and Fields 2004), and high acidity (Mollapour et al. 2004). TPS1 and TPS2 are also reported to have an additional function in the regulation of sugar influx into glycolysis (Hohmann et al. 1996, Thevelein and Hohmann 1995). Trehalose is synthesised from glucose-6-phosphate (G6P) via trehalose-6-phosphate (Tre6P). Cells lacking TPS1 showed a high accumulation of sugar phosphate, G6P whilst Tre6P, the product of trehalose synthase (encoded by TPS1), was previously shown to inhibit hexokinase, and has been postulated to prevent glycolysis (Thevelein and Hohmann 1995). In the experiment conducted by Thevelein and Hohmann (1995), the Δtps2 mutant was shown to accumulate Tre6P, which was considered responsible for the inhibition of hexokinase. Hohmann et al. (1996) proposed that inhibition of hexokinase by Tre6P may be a means of controlling sugar influx into glycolysis. In this study, we measured the residual sugar in the medium at the time of fermentation arrest in both Δtps1 and Δtps2 deletants (Table 3.3). ΔTps2 was shown to have much higher residual sugar than the parent and Δtps1. We can only speculate that the arrest of sugar catabolism is due to the accumulation of trehalose-6-phosphate in Δtps2

mutant, at levels inhibitory to hexokinase. The cellular amounts of glucose, G6P, Tre6P and trehalose remain to be determined to confirm this hypothesis.

3.4.3 Other cellular processes (autophagy, membrane invagination) of importance to fermentation

The processes of autophagy and membrane invagination are also indicated to be required for yeast to successfully complete fermentation. Figure 3.3 shows that genes annotated to these processes are over-represented (or enriched) within the 93 FEG dataset. Gene Ontology analysis of the original 336 genes identified in the micro-scale fermentation screen conducted in this laboratory (Walker et al. 2013) revealed that 11 genes were annotated in autophagy and membrane invagination. Eight of these 11 genes were further shown to have protracted fermentation when evaluated in 100 mL scale (*SLM4*, *ATG31*, *ATG7*, *VPS41*, *VAC8*, *SNX4*, *MEH1* and *VAM3*).

Autophagy and membrane invagination have been previously implicated as being important for growth under low temperature and high pressure, whereby growth sensitivity was observed in Δgtr1, Δgtr2, Δmeh1, and Δslm4 deletants under these conditions (Abe and Minegishi 2008). In this study, *GTR1* and *GTR2* do not appear to be required for high sugar fermentation, as the Δgtr1, and Δgtr2 deletants were able to complete fermentation at times similar to the parent (BY4743) or only 10% longer than the parent. The genes *VPS41*, *MEH1* and *VAM3* were shown to extend fermentation when deleted, with Δvps41, Δmeh1 and Δvam3 completing fermentation 71%, 65% and 78% more slowly than the parent, respectively. These genes are annotated to and required for vacuole function. *VPS41* encodes for a vacuolar membrane protein required for homotypic vacuole fusion (Seals et al. 2000). *MEH1* is a component of the EGO complex which is required for proper sorting of general amino acid permease, Gap1p; the loss of Meh1p has been shown to result in defective vacuolar acidification (Gao and Kaiser 2006), whilst *VAM3* is required for vacuole assembly (Sato et al. 1998).

3.5 Summary and conclusion

The laboratory scale (100 mL) fermentation screening of 181 gene deletants conducted as part of this PhD project, identified 93 gene deletants having protracted or unfinished fermentation. The role of these genes in relation to fermentation was highlighted by Gene Ontology (GO), whereby genes were classified based on their annotation to specific GO terms related to biological process, function and cellular location. GO terms relating to cellular homeostasis, vacuolar acidification, cellular pH reduction, chemical/ion/cation transport, regulation of transcription, autophagy and membrane invagination were identified as shown in Figure 3.3. Furthermore, nine genes within the 93 FEG dataset were observed to lead to incomplete or 'stuck' fermentation when deleted.

It is evident that there are a myriad of cellular processes and mechanisms, represented by genes within the FEG dataset, which are involved in the adaptive response to the four main stresses during fermentation; namely hyperosmolarity (high concentration of sugars), high acidity, toxicity from increasing ethanol content and nutrient depletion. Investigation of some of these genes and related cellular mechanisms was the main subject of this project.

The identification of such genes and processes is useful in extending our knowledge on the cellular processes behind yeast's adaptation to harsh conditions such as those encountered in an anaerobic wine fermentation. Such information is valuable in terms yeast strain optimisation programs, whether using genetic engineering (gene modification) or non-recombinant means and traditional approaches. The ability to predetermine the outcome of specific selection pressures during adaptive evolution strategies will reduce the need for screening for desirable phenotypes in what is usually a random approach for producing new yeast strains. Furthermore, recombinant technology, when finally accepted by the wine industry, will provide the best approach of generating industrial yeast with tailored fermentation phenotypes.

CHAPTER 4 APPROPRIATE VACUOLAR ACIDIFICATION IN *SACCHAROMYCES CEREVISIAE* IS REQUIRED FOR EFFICIENT HIGH SUGAR FERMENTATION

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Author contributions: TDN developed and conducted all experiments, analysed data, and drafted/constructed the manuscript. MEW, JMG and VJ supervised all research, contributed ideas and design and editing of the manuscript.

The following authors agree that the statement of the contribution of jointly authored paper accurately describes their contribution to research manuscript and give their consent to their inclusion in this thesis.

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Journal of Biological Chemistry, (in print)

CHAPTER 5 CONSTRUCTION OF AN OVER-EXPRESSION LIBRARY IN A HAPLOID WINE YEAST

5.1 Introduction

Gene functionality has been extensively investigated in whole genome studies using yeast collections of specific gene deletions in laboratory yeast (Auesukaree et al. 2009, Brett et al. 2011, Erasmus et al. 2003, Teixeira et al. 2009, van Voorst et al. 2006, Walker et al. 2013, Winzeler et al. 1999). Alternatively, over-expression studies have been conducted using libraries whereby individual genes (Gelperin et al. 2005) or genomic fragments containing several genes (Jones et al. 2008), are cloned into either episomal (multiple copy, 2 micron) or centromeric (single copy) plasmid vectors. Gene expression is either under the control of a heterologous promoter such as *GAL1* (Gelperin et al. 2005) or under the control of the endogenous promoter (Ho et al. 2009). Whilst these libraries are commercially available in a laboratory yeast background, there is no equivalent construction in wine yeast that is readily available. Furthermore, over-expression libraries have not typically been used for investigations related to industrial (alcoholic) fermentation.

In this study, the use of the homozygous laboratory yeast deletion library to identify genes required for the successful completion of high sugar fermentation (Chapter 3) was extremely valuable, albeit it has been performed in a wine yeast background. This work was however expanded to include over-expression studies using a commercially available library suitable for fermentation research. The yeast genomic tiling collection constructed by the Prelich laboratory (Jones et al. 2008) was considered suitable as gene expression was dependent on the endogenous promoter and not an inducible promoter such as that of *GAL1*, which is glucose repressed (Gelperin et al. 2005). The yeast collection includes ~1500 individual plasmids; each with a genomic fragment (encompassing 3-5 genes), totalling ~97% of the yeast genome as overlapping clones. The library, in the episomal *E. coli*-yeast shuttle vector, pGP564, was sourced from Millenium Science, USA as glycerol stocks of the transformed bacteria. Plasmid pGP564 has a yeast *LEU2* selectable marker (Appendix Figure 4.1), which is able to complement *leu2* auxotrophy in the host

yeast strain. Wine yeast is typically diploid and prototrophic. It can be aneuploid and thus not suitable for plasmid borne overexpression studies using this plasmid system. An isogenic derivative of the haploid wine strain C9 (Walker et al. 2003) was constructed in the laboratory by Dr Jennie Gardner. This strain was used in the present study as the background to construct a △leu2 auxotrophic mutant, for use as the host strain to construct the wine yeast overexpression library. This chapter describes the yeast strain construction, and high-throughput transformation of the library as individual plasmids.

The application of the *KanMX* cassette, which contains the known *kan* open reading frame from *E. coli* transposon *Tn*903 cloned between the *TEF* 5' and 3' regulatory sequences from the filamentous fungus *Ashbya gossypii (*Wach et al. 1994), has been very useful in the disruption of genes in wine yeast (Walker et al. 2003). The Kan^R gene confers resistance to geneticin (G418) in yeast, and as such is a dominant selectable marker, suitable for selection of transformants in prototrophic yeast, such as wine strains. Gene disruption with this antibiotic resistance marker, unlike an auxotrophic marker, has little impact on the growth and fermentation profile of transformants (Baganz et al. 1997).

The use of the over-expression library in a wine yeast background allows a comprehensive screening under various fermentative conditions, i.e. high sugars (> 200 g L^{-1}) and variable amounts of nitrogen. Laboratory yeast strains are not ideal for this type of fermentation study. They often possess multiple auxotrophic requirements making investigations under low nitrogen conditions difficult because of growth problems. Also, laboratory strains generally having a reduced fermentation capability in industry-like conditions. Therefore, a haploid wine yeast strain was chosen as the background for this study. The isogenic strain ISOC9d was constructed from C9, a haploid derivative of wine strain Lalvin L2056 (Walker et al. 2003). The fermentation growth and metabolite profile of this strain is similar to L2056 and C9 (Walker, *pers. comm.*). A leucine auxotroph of ISOC9d (L-2056 *ho∆ MATα*) was constructed as the background strain for the overexpression library. Transformation and plasmid maintenance within the yeast was dependent on the complementation of

△leu2 auxotrophy by the plasmid-borne *LEU2* gene. As the *leu2::KanMX* disruption cassette was not available as a deletion clone, it was constructed as part of the study, and used to generate the leucine auxotrophic mutant, ISOC9d△leu2 (L-2056 *hoΔ MATα, △leu2::KanMX*) which was selected for resistance to geneticin (G418) and its inability to grow on minimal drop-out medium without leucine.

5.2 Materials and Methods

5.2.1 Yeast strain

The haploid wine yeast strain used for over-expression construction is ISOC9d (L-2056 *hoΔ MATα*). Culture conditions are described in Section 2.1.

5.2.2 PCR amplification of 5' and 3' UTR sequences of LEU2 *and cloning into plasmid pGEMT*

The 3'UTR (228 bp) and 5'UTR (250 bp) of the *LEU2* gene were amplified separately using genomic DNA isolated from ISOC9d as the template DNA. The 228 bp 3' UTR fragment was amplified using primers 3'UTRleu2F and 3'UTRleu2R, and the 250 bp 5' UTR fragment with 5'UTRleu2F and 5'UTRleu2R. The primers listed in Table 5.1 were designed according to the *Saccharomyces cerevisiae Genome Database* (SGD) http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=Leu2). PCR reactions were performed in 1x Dynazyme EXT buffer with 0.5 mM dNTPs, 0.5 mM $MgCl_2$ and 1 U of DNAzyme EXT polymerase (Finnzymes). Cycling conditions were as follows: 32 cycles of 94^{o}C, 30 s; 58^{o}C, 30 s; 72^{o}C, 30 s and 72^{o}C, 5 min. The A-tailed PCR products were gel purified before cloning into pGEM®T easy vector (Promega) using TA cloning (Promega, Cat # A1360) as per the manufacturer's instructions. The plasmids used in this study are described in Table 5.2.

The standard ligation reaction consisted of 5 µl of 2x ligation buffer, 1 µl of pGEM®-T easy vector (50 ng), 3 µl of 3'UTR (or 5'UTR) and 1 µl of T4 DNA ligase in a 10 µl reaction volume. The ligation was incubated overnight at 4°C. The ligated DNA was transformed into *E. coli* DH5α and transformants selected on LB agar plates

containing 50 mg L^{-1} of ampicillin, 100 µl of 100 mM IPTG and 50 µL of X-Gal (20 mg mL^{-1}) after growing overnight at 37°C and 1 day at 4°C. The individual 3'UTR (228 bp) and 5'UTR (250 bp) sequences were verified by DNA sequence analysis in the intermediate plasmids pGEM-T3'UTR and pGEM-T5'UTR.

Table 5.1 Primers used in this study

Name	Sequence (5'-3')*	References
5'UTRleu2F[1,2]	CCGAGCGGTCTAGAGCGCCTGATTCAAG	This study
5'UTRleu2R[1]	CCGGATCCGGCGCTCTCACCTTTCCTT	This study
3'UTRleu2F[1]	CGGAATTCTAATGGCTCAACGTGATAAGGAA	This study
3'UTRleu2R[1,2]	CTTTCTCGAGTATGGGAAATGGTTCAAGAAGG	This study
KanMX3rev[2]	CATCCATGTTGGAATTTAATCGCGGCCTCG	Gardner et al. 2005
Leu2interF[2]	GTGCTGTGGGTGGTCCTAAA	This study
Leu2interR[2]	TTGCACTTCTGGAACGGTGT	This study
KanR_167bp_F[2]	CAGTCGTCACTCATGGTGATTTC	This study
KanR_167bp_R[2]	GCCGTTTCTGTAATGAAGGAG	This study
KanR_ORF_F[2]	ATGAGCCATATTCAACGGGAAACG	This study
KanR_ORF_R[2]	TTAGAAAAACTCATCGAGCATC	This study

[1] Primers for PCR; [2] Primers for sequencing; * bases (underlined font) are restriction sites;
[a] *http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html*

Table 5.2 Plasmids used in this study

Plasmid name	Selection media	Source
pBS418	LB kanamycin*	Jeff Eglinton, AWRI
pGEM-T easy	LB kanamycin*	Promega
pGEM-T-3'UTR	LB kanamycin*	This study
pGEM-T-5'UTR	LB kanamycin*	This study
pBS418-LEU2UTRs	LB kanamycin*	This study
pGP564	LB kanamycin*	Millenium Science
	Minimal media without leucine**	

*used for *E. coli*; ** used for *S. cerevisiae*

5.2.3 Cloning 5' and 3' UTR sequences of **LEU2** *into plasmid pBS418*

The cloned *LEU2* UTR sequences in pGEM-T easy contain unique restriction sites engineered into the sequence during amplification, for the purpose of cloning into the plasmid pBS418 (Walker et al. 2005). The plasmids pGEM-T5'UTR and pGEM-T3'UTR were digested with *Bam*HI, *Xba*I and *Eco*RI, *Xho*I, respectively, and the 3' and 5' UTR DNA fragments gel isolated and purified using a PCR gel purification kit (Promega, Cat # A9282).

The recombinant plasmid (pBS418-LEU2UTRs) was constructed by sequential cloning of 3'UTR and 5'UTR fragments into the multiple cloning site of pBS418 using standard molecular techniques (Sambrook & Russell 2001, Walker et al. 2005). Figure 5.1 describes briefly the steps of plasmid construction. The purified plasmid (~4.84 kb) isolated from *E. coli* transformants was characterised by restriction digestion with *Xba*I and *Xho*I. The plasmid was then transformed in to wine yeast ISOC9d to construct the ISOC9dΔleu2 strains as described in Section 2.7.6. Geneticin (G418) was added in YEPD at concentration of 200 µg mL^{-1} to select transformants.

5.2.4 Preparation of over-expression plasmids from Yeast Genomic Tiling Collection on pGP564

Individual *E. coli* clones, each containing the pGP564 plasmid with a unique region of *S. cerevisiae* genomic DNA, were inoculated into 5 mL of LB medium (low salt) with the addition of kanamycin (final concentration of 50 µg mL^{-1}) and grown at 37°C overnight, shaking at 250 rpm. The plasmid preparation was performed using Wizard® Plus SV Minipreps DNA Purification System (Promega, Cat # A1125), as per the manufacturer's instructions. The purified plasmids were then pipetted into new 96 well microtitre plates in the same order as the corresponding *E. coli* cultures (sourced from Thermo Scientific), and kept at -20°C for transformation.

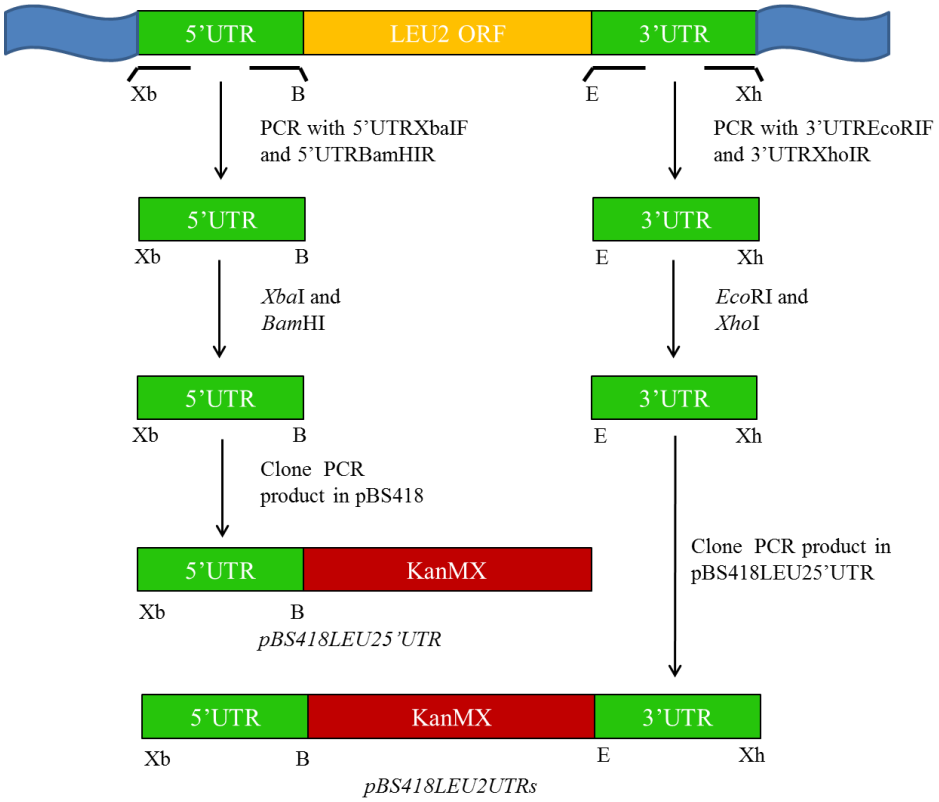


Figure 5.1 Construction of *LEU2::KanMX* cassettes in plasmid pBS418-LEU2UTRs. The *LEU2* ORF is shown in yellow and the corresponding 5' and 3' UTRs in green. The UTRs were amplified using primers as indicated in solid black lines. Letters indicate the engineered restriction sites within the DNA sequence: Xb (*Xba*I), B (*Bam*HI), E (*Eco*RI) and Xh (*Xho*I).

5.2.5 Transformation of Yeast Genomic Tiling Collection on pGP564 in wine yeast ISOC9dΔLeu2 (over-expression library construction)

A 96-well plate yeast transformation method was tested using the ISOC9dΔleu2 yeast strain developed in this laboratory. Each transformation used ~50 ng of plasmid DNA per well. The method is described on the "Prelich Lab Yeast Systematic Over-expression Library Methods" website (http://steelhead.aecom.yu.edu/SystematicLibrary.html#Library_transformation_and_screening.). In brief, a TRAFO mix (Table 5.3) was initially prepared and pipetted into each well of a 96 well plate using an 8-channel pipette. Purified plasmids (~50 ng of plasmid DNA per well) from source plates were pipetted into columns 1 to 11 of the transformation plates whilst column 12 was used for the negative control. Plates were shaken at 200 rpm for 2 minutes and incubated at 42°C for 1 hour. Cells were pelleted and resuspended in 14 µL of sterile water. Then 7 µL of resuspended cells was spotted using an 8-channel pipette onto minimal drop-out medium without leucine to select transformants. Following growth, the transformants were transferred into 100 µL of sterile water. The cells were dispersed in the water by gentle shaking for 2 minutes and pinned to selective plates in duplicate using an 8-channel pipette.

Table 5.3 TRAFO mix recipe used for over-expression library construction

Number of plates	1 (mL)	2 (mL)	4 (mL)	9 (mL)	Per well (µL)
50% PEG 3350	10	20	40	90	100
1M LiAc	1.5	3	6	13.5	15
H_2O	0.5	1	2	4.5	5
2 mg mL^{-1} ssDNA*	2	4	8	18	20
Competent cells (ISOC9dΔleu2)	1	2	4	9	10
Total	**15**	**30**	**60**	**135**	**150**

* ssDNA is a 2 mg mL^{-1} stock of salmon sperm carrier DNA (Sigma D1626), which was boiled for 10 minutes and then put on ice for 5 minutes.

5.3 Results

5.3.1 Construction of leu2::KanMX *disruption cassette*

A *leu2::KanMX* disruption cassette was constructed in this study. Plasmid pBS418, a pBluescriptSK (Stratagene) vector (Walker et al. 2005) was modified in a two-step process as shown in Figure 5.1. Short sequences (~220-250 bp) of the untranslated regions (UTR) of the *LEU2* gene were amplified from ISOC9d genomic DNA and inserted either side of the *KanMX* module. The individual amplified sequences were initially cloned in pGEMT-easy vector (Promega), and verified by DNA sequence analysis. The insertion of the individual 5' and 3' PCR fragments into pBS418 was confirmed by DNA sequencing and restriction digestion as shown in Figure 5.2. The recombinant plasmid pBS418LEU2UTRs was shown to have the correct insertion of the *LEU2* UTR sequences adjacent to the *KanMX* module, as represented by the increase in size of the ~1.4 kb *KanMX Xba*I-*Xho*I fragment (as in pBS418) to ~1.9 kb to include the two short *LEU2* fragments.

5.3.2 Generation of a leucine auxotroph of the haploid wine yeast (ISOC9dΔLeu2)

Transformation was conducted using the lithium acetate method (Gietz and Schiestl 2007). The wine strain ISOC9d was transformed with 1926 bp of *KanMX* flanked 5'UTR and 3'UTR fragment isolated from pBS481-LEU2UTRs (Figure 5.3). Geneticin resistant transformants were isolated on YPD containing 200 µg mL^{-1} G418. Three transformants were isolated and confirmed by their inability to grow on minimal drop-out medium in the absence of leucine (Figure 5.4).

The transformants of ISOC9dΔLeu2 were analysed by PCR using primers of 5'UTRleu2F and KanMX3rev (Table 5.1). KanMX3rev is internal to the *KanMX* module, whilst 5'UTRleu2F is complementary to the 5'UTR. The PCR was conducted as described in the Materials and Methods. PCR analysis using genomic DNA isolated from the yeast transformants and the parent strain ISOC9d as shown in Figure 5.5 confirmed the replacement of the endogenous *LEU2* gene with the *⊿leu2::KanMX* cassette. The absence of the *KanMX* sequence in the parent strain was demonstrated by the lack of the 644 bp leu2-KanMX PCR fragment. The

amplified DNA was excised from the gel, purified and sequenced. Comparison of the sequenced DNA to the reference *KanMX* (788 bases) and *LEU2* gene (207 bases) using BLAST software (http://lastweb.cbrc.jp/) verified the sequence as correct (data not shown).

Three of ISOC9dΔleu2 isolates were evaluated for fermentation performance in CDGJM and were able to complete fermentation in leucine supplemented media at a similar time as the parental strain ISOC9d (unsupplemented CDGJM) (Forester J., *pers. comm*). ISOC9dΔleu2 transformant I was chosen as the genetic background for the Yeast Genomic Tiling Collection in plasmid pGP564 (Jones et al. 2008).

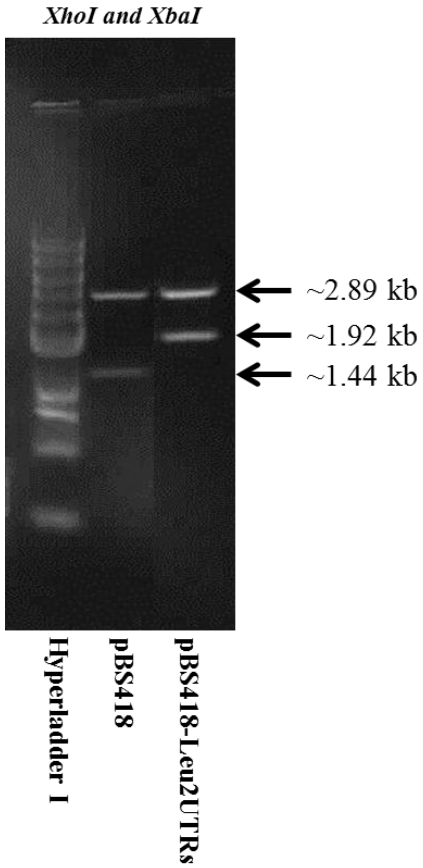


Figure 5.2 Gel image of plasmids pBS418 and pBS418-LEU2UTRs digested with restriction endonucleases *Xho*I and *Xba*I.

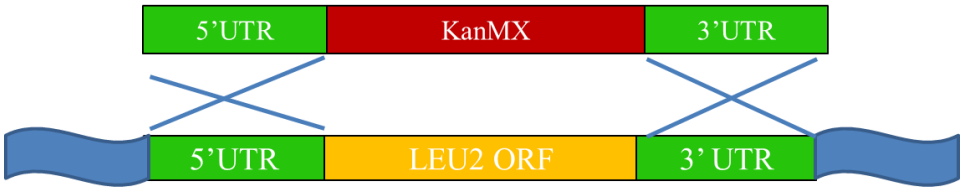


Figure 5.3 Schematic diagram of the disruption of the native *LEU2* gene in wine yeast. The *LEU2* gene in ISOC9d was replaced with the *KanMX* resistance marker, flanked by direct repeats of 5'UTR and 3'UTR of the *LEU2* gene.

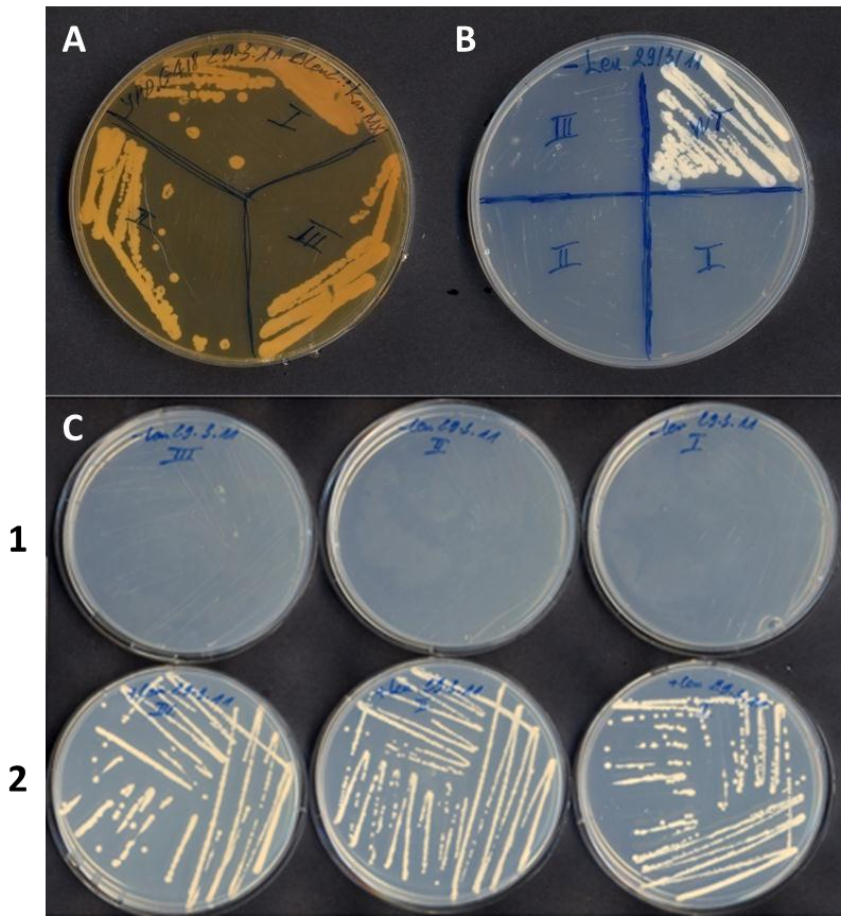


Figure 5.4 Growth of 3 transformants (I, II and III) of the putative *leu2::KanMX* strains (ISOC9dΔleu2) on YPD containing 200 µg mL^{-1} of geneticin or G418 (A) and minimal drop-out medium without leucine (B). Auxotrophic requirement of leucine for growth is demonstrated by the inability to grow on minimal drop-out media without leucine (C-1) and growth when supplemented with leucine (C-2).

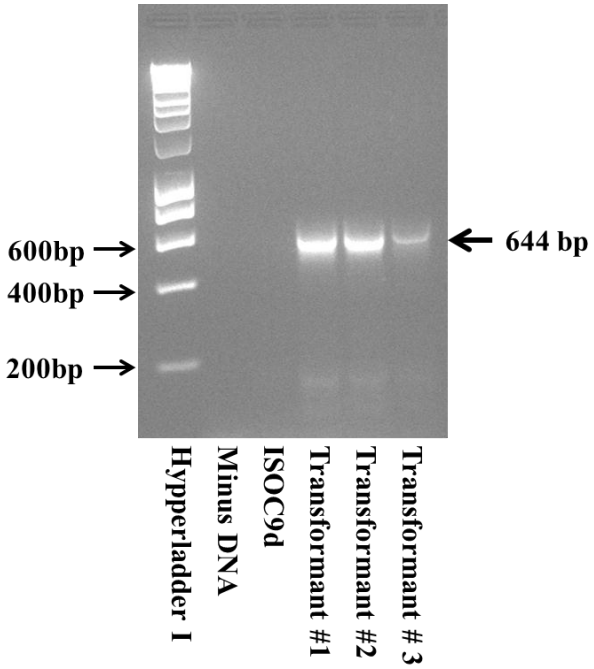


Figure 5.5 PCR determination of the presence of *KanMX* flanked with UTRs of *LEU2* gene in ISOC9dΔleu2 (I, II and III) strains. Primers 5'UTRleu2F and KanMX3rev were used for amplification.

5.3.3 Construction of an over-expression library by transformation of Yeast Genomic Tiling Collection in plasmid pGP564 in ISOC9dΔleu2

The Yeast Genomic Tiling Collection (Jones et al. 2008, Millennium Science, USA) is comprised of ~1500 *E. coli* clones, each containing the pGP564 plasmid with a unique region of *S. cerevisiae* genomic DNA. Plasmid purification of the individual clones was performed with the cooperation of other two PhD students (Mrs Jade Haggerty and Ms Jin Zhang), who's research included the use of this library to identify genes associated with yeast aroma compounds and nitrogen utilisation, respectively.

Each culture was inoculated into 5 mL of LB medium (low salt; 5 mg L^{-1} of NaCl) with the addition of kanamycin (final concentration of 50 mg mL^{-1} for plasmid selection) and grown at 37°C overnight with shaking at 250 rpm. Plasmid preparation was performed using Wizard® Plus SV Minipreps DNA Purification System (Promega), as per manufacturer's instructions. The purified plasmids were pipetted into new 96 well plates with the same order as the corresponding source *E. coli* cultures, and stored at -20°C prior to transformation.

A 96-well plate yeast transformation method was tested using the ISOC9dΔleu2 yeast strain. Each transformation used ~50 ng of plasmid DNA per well. The transformation method is described on the "Prelich Lab Yeast Systematic Over-expression Library Methods" website, with some modifications (refer to Materials and Methods). A control was included, namely the parent strain ISOC9dΔleu2 transformed with the 'backbone' plasmid pGP654.

5.3.4 Initial trial of screening of the over-expression library

Two random strains (clone YGPM6c02 and YGPM14p14 from the over-expression library; Table 5.4) and the parent strain (ISOC9dΔleu2 + pGP564) were used to test for retention of the individual plasmids during fermentation. Three different starter media were used as test media for propagation of the clones from glycerol stocks, namely, minimal drop-out without leucine (Min DO-Leu), CDGJM without leucine

(CDGJM-Leu) and 50% of Min DO-Leu and 50% CDGJM-Leu. Plasmid retention (as a percentage) was based on the number of cells counted (colony forming units or cfu) after replica plating (at a density of ~200 cells per plate) on YEPD and minimal DO-Leu agar. Plasmid retention in cells inoculated in CDGJM starter medium without leucine was observed not to be significantly different from cells grown in minimal DO-Leu (data not shown). The retention of plasmid was confirmed by PCR using primers Kan_167bp_F and Kan_167bp_R, which was used to amplify 167 bp of the Kanamycin resistance gene, *KAN*^{r}, within the plasmid sequence (data not shown).

Plasmid retention during extended fermentation was also tested in various media. Three media without leucine were used to conduct fermentation including minimal DO-Leu, CDGJM-leu and a combined medium (with 50% of minimal DO-Leu medium and 50% of CDGJM-leu, designated 50-50). The plasmid retention of the cells was measured by comparing the number of colonies formed after 2-3 days growth on minimal DO-Leu from appropriately diluted cultures (~200 cells per plate) to the number of colonies on YEPD at the end of fermentation (Figure 5.6). The comparison between two over-expression (OE) mutants and the parent strain demonstrated the dependency of plasmid maintenance on the cloned gene. However, the choice of media did not appear to significantly affect (using t-test data analysis) the final outcome with regards to the number of cells on both YPD and minimal DO medium, nor the volume used to plate out the cells (data not shown). These results suggest that plasmid stability may affect the final outcome of the fermentation, in terms of duration although this has not been extensively tested.

Table 5.4 Description of two strains using for initial trial of over-expression library screening

Clone: YGPM14p14	**Position:G9**	**CHr:VI**	**Size of insert: 9875**
Genes present	**SGD explanation**		**Overexpression**
[*AUA1*]&	Protein required for the negative regulation by ammonia of Gap1p, which is a general amino acid permease		vegetative growth: arrested
WWM1	WW domain containing protein of unknown function; binds to Mca1p, a caspase-related protease that regulates H2O2-induced apoptosis; overexpression causes G1 phase growth arrest and clonal death that is suppressed by overexpression of MCA1		vegetative growth: decreased cell cycle progression: abnormal
CDC4	F-box protein required for G1/S and G2/M transition, associates with Skp1p and Cdc53p to form a complex, SCFCdc4, which acts as ubiquitin-protein ligase directing ubiquitination of the phosphorylated CDK inhibitor Sic1p		N/A
SMC1	Subunit of the multiprotein cohesin complex, essential protein involved in chromosome segregation and in double-strand DNA break repair; SMC chromosomal ATPase family member, binds DNA with a preference for DNA with secondary structure		N/A
[*BLM3*]*	Ubiquitin-specific protease involved in transport and osmotic response; interacts with Bre5p to co-regulate anterograde and retrograde transport between the ER and Golgi; involved in transcription elongation in response to osmostress through phosphorylation at Ser695 by Hog1p; inhibitor of gene silencing; cleaves ubiquitin fusions but not polyubiquitin; also has mRNA binding activity		vegetative growth: decreased

Clone: YGPM6c02	**Position:E6**	**CHr:IV**	**Size of insert: 11912**
Genes present	**SGD explanation**		**Overexpression**
[*YDR169C-A*]	Putative protein of unknown function; identified by fungal homology and RT-PCR		N/A
SEC7	Guanine nucleotide exchange factor (GEF) for ADP ribosylation factors involved in proliferation of the Golgi, intra-Golgi transport and ER-to-Golgi transport; found in the cytoplasm and on Golgi-associated coated vesicles		resistance to Brefeldin A: increased
tQ(UUG)D3	Glutamine tRNA (tRNA-Gln), predicted by tRNAscan-SE analysis; thiolation of uridine at wobble position (34) requires Ncs6p		N/A
YDR170W-A	Retrotransposon TYA Gag gene; Gag is a nucleocapsid protein that is the structural constituent of virus-like particles (VLPs); similar to retroviral Gag; YDR170W-A is part of a mutant retrotransposon		N/A

Notation for the genes on the plasmids:

[ ] indicate that the ORF is intact, but may be missing necessary upstream or downstream sequences.

[ ]* indicates that the 3’ end of the gene is missing.

[ ]& indicate that the 5’ end of the gene is missing.

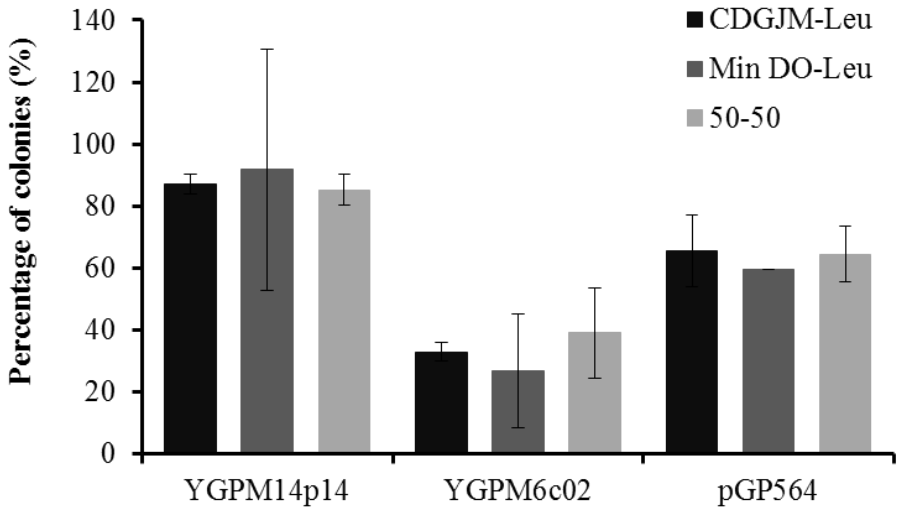


Figure 5.6 Plasmid retention of two randomly selected strains (YGPM14p14 and YGPM6c02) and the parent ISOC9dΔleu2 + pGP564 after growing in Chemically Defined Grape Juice Medium without leucine (CDGJM-leu), Minimal drop-out without leucine (Min DO-Leu) and 50-50 (50% CDGJM-leu and 50% Min DO-Leu). The fermentations were conducted as described in Section 2.5.1. Cells were harvested at the end of fermentation (no residual sugar), counted using a haemocytometer and plated at a density of 200 cells on YEPD and Min DO-leu agar. Plasmid retention is shown as a percentage of colonies on Min DO-leu compared to YEPD agar. Each sample was conducted in duplicate. Error bars represent the standard deviation.

5.4 Discussion and Conclusion

The *LEU2* selection marker for the propagation of the pGP564 based over-expression library in yeast required a recipient strain that had an auxotrophic growth requirement for leucine. We successfully constructed a haploid deletion strain (ISOC9dΔleu2) from haploid strain (ISOC9d) which was indirectly generated from commercial strain L2056 (Jiranek et al. 2013). The ISOC9dΔleu2 strain was unable to grow on minimal medium lacking leucine and has been used in the construction of an over-expression library where the *LEU2* marker is required. The successful generation of haploid derivatives of wine yeast strains that are amenable to classical and recombinant genetic manipulation have helped to overcome the limitation of using laboratory strains to study yeast physiology during oenology fermentation (Walker et al. 2003). The disruption of the *LEU2* gene in ISOC9d using a *LEU2::KanMX* cassette was based on a previous described system (Wach et al. 1994). ISOC9d is an isogenic derivative of C9 (Walker et al. 2003), in which the deletion cassette used for disruption of *HO* and removal during meiotic recombination allowed the generation of a *⊿ho* mutant. *HO* functionality was restored using a plasmid based *HO* gene, to allow for switching of mating type. Subsequent curing of the plasmid and sporulation resulted in the generation of stable homozygous (isogenic) haploids, one of which was ISOC9d. The deletion cassette used in this study did not allow for removal of the *KanMX* module following integration into the chromosome. In this case, the flanking sequences of *LEU2* (5'UTR and 3'UTR) which were amplified from genomic DNA as template originating from parental strain (ISOC9d) allowed only the correct targeting of the *leu2::KanMX* deletion cassette to the *LEU2* locus in the chromosome.

The over-expression library constructed from the Yeast Genomic Tilling Collection containing of over ~1500 unique clones of the yeast *S. cerevisiae* genome segments will be useful in identifying genes that are required for a number of fermentation traits including yeast robustness. The screening of the entire library is of course important to determine which genes have a role in the completion of high sugar fermentation. However, the evaluation of the over-expression strains within this project finished at this point due to time constraints. Further work in the project

instead focussed on a set of candidate genes identified from amongst the homozygous deletions as detailed in the previous chapters. This subset of yeast deletants, *TPS1* and *TPS2* are the subject of Chapter 6.

The plasmid pGP564 is based the 2-micron vector which is well known for its instability. Plasmid retention during extended fermentation was also tested in various media without leucine. The percentage of colonies of three random transformants showed that the plasmid retention could be different in transformants (Figure 5.6). So it would be ideal to evaluate the plasmid retention of individual over-expression mutants when screen their fermentation performance.

The plasmid pGP564 used in the over-expression library (Jones et al. 2008) was however used to construct over-expression strains for some of the genes identified in Chapter 4, so as to evaluate their fermentation performance (see Chapter 6). At the time of this study, the Yeast Tiling Collection was the only commercially available over-expression library (only available as bacterial clones) suitable for investigation of extended growth in industrial fermentations containing hexose sugars such as glucose and fructose. Since then, a centromeric based system has become available which affords greater plasmid stability within the yeast (Ho et al. 2009, Millennium Science, USA). Although developed for screening of bioactive compounds (drugs), it is yet to be tested in fermentation.

The wine yeast OE library based on the episomal plasmid pGP564, whilst not ideal because of limitations of plasmid stability, has been useful not only in this study (Chapter 6) but also the investigation of other genes identified in related projects being conducted in the Jiranek laboratory.

CHAPTER 6 THE LINK BETWEEN THE TREHALOSE BIOSYNTHESIS PATHWAY AND THE COMPLETION OF FERMENTATION

6.1 Introduction

Trehalose biosynthesis pathways are widely present in nature, occurring in eubacteria, archaea, plants, fungi and animals (Avonce et al. 2006). In the yeast, *Saccharomyces cerevisiae*, trehalose is synthesised through two steps. In the first, UDP-glucose and glucose-6-phosphate combine to form trehalose-6-phosphate (Tre6P) under the catalysis of trehalose-6-phosphate synthase (TPS) encoded by *TPS1*. Trehalose-6-phosphate is then dephosphorylated in a second step mediated by trehalose-6-phosphate phosphatase (TPP), encoded by *TPS2*, to form trehalose (Figure 6.1). The accumulation of trehalose can help cells resist stresses such as the presence of ethanol, temperature extremes and high osmolarity due to salt (Mahmud et al. 2010, 2009a, 2009b). Concomitant with trehalose synthesis, Tps1p is additionally involved in the regulation of sugar influx in glycolysis (Hohmann et al. 1996, Thevelein and Hohmann 1995). The deletion of *TPS1* resulted in the accumulation of glucose-6-phosphate, as also caused by the overexpression of hexokinase in yeast (Ernandes et al. 1998). Different models have been proposed to explain the involvement of Tps1p in the regulation of sugar influx given the product Tps1p, trehalose-6-phosphate (Tre6P), was shown to inhibit hexokinase (Van Vaeck et al. 2001). Deletion of *TPS2* resulted in accumulation of trehalose-6-phosphate, since lack of the enzyme abolishes conversion to trehalose (Devirgilio et al. 1993). From these studies, Tre6P is speculated to play an important role in controlling influx from various sugar metabolic pathways into glycolysis (Teusink et al. 1998).

In *Saccharomyces cerevisiae*, three glucose phosphorylating enzymes are responsible for the generation of glucose-6-phosphate (G6P) from glucose. Glucokinase encoded by *GLK1*, phosphorylates glucose in the first irreversible step in glucose metabolism. Hexokinase isoenzymes, Hxk1p and Hxk2p, also catalyse phosphorylation during glucose metabolism. They are encoded by *HXK1* and its paralog *HXK2*, respectively

(Walsh et al. 1983). Hxk2p is the predominant hexokinase during growth on glucose, since the nuclear located enzyme functions to repress *HXK1* and *GLK1* and to induce expression of its own gene. *HXK1* and *GLK1* expression is highest during growth on non-fermentable carbon sources (Herrero et al. 1995, Rodriguez et al. 2001). However, in Δtps2 deletants that lack trehalose-6-phosphate phosphatase, there is an accumulation of Tre6P and inhibition of hexokinase (Hohmann et al. 1996). This chapter evaluates the importance of trehalose biosynthesis in relation to glycolysis and a high sugar fermentation by genetic manipulation of key genes related to trehalose biosynthesis and sugar metabolism.

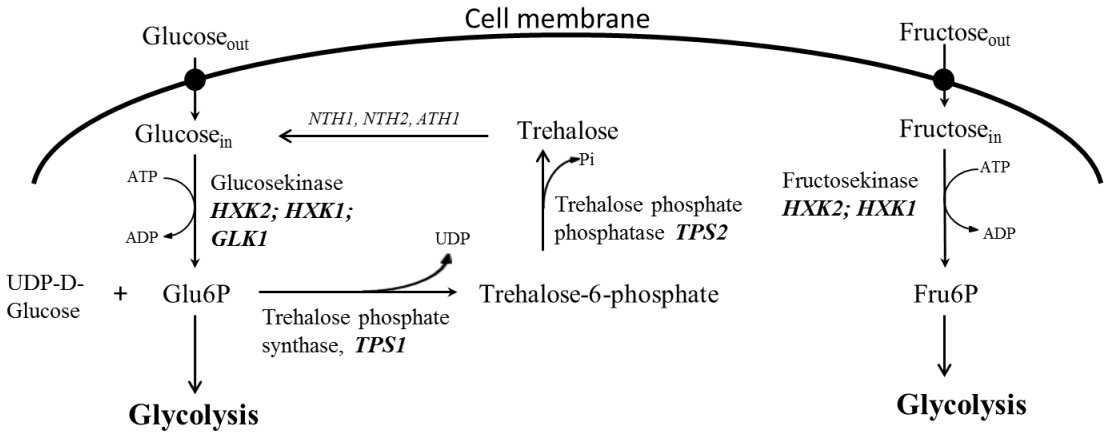


Figure 6.1 Pathway of trehalose biosynthesis. Trehalose is formed from in two steps: glucose-6-phosphate (Glu6P) is converted to trehalose-6-phosphate (Tre6P) and Tre6P is then dephosphorylated to trehalose. The trehalose pathway is catalysed by Tps1p and Tps2p enzymes encoded by *TPS1* and *TPS2*.

6.2 Materials and Methods

6.2.1 Yeast strains and plasmids

The yeast strains and plasmids used in this study are described in Table 6.1 and Table 6.3, respectively. Plasmid maps of pGP564 (Jones et al. 2008) and pYX212 (Van Vaeck et al. 2001) are described in Appendix Figure 3.1. Plasmid pYX212TreA was kindly provided by Prof. Johan Thevelein (Laboratory of Molecular Cell Biology, Katholieke University of Leuven, Belgium) whilst pGP564 was sourced from Millennium Science. The control plasmid pYX212 (Appendix 5, Figure 5.1) used in this study was generated by removal of the 1.67 kb TreA gene (Van Vaeck et al. 2001) following digestion with *BamH*I and *Xho*I (New England Biolabs), and removal of the 5' overhangs with Klenow DNA polymerase and subsequent ligation of the purified plasmid DNA fragment (Sambrook and Russell 2001). These *E. coli*-yeast shuttle plasmids have the following selectable markers for transformation and maintenance in yeast: leucine (pGP564) and uracil (pYX212).

6.2.2 Purification of HXK2 *gene from* Saccharomyces cerevisiae

A 1.99 kb PCR fragment encompassing a functional *HXK2* gene encoding hexokinase Hxk2p was synthesized. The DNA sequence included 386 bp 5' untranslated DNA sequence, 1461 bp coding sequence and 143 bp 3' untranslated DNA sequence. The gene was PCR amplified using the *HXK2* containing pGP564 plasmid YGPM24j02 (Genome Tiling library; Millennium Science) as a DNA template and the primers HXK2fw; 5'-GGGATCCATTCGTACGGTACTTTCTCTGTTT-3' (and HXK2rv; 5'-CCTCGAGTACGCAAGCTATCTAGAGGAAGTGT-3' (Table 6.2). The engineered *BamH*I and *Xho*I sites are underlined in the HXK2fw and HXK2rv primer sequences respectively. The PCR amplification was performed in 1x Velocity buffer with 0.5 mM dNTPs, 0.5 mM $MgCl_2$ and 1 U of Velocity polymerase (supplier??). Cycling conditions were as follows: 35 cycles of 98°C, 30 s; 56°C, 30 s; 72°C, 90 s and 72°C, 5 min.

The 1.99 kb PCR product containing the ORF and promoter region of *HXK2* gene was cloned into the *BamH*I and *Xho*I sites of pGP564 vector (Millennium Science) using T4 ligase enzyme (Cat # M0202T, New England Biolabs). The ligation was conducted at 4°C overnight as according to manufacturer's instructions. Previously prepared calcium chloride treated competent cells of *E. coli* DH5α (as described in Section 2.7.4) were transformed with the ligation mixture. DH5α transformants were selected on Luria-Bertani (LB) agar containing 50 µg mL^{-1} of Kanamycin. The resultant recombinant plasmid pGP564HXK2 was verified by restriction digestion and DNA sequence analysis, following plasmid DNA isolation from transformants grown in liquid culture (Sambrook and Russell 2001).

Table 6.1 Yeast strains used in this study

Strain	Genotype	Source
BY4743 diploid *MATa/α*	*MATa/α his3Δ1/his3Δ1 leu2Δ0 /leu2Δ0 lys2Δ0/LYS2*	SGD project
Δtps1 diploid *MATa/α*	BY4743*Δybr126c::KanMX4*	SGD project
Δtps2 diploid *MATa/α*	BY4743*Δydr074w::KanMX4*	SGD project
Δtps1(pGP564HXK2)	BY4743*Δybr126c::KanMX4* + pGP564HXK2	This study
Δtps1(pGP564)	BY4743*Δybr126c::KanMX4* + pGP564	This study
Δtps2(pGP564HXK2)	BY4743*Δydr074w::KanMX4* + pGP564HXK2	This study
Δtps2(pGP564)	BY4743*Δydr074w::KanMX4* + pGP564	This study
BY4743(pGP564HXK2)	BY4743 + pGP564HXK2	This study
BY4743(pGP564)	BY4743 + pGP564	This study
Δtps2(pGP564HXK2+TreA)	BY4743*Δydr074w::KanMX4* + pGP564HXK2 + pYX212TreA	This study
Δtps2(pGP564HXK2+pYX212)	BY4743*Δydr074w::KanMX4* + pGP564HXK2 + pYX212	This study
Δtps2(pGP564+TreA)	BY4743*Δydr074w::KanMX4* + pGP564 + PYX212TreA	This study
Δtps2(pGP564+pYX212)	BY4743*Δydr074w::KanMX4* + pGP564 + pYX212	This study
BY4743(pGP564HXK2+TreA)	BY4743 + pGP564HXK2 + pYX212TreA	This study
BY4743(pGP564HXK2+pYX212)	BY4743 + pGP564HXK2 + pYX212	This study
BY4743(pGP564+TreA)	BY4743 +pGP564 + pYX212TreA	This study
BY4743(pGP564+pYX212)	BY4743 +pGP564 + pYX212	This study

Table 6.2 Primers used in this study

Name	Sequence (5'-3')*	Source
HXK2-fw[1,2]	GGGATCCATTCGTACGGTACTTTCTCTGTTT	This study
HXK2-rv[1,2]	CCTCGAGTACGCAAGCTATCTAGAGGAAGTGT	This study
KanMX3 rev[2]	CATCCATGTTGGAATTTAATCGCGGCCTCG	Gardner et al. (2005)
HXK2_inter-F[2]	TACACTGACCCAGAAACTAA	(Kummel et al. 2010)
HXK2_inter-R[2]	ATATCGGAACAAACATCGTA	Kummel et al. (2010)
pGP564-F[2]	GCTATGACCATGATTACGCCAAGC	This study
pGP564-R[2]	CGACTCACTATAGGGCGAATTG	This study
Leu2_inter-F[2]	GTGCTGTGGGTGGTCCTAAA	This study
Leu2_inter-R[2]	TTGCACTTCTGGAACGGTGT	This study
TreAinF[2]	TTTGCGTATCTCAGACACGG	This study
PYXCR[2]	GGGATGTATCGGTCAGTCAT	This study
PYXCF[2]	GCGGTATTTCACACCGCATA	This study
URACR[2]	TGTGGTCTCTACAGGATCTGAC	This study

* underlined bases represent the restriction endonuclease sites. [1] Primers used for PCR amplification. [2] Primers used for PCR checking and sequencing.

Table 6.3 Plasmids used in this study

Plasmid	Selection Medium	Source
pGP564	minimal media without leucine	Millenium Science
pGP564HXK2	minimal media without leucine	This study
pYX212TreA	minimal media without uracil	Van Vaeck et al. (2001)
pYX212	minimal media without uracil	This study

6.2.3 Transformation of the over-expression plasmid pGP564HXK2 and pYX212TreA in Δtps1 and Δtps2 deletants

The lithium acetate transformation method was used to transform plasmids in these laboratory yeast strains (as described in Section 2.7.5). A minimal drop-out medium without leucine was used to select yeast transformants harbouring pGP564 or pGP564HXK2. Transformants harbouring pYX212 or pYX212TreA were selected on minimal drop-out medium without uracil. Plasmids pGP564HXK2 and pYX212TreA were also co-transformed, and transformants selected on minimal drop-out medium lacking leucine and uracil. The various yeast transformants are described in Table 6.1.

6.2.4 Confirmation of transformed yeast strains

Yeast transformants were purified as single colonies, then each clone was inoculated into 25 mL of minimal drop-out without leucine and/or uracil medium and incubated for 12-16 hours at 28°C. Genomic DNA (including plasmid DNA) was extracted according to Adams et al. (1998). A PCR procedure was performed to verify the presence of plasmids by using primers listed in Table 6.2. PCR was performed in 1x MangoTaq buffer with 0.5 mM dNTPs, 1.5 mM $MgCl_2$ and 1 U of MangoTaq DNA polymerase (Cat no: BIO-21083, Bioline). Cycling conditions were as follows: 32 cycles of 94°C, 30 s; 58°C, 30 s; 72°C, 45 s and 72°C, 5 min.

6.2.5 Laboratory-scale fermentation and monitoring of key metabolites

Fermentations were conducted in triplicate (100 mL) in Chemical Defined Grape Juice Medium (CDGJM) containing 200 g L^{-1} of glucose and fructose, 450 g L^{-1} of FAN and sufficient nutrients. Plasmids were retained in the yeast transformants through the exclusion of leucine and/or uracil from the medium. The fermentation profiles were monitored as described in Section 3.2.2. Sugar analysis is detailed in Section 2.5.2.

6.3 Results

6.3.1 Fermentation profiles of TPS1 *and* TPS2 *gene deletants*

The screening of a subset of yeast deletants from the homozygous diploid deletion library in laboratory-scale fermentation (described in Chapter 3) identified nine deletants unable to finish fermentation, and which are known as 'stuck' mutants (Walker et al. 2013). Of these 'stuck' mutants, Δtps1 exhibited ~98% of the parent biomass (as estimated by optical density; OD_{600}) whilst Δtps2 was only ~72%. The fermentation profile and growth of the Δtps1 and Δtps2 mutants are reported in Figure 6.2. The residual glucose and fructose in the fermentation medium at the time of fermentation arrest in these mutants was determined (Figure 6.3). These results indicate that trehalose metabolism may play an important role in stress resistance and the ability of yeast cells to finish alcoholic fermentation. These results were consistent with previous findings that the deletion of *TPS2* caused accumulation of trehalose-6-phosphate (Tre6P) (Devirgilio et al. 1993), which inhibits hexokinase activity and consequently affects glycolysis (Hohmann et al. 1996). The residual sugar content in terminal samples from Δtps2 fermentations was significantly higher than that of the parent (Figure 6.3).

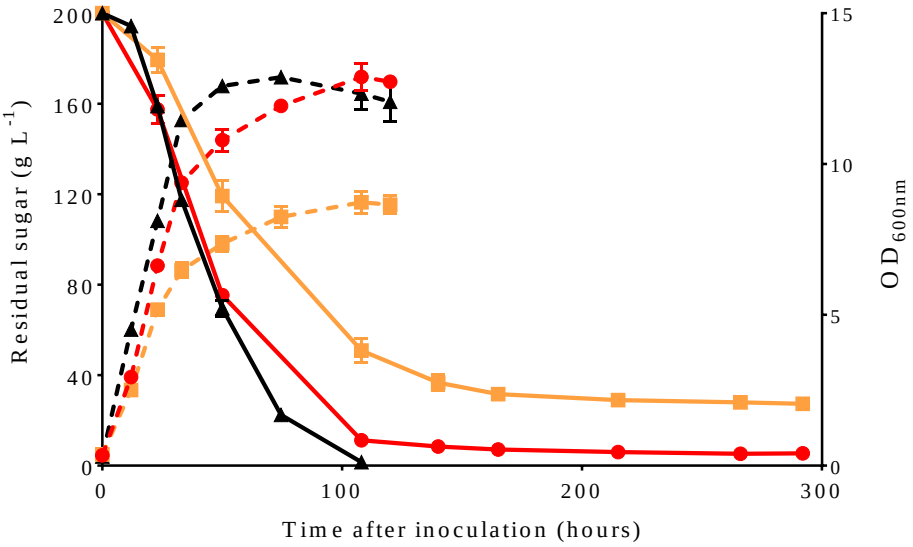


Figure 6.2 Fermentation profiles of Δtps1 (●), Δtps2 (■) and the parent (▲) in 100 mL of CDGJM containing 200 g L^{-1} sugar. Samples were regularly taken for growth (optical density; broken lines) and residual sugar (solid lines) analysis. Triplicate fermentations were conducted at 28°C with shaking at 120 rpm. Values represent the mean and include the standard deviation (error bars).

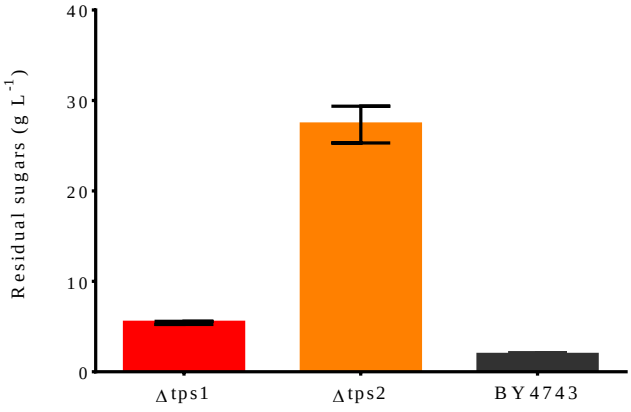


Figure 6.3 Residual sugars (g L^{-1}) of Δtps1 (■), Δtps2 (■) at the time of fermentation arrest as compared with the parent (■) at the end of fermentation. Standard deviation of the mean of triplicate fermentations is included (error bars).

*6.3.2 Determination of fermentation profiles of Δtps1 and Δtps2 with over-expression of hexokinase (***HXK2***)**

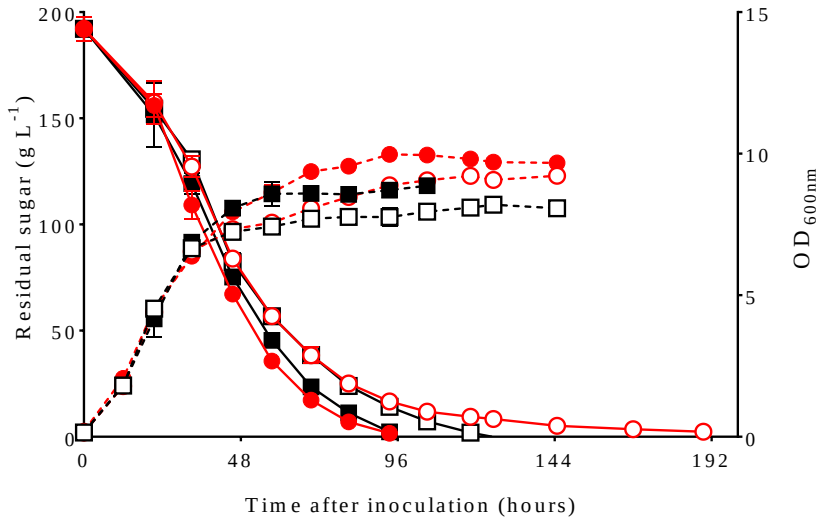
Glycolysis during alcoholic fermentation is regulated by hexokinase (Hxk2p) through its influence on the expression of *HXK1* and *GLK1* encoding hexokinase 1 (Hxk1p) and glucokinase (Glk1p), which are involved in the first irreversible step in the intracellular metabolism of glucose (Rodriguez et al. 2001). Hexokinase 2 is the predominant hexokinase during growth on glucose and is responsible for fermentable carbon utilisation (glucose and fructose) during alcoholic fermentation in yeast (Herrero et al. 1995). The influx of sugars into the glycolytic pathway is restricted by one of the intermediates of the trehalose biosynthetic pathway, trehalose-6-phosphate, which inhibits hexokinase (Hohmann et al. 1996). To investigate the regulatory role of the trehalose biosynthetic pathway on hexokinase activity, we over-expressed *HXK2* encoding for the hexokinase isomer 2. The over-expression system was based on an episomal *LEU2* plasmid, pGP564 (as described in Chapter 5), in which the complete *HXK2* gene including the 5' and 3' untranslated sequences were cloned.

The transformation of plasmids into the parent strain and Δtps1 and Δtps2 mutants was confirmed by PCR (Appendix 5, Figure 5.2) and sequencing (data not shown). The empty plasmid pGP564 was transformed into the above strains to ensure that all strains were grown in the same medium conditions to enable a direct comparison between transformants containing the empty plasmid (controls) and those overexpressing *HXK2*. The results from laboratory scale (100 mL) fermentations of CDGJM without leucine (Figure 6.4) showed that the Δtps2 mutant (Δtps2(pGP564)) was still unable to complete fermentation even when *HXK2* was overexpressed. Sugar utilisation in Δtps2(pGP564) and Δtps2(pGP564HXK2) was arrested contrary to the parent BY4743(pGP564), which catabolised all the sugar in 125 hours. In the case of the mutants, fermentation was considered 'stuck' when the residual sugar content remained unchanged for 72 hours.

The expression of *HXK2* was effective in reversing the arrested phenotype observed in the Δtps1 mutant as shown in Figure 6.4A. The fermentation profile of

Δtps1(pGP564HXK2) was comparable to that of the parent strain overexpressing *HXK2* (BY4743(pGP564HXK2). Both overexpression strains were observed to be 25% faster than the control strain BY4743(pGP564), whilst the Δtps1 mutant with the empty vector (Δtps1(pGP564)) was markedly extended fermentation (by 103%) as compared to the expression strain, Δtps1 (pGP564HXK2). The fermentation of Δtps2 either with over-expression of *HXK2* (Δtps2(pGP564HXK2)) or with an empty control vector (Δtps2(pGP564)) was observed to be incomplete. Residual sugar at the time of fermentation arrest totalled 20.5 ± 1.3 g L^{-1} and 19.5 ± 1.1 g L^{-1}, respectively (Figure 6.4B). The growth of Δtps1 was also similar to the parent whilst Δtps2 growth was lower than the parent (76 %) and Δtps1 (73 %). Therefore it is apparent from these results that the over-expression of *HXK2* gene in Δtps2 mutant does not help to overcome the inhibition of trehalose-6-phosphate, particular later in fermentation, as indicated by the arrest in fermentation of the Δtps2 mutant (Figure 6.4B).

A



B

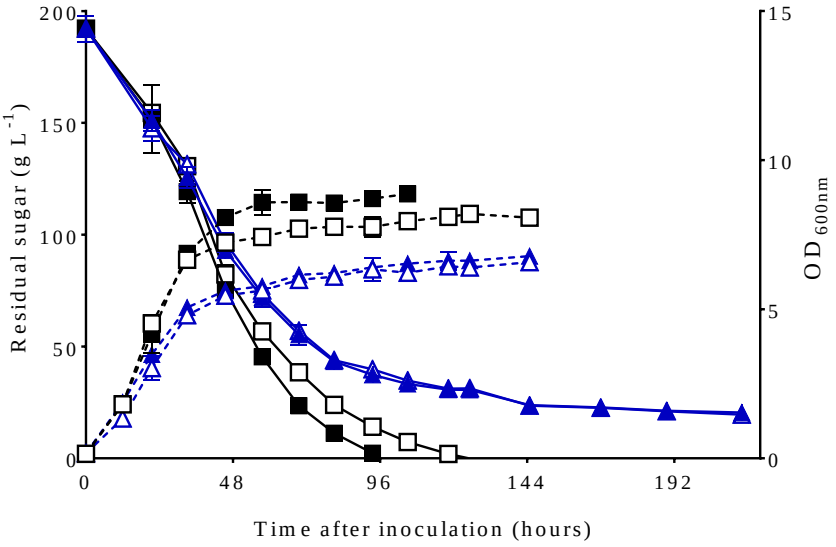


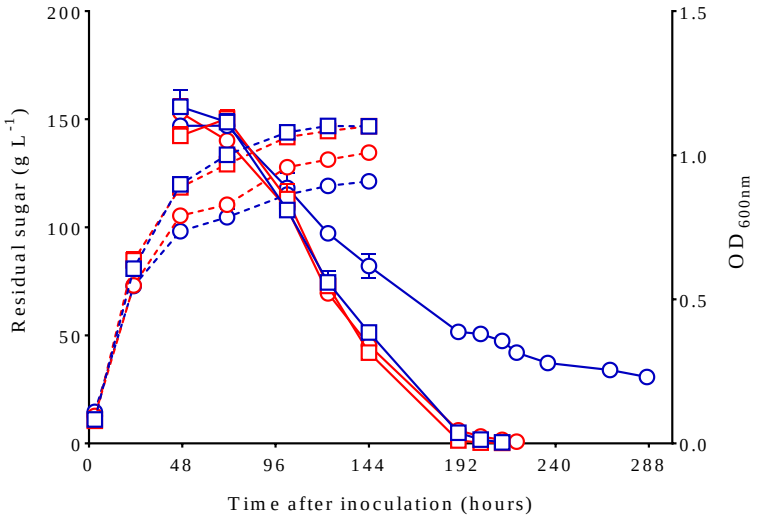
Figure 6.4 Fermentation and growth profiles of parent and Δtps1 (**Panel A**) and parent and Δtps2 strains (**Panel B**), either transformed with the empty plasmid, pGP564 (**□, ○, △**) or with the hexokinase over-expression plasmid, pGP564HXK2 (**■, ●, ▲**). The fermentation was conducted in 100 mL of CDGJM without leucine. At the indicated time points samples were taken and analysed for growth (OD_{600}) and residual sugar. Standard deviation of the mean of triplicate fermentations is included (error bars).

*6.3.3 Evaluation of fermentation performance of Δtps2 mutant when hexokinase (***HXK2***) and phosphotrehalase (TreA) genes are overexpressed***

In further investigation of the regulatory role of Tre6P in yeast, we investigated whether heterologous expression of a non-*Saccharomyces* gene encoding the phosphotrehalase gene (TreA), from *Bacillus subtilis*, would result in reduction of intracellular Tre6P. Phosphotrehalase hydrolyses Tre6P (a disaccharide) to its monosaccharide constituents glucose and glucose-6-phosphate. Plasmid pYX212TreA, was co-transformed into Δtps2 and BY4743 together with pGP564 (empty plasmid) or the *HXK2* overexpression plasmid, pGP564HXK2. Transformants were selected by growth on minimal medium without leucine and uracil. The presence of the plasmids was confirmed in the transformed strains by PCR (Appendix 5, Figure 5.3). The primers, TreAinF, URACR, PYXCF and PYXCR listed in Table 6.2, and designed to target sequences within the TreA gene, *URA3* and vector pYX212, respectively, were used.

The introduction of the TreA gene into the Δtps2 mutant (i.e. vectors resulted in the rescue of alcoholic fermentation (Figure 6.5). Accordingly, there was no significant difference in sugar utilisation between Δtps2 (pGP564 + pYX212TreA) over-expressing TreA and the parent, BY4743 bearing the corresponding empty vectors (pGP564 + pYX212). The growth of Δtps2 with over-expression of the TreA gene increased to 92% of the parent. In this experiment, additional over-expression of *HXK2* did not enhance fermentation further.

A



B

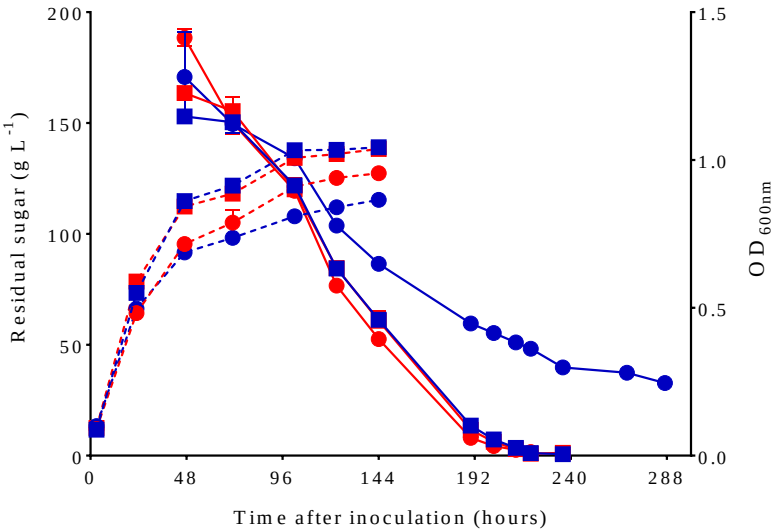


Figure 6.5 Fermentation profile of the parent (■, □, ■, □) and Δtps2 (●, ○, ●, ○) strains transformed with the empty pYX212 plasmid (blue) or with the TreA expression plasmid (red). Panel **A** depicts the transformed strains with the empty pGP564 plasmid and panel **B** depicts transformed strains with the *HXK2* over-expression plasmid (pGP564HXK2). Triplicate fermentations were conducted in 100 mL of CDGJM without leucine and uracil at 28°C with shaking at 120 rpm. Standard deviations are included as error bars.

6.4 Discussion

6.4.1 The trehalose pathway may play an important role in the completion of fermentation

Trehalose is well known as serving a unique role as a stress metabolite (Arguelles 1994, Fillinger et al. 2001, Hounsa et al. 1998, Majara et al. 1996, Thevelein and Hohmann 1995). Recently, one of the cellular processes in which trehalose biosynthesis has been implicated is the regulation of glycolysis (Francois et al. 2012, Gancedo and Flores 2004, Hohmann et al. 1996). Experimental evidence has implicated trehalose-6-phosphate (Tre6P), an intermediate of trehalose biosynthesis in regulating glucose metabolism via inhibition of glycolysis (Hohmann et al. 1996).

In this study, deletion of either *TPS1* or *TPS2* resulted in incomplete high sugar fermentation. Some models have been put forward trying to explain the involvement of *TPS1* and *TPS2* in the regulation of glycolysis, particularly in the control of the sugar influx, with the resultant effect on fermentation completion. The first model is based on the finding that Tre6P, the product of a Tps1p reaction inhibits the main hexokinase in yeast encoded by *HXK2* (Blazquez et al. 1993). Interestingly, the Tre6P inhibition model of hexokinase is probably incomplete since yeast cells growing exponentially on glucose (and thus in a highly glycolytic state) or overexpressing *TPS2* that encodes the trehalose 6-P phosphatase have barely detectable Tre6P levels and growth on glucose is not impaired (Hohmann et al. 1996). More importantly, deletion of *TPS2* was known to cause the accumulation of Tre6P and loss of trehalose 6-P phosphatase activity (Devirgilio et al. 1993). The accumulation of Tre6P is hypothesized to inhibit hexokinase (Figure 6.6). The second hypothesis proposes that the trehalose biosynthesis pathway can serve an additional function, for example, one involving a requirement for inorganic phosphate (Pi) for the functioning of glycolysis. The importance of Pi in rescuing the growth of a △tps1 mutant on fermentative sugars has been illustrated by hyperactivity of glycerol-3-phosphate dehydrogenase, Gpd1p and/or the glycerol facilitator encoded by *FPS1* (Vanaelst et al. 1991). Another model has been shown to indicate the regulation of *TPS1* on the restriction of sugar influx through an

unidentified protein interaction (Francois et al. 2012). In yeast there is no direct evidence for this hypothesis. However, the fact that the Δtps1 mutant is unable to undergo sporulation, a necessary process that occurs during the absence of fermentable carbon sources, supports the idea that Tps1p has an additional function rather than simply formation of Tre6P (reviewed in Francois et al. 2012).

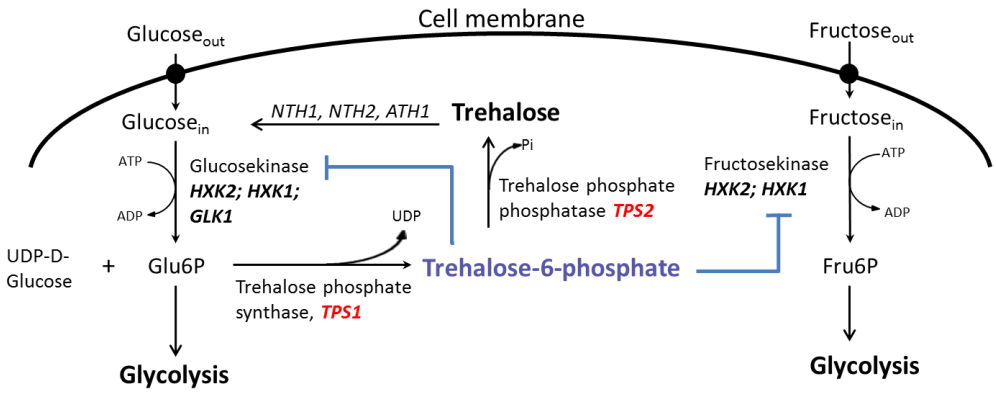


Figure 6.6 The link between the trehalose pathway and glucose and fructose influx into glycolysis. Blue lines indicate the inhibition of hexokinases by trehalose-6-phosphate (Tre6P). Deletion of the *TPS2* gene results in high accumulation of Tre6P that is purported to causes increased inhibition of hexokinase activity.

6.4.2 Over-expression of the HXK2 *gene rescued the fermentation performance of Δtps1 but not in Δtps2 mutant*

In order to evaluate the importance of inhibition by the intermediate Tre6P, an attempt to overcome such inhibition of hexokinase was made by the over-expression of hexokinase genes in Δtps1 and Δtps2 mutants, during high sugar fermentation. *HXK2* gene was used for overexpression studies because it encodes for hexokinase 2 (Hxk2p), and is repressed when cells of *Saccharomyces cerevisiae* are grown on media containing glucose and fructose (Rodriguez et al. 2001), and Hxk2p contributes to the utilisation of fermentable sugar in yeast (Herrero et al. 1995). Other hexokinase genes were also considered for use in this investigation, however, *HXK1* overexpression improved fermentation in lab strain but not in a wine strain (Vin13) (Berthels et al. 2007). The absence of stimulation of fermentation in Vin13 by overexpression of *HXK2* may be due to its higher sensitivity to the accumulation of Tre6P (Blazquez et al. 1993). In addition, *HXK2* regulated the expression of *HXK1* and *GLK1*. Thus *HXK2* was chosen for over-expression in Δtps1 and Δtps2 mutants. The expression of the hexokinase gene in Δtps1 was demonstrated to improve the fermentation performance whilst the fermentation performance of Δtps2 was largely unaffected. It seems that the expression of *HXK2* is activated in the absence of Tre6P and repressed in the presence of accumulating Tre6P, as in the case of the △tps2 strain (Figure 6.4B). It is postulated that the over-expression of the *HXK2* gene delayed the inhibition by Tre6P as implied by the lower residual sugar content of the medium from the Δtps2 mutant overexpressing the hexokinase 2 gene in comparison to the Δtps2 mutant alone (20.5 ± 1.3 g L^{-1} vs 27.3 ± 2 g L^{-1}). The similar growth behaviour of both the parent strain BY4743 and the Δtps1 mutant overexpressing *HXK2*, together with the shortened fermentation duration (25% faster than BY4743 (pGP564 + pYX212)) suggests that the *TPS1* gene is not influenced by the accumulation of hexose phosphates (Figure 6.4A).

The increased Tre6P concentration in the cell could be explained through the relationship between the substrates in the trehalose biosynthesis pathway and sugar influx in glycolysis. Tps1p has been shown to be highly sensitive to the inhibition of Pi which is a non-competitive inhibitor to both glucose-6-phosphate (Glu6P) and

UDP-glucose (UDP-Glu), and is stimulated by fructose-6-phosphate, Fru6P (Vandercammen et al. 1989). Tps2p instead requires Pi for full activity. On the other hand, fructose-6-phosphate (Fru6P) acts as an allosteric factor in reducing Glu6P (Vandercammen et al. 1989). Taking into account these findings, the accumulation of Glu6P as observed in the fermentation of the parent strain, would stimulate Tre6P synthase and reduce Tre6P phosphatase activity (Hohmann et al. 1996). Another aspect concerning the inhibition of hexokinases by Tre6P is that it would lead to increased sugar concentrations (Hohmann et al. 1996). The increasing concentration of hexose sugar phosphates is thought to alleviate inhibition by Tre6P on hexokinase (Ernandes et al. 1998). However, the concentration of free sugars in the cell varies only by a few mM (Hohmann et al. 1996, Neves and Francois 1992, Neves et al. 1995) and is well above the concentration of Tre6P (Blazquez et al. 1993, Hohmann et al. 1996). Hohmann et al. (1996) indicated that 1 mM glucose can outcompete the inhibitory effect of 0.4 mM Tre6P *in vitro*, and proposed that the regulatory system was only effective if the concentrations of Tre6P are as high as the sugar concentrations. Thus these findings support the hypothesis that the accumulation of Tre6P can inhibit hexokinases, and result in reduction of sugar utilisation.

6.4.3 The introduction of phosphotrehalase (TreA), from Bacillus subtilis *improved fermentation performance of* Δ*tps2 mutant*

Heterologous expression of the bacterial phosphotrehalase (TreA) was an approach undertaken in an effort to get a clearer understanding of the relationship between trehalose biosynthesis and glycolysis through the competitive regulation of trehalose biosynthesis intermediates on the hexokinases. The expression of hexokinase, Hxk2p was demonstrated to improve fermentation performance of the Δtps1 mutant but not the Δtps2 mutant (Figure 6.4). The sugar utilisation of both the Δtps2 mutant expressing *HXK2* and the Δtps2 mutant alone was arrested at the late stage of fermentation. These observations led to the question as to whether hexokinase activity would be maintained when the content of Tre6P was artificially reduced through genetic engineering.

In this study, both hexokinase 2 and phosphotrehalase genes were expressed in a Δtps2 mutant. A phosphotrehalase gene from *Bacillus subtilis* was introduced into the Δtps2 mutant in order to reduce Tre6P concentration and to evaluate the fermentation performance of this mutant. This enzyme is functional in yeast because it was demonstrated to reduce Tre6P levels in a Δtps2 mutant (Van Vaeck et al. 2001). Tre6P is hydrolysed to glucose and Glu6P by phosphotrehalase (Gotsche and Dahl 1995). The expression of TreA in a Δtps2 mutant was shown to improve fermentation performance (Figure 6.5). Taking this finding with previous enzymatic results, it is concluded that Tre6P has a significant inhibition on the hexokinase activity that results in repression of sugar influx to glycolysis and thus affects fermentation completion.

As mentioned previously, the fermentation performance of the Δtps2 mutant was highly reduced at the later stages of fermentation (Figures 6.2 and 6.4B). This result is consistent with previous studies that the greater accumulation or high level of Tre6P results in inhibition of hexokinases, causing cessation of glycolysis. Tre6P concentrations in a Δtps2 mutant are ~1.5 mM (Van Vaeck et al. 2001), which is almost 10-fold higher than the ~0.2 mM present in a wild-type yeast (Hohmann et al. 1996). In contrast, the restoration of sugar catabolism when phosphotrehalase is expressed in both the Δtps2 mutant and parent strain, as shown in Figure 6.5B, is indicative of the reduction in Tre6P concentrations in these strains, although these were not measured. This presumed low level of Tre6P might release inhibition of hexokinase, and thus enhance fermentation performance. In addition, the expression of phosphotrehalase was shown, not surprisingly, to rescue the growth defect in a Δtps2 mutant, which was not dissimilar from the phenotype of the parent cells over-expressing *TPS2* (Van Vaeck et al. 2001). This indicates that low levels of Tre6P are sufficient to sustain the growth of cells and improve growth of a Δtps2 mutant (Figure 6.5).

6.5 Summary and conclusion

Two genes, *TPS1* and *TPS2*, which encode for two steps in the trehalose biosynthetic pathway, were identified as contributing to incomplete fermentation when deleted.

The trehalose biosynthetic pathway consists of two consecutive enzymatic reactions catalysed by a trehalose-6-phosphate-synthase (TPS) enzyme, which produces the intermediate trehalose-6-phosphate (Tre6P), and a trehalose-6-phosphate - phosphatase (TPP) enzyme, producing trehalose. In *S. cerevisiae*, the TPS/TPP complex is encoded by *TPS1* and *TPS2*, respectively. The deletion of these genes results in the lack of TPS and TPP activity and incomplete fermentation. The intermediate of the two reactions catalysed by these genes, Tre6P, is absent in a Δtps1 mutant and exhibits marked accumulation in a Δtps2 mutant. The accumulation of Tre6P has been found to inhibit hexokinase activity, affecting sugar influx to glycolysis (Hohmann et al. 1996). This study sought to determine the importance of such inhibition by the intermediate Tre6P by attempting to overcome hexokinase inhibition through over-expression of a main hexokinase gene (*HXK2*) in Δtps1 and Δtps2. Over-expression of *HXK2* in Δtps1 rescued the extended fermentation phenotype, with the fermentation duration comparable to the control strain BY4743. However, overexpression of *HXK2* was ineffective in Δtps2, with the mutant still unable to complete fermentation. An alternative approach was used to determine whether glycolysis could similarly be restored by directed degradation of Tre6P through the introduction of phosphotrehalase (TreA), from *Bacillus subtilis*, in a Δtps2 mutant. The results of this study demonstrated that the fermentation phenotype of Δtps2 mutant was similarly rescued, to that of the parent, with the mutant having similar growth and fermentation kinetics.

The effect of intermediates relating to trehalose biosynthesis (Tre6P) on hexokinase activity would be worth evaluating in greater detail, specifically the levels of the intracellular intermediates Tre6P, Glu6P and Fru6P. Moreover, the level of expression of the *HXK2* and TreA genes in experimental strains (Δtps1, Δtps2p and parent BY4743) would also be interesting to examine. Because of time constraints the relationship between the trehalose biosynthesis pathway and sugar utilisation was only evaluated by examination of fermentation profiles of key mutants. Other functional studies would also be required to evaluate the inhibition of hexokinase by Tre6P. However, the results in this study are largely consistent with those obtained previously (Blazquez et al. 1993, Hohmann et al. 1996, Thevelein and Hohmann

1995), and together give a better understanding of the relationship between these cellular processes (Figure 6.1). Such information is valuable in yeast strain optimisation for improved fermentation performance and inclusion of these findings would help meet expectations of the wine industry in efforts to overcome stuck or sluggish fermentations.

CHAPTER 7 GENERAL DISCUSSION AND FUTURE DIRECTIONS

The complete consumption of essentially all sugars by yeast is vital for successful production of a 'dry' wine. A completed alcoholic fermentation is one where residual sugars are usually less than 2 g L^{-1} (Bisson 1999). The successful completion of alcoholic fermentations, particularly those typical of industrial winemaking, depends on the ability of yeast to cope with stresses imposed by initially high sugar concentrations and rapid oxygen depletion as well as others factors such as nutrient deficiency and the accumulation of ethanol in the later stages of fermentation. Studies on improvement of fermentation performance of wine yeast have been carried out previously (Alper et al. 2006, Gardner et al. 2005, Jiranek et al. 2013, McBryde et al. 2006, Teixeira et al. 2010,). These involve a number of studies conducted using yeast deletion collections to identify genes involved in responses to the individual stresses including many well known to be present during fermentation (Abe and Minegishi 2008, Ando et al. 2006, Fujita et al. 2006, Mollapour et al. 2004, Reiner et al. 2006, Teixeira et al. 2009, 2010, Tucker and Fields 2004, van Voorst et al. 2006, Yazawa et al. 2007). However, industrial wine fermentations are substantially more complex with yeast being exposed to multiple stresses of which the type, combination and intensity changes over the course of fermentation. This is exacerbated in high sugar fermentation, particularly due to the impact of higher osmolarity, increased concentrations of ethanol and longer periods of nutrient deficiency.

We sought to identify genes which are essential for the successful completion of fermentation under such conditions by screening a yeast deletion library in Chemically Defined Grape Juice Medium with relatively high sugar content (200 g L^{-1}). Laboratory scale (100 mL) fermentations were conducted to re-screen 336 gene deletants, previously identified by this group from a micro-scale fermentation library screen (Walker et al. 2013). From these, 93 gene deletants were identified as having protracted or incomplete fermentation and these were named the Fermentation

Essential Gene (FEG) dataset. The deletants of these genes were chosen for further study because they were largely shown to have little or no effect on growth, but were clearly affected in their ability to complete a timely fermentation. The FEG dataset was then analysed using Gene Ontology classifications and genes were annotated according to their known role(s) in biological processes. Enrichment of genes within the FEG dataset for particular GO terms was revealed by comparison to their occurrence within the genome and this highlighted the potential role of particular cellular mechanisms important for completion of high sugar fermentation, such as in winemaking. As described in Chapter 3 these include responses to the yeast's exposure and adaptation to fermentation stress. Of these 93 FEG deletants, nine deletants exhibited an extreme phenotype of incomplete or 'stuck' fermentation. These genes were involved in ion homeostasis (*SSQ1*, *PTK2* and *ZAP1*), signalling (*PCL1*), response to heat (*SIN3*), NAD recycling (*NPT1*), trehalose synthesis (*TPS1* and *TPS2*) and ubiquitin recycling (*DOA4*). More details on these have been recently submitted for publication by our group; Walker et al. (2013).

Further investigation of common functions of genes of the FEG dataset using Gene Ontology analysis revealed that a number of biological processes are important for completion of fermentation under the conditions used, for example; vacuolar acidification, cellular (ion) homeostasis, autophagy and membrane invagination. Of these biological processes, vacuolar acidification was further analysed especially since a large proportion of genes known to be important for this process were found in the FEG dataset. For instance, those that encode the eight subunits of the V_1 peripheral domain and five subunits of the V0 internal membrane domain of the V-ATPase complex were all present. Acidification of the vacuole occurs by the transport of protons through the V-ATPase located on the vacuolar membrane. This process has previously been identified to be involved in various cellular processes including pH regulation, chemical (ion) regulation, amino acid storage and metal detoxification (Anraku et al. 1989, 1992, Corbacho et al. 2012, Cyert and Philpott 2013, Forgac 1999, 2007, Graham et al. 2003, Nass and Rao 1999, Ungermann et al. 1999). In this study we also analysed the complete FEG dataset for involvement in vacuolar acidification by both measurement of vacuolar acidity during fermentation

and the ability to grow on neutral media and discovered 18 more genes not previously known to be annotated to this term. This further supports our hypothesis that this process is vital for yeast to complete fermentation under these conditions. Otherwise, the investigation of other function gene ontologies such as autophagy or membrane invagination in further work would be valuable to get better understanding of the stress response of yeast cell and would be helpful in the construction of robust yeast strains for successful fermentation.

Gene Ontology analysis also revealed the importance of trehalose biosynthesis for high sugar fermentation and thus this was also analysed further. The trehalose biosynthesis pathway has a role not only in stress resistance but also in regulation of sugar influx to glycolysis through the inhibitory action of the intermediate, trehalose-6-phosphate (Tre6P). Deletion of *TPS1* (encoding trehalose-6-phosphate synthase) and *TPS2* (encoding trehalose-6-phosphate phosphatase) in a laboratory strain resulted in incomplete or 'stuck' high sugar fermentation. The deletion of *TPS2* has previously been shown to result in a marked accumulation of Tre6P (Devirgilio et al. 1993), which is thought to inhibit the activity of hexokinases and consequently affect sugar influx into glycolysis (Hohmann et al. 1996). Strains were constructed to evaluate the effect of a subset of genes on the fermentation phenotype of the Δtps1 and Δtps2 mutants and thus provide some insight into the contribution of each gene toward their fermentation phenotype. Excitingly, the over-expression of hexokinase 2 in Δtps1 mutant was able to rescue the slow fermentation phenotype of Δtps1 and resulted in a fermentation phenotype similar to the parent. The fermentation of Δtps2 with over-expression of *HXK2* was however still incomplete. The *Bacillus subtilis* phosphotrehalase gene (TreA) was then introduced in the Δtps2 mutant in an attempt to reduce the accumulation of the Tre6P intermediate and thus release its inhibition on hexokinase. This approach was successful as the fermentation phenotype of Δtps2 mutant with expression of TreA was significantly improved (Chapter 6). This supports our hypothesis that the trehalose biosynthesis pathway affects hexokinase activity through its intermediate, Tre6P, and thus has an important role in fermentation completion via not only stress cell protection but also the likely control of sugar influx through glycolysis. Further experiments on characterization of the

physiology of the mutants generated in this study are essential. It has been shown that these mutants had a faster fermentation compared to the parent. To explain this, a determination of trehalose and Tre6P contents in the experimental strains (Δtps2 and BY4743) with overexpression of *HXK2* and/or TreA genes would give a clearer understanding of the physiological characters of these mutants. An experiment on the expression of *HXK2* and TreA genes in these experimented strains, otherwise, would be valuable to determine the expression level of these genes in the accumulation of Tre6P.

In this study genes essential for the completion of a high sugar fermentation, have been successfully defined through the use of a commercial laboratory yeast deletion library. Such genes when deleted result in the inability of yeast to complete fermentation under these conditions. We also then sought to find genes that when overexpressed also resulted in the same phenotype. This gene set would presumably involve mostly regulators, likely suppressors, of the biological pathways already highlighted in this study as well as others not yet found. To achieve this we sought to construct an overexpression library in an appropriate background since none are currently available commercially. An over-expression library was constructed in the wine yeast strain (ISOC9d) with a Yeast Genomic Tilling Collection (Jones et al. 2008). A derivative of this strain bearing a leucine auxotrophy was successfully constructed in this study (Chapter 5). Validation and optimisation of the screening of the over-expression library was also conducted, however, due to time constraints, evaluation of this library was not possible within the scope of this project and is on-going within our laboratory by others. Access to some of the overexpression constructs was beneficial in other parts of this project. However, the stability of the plasmid used to construct this overexpression library should be evaluated in further investigations.

Since sugar concentrations in grape juice have been steadily increasing in Australia, in part due to climate change (shortened, drier vintages) and altered harvesting and vinification practises (Godden and Muhlack 2010), the development of efficient yeast strains able to meet specific winemaking targets with such fruit is vital. This

study has advanced understanding of the genetic basis for successful fermentation, particular in the presence of high sugar concentrations and thus will be very useful for designing strategies to generate robust yeast strains. Furthermore, the up-coming complementary dataset from the over-expression library screening together with this data will provide a greater depth of knowledge of the mechanisms behind such traits. Information gained on the genes (and processes) involved may provide the basis for further strain improvement through a number of non-recombinant approaches such as directed evolution and classical breeding. Whilst gene deletions and to a lesser extent, gene overexpression are considered radical modifications, naturally occurring genetic mutations (as single nucleotide polymorphisms or small insertions and deletions) with the potential to alter fermentation outcome have been identified in commercial wine yeasts (Borneman et al. 2008, Marullo et al. 2007, Novo et al. 2013, Steyer et al. 2012, Walker et al. 2005). Genetic heterogeneity within such genes as those identified in this study, as well as the recent genetic linkage to specific fermentation attributes such as hydrogen sulphide production (Linderholm et al. 2010), presents new opportunities for strain improvement by classical breeding programs analogous to those used in cereal production (Marullo et al. 2006, Timberlake et al. 2011).

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Appendices

Appendix 1

Starter Chemical Defined Grape Juice Medium (Starter-CDGJM) (1 Litre)

Nutrient supplementary stocks used for Starter-CDGJM and CDGJM were made according to McBryde et al. (2006) and Jiranek and Henschke (1995).

Glucose	50 g
Fructose	50 g
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.44 g
Potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6.4H_2O$)	3.14 g
100 x salts	10 mL
500 x trace minerals	2 mL
80 x vitamins	12.5 mL
25 x amino acids	22.2 mL
500 x Tween 80/ergosterol	2 mL

1.5 g Polyphenol was dissolved in 3 mL of absolute ethanol overnight before adding to medium (if needed).

pH is adjusted to 3.5

Filter sterilized (0.22 µm)

Chemical Defined Grape Juice Medium with polyphenols (CDGJM+PP) (1 Litre)

Glucose	100 g
Fructose	100 g
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.44 g
Potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6.4H_2O$)	3.14 g
100 x salts	10 mL
500 x trace minerals	2 mL
80 x vitamins	12.5 mL
25 x amino acids	22.2 mL
500 x Tween 80/ergosterol	2 mL

1.5 g Polyphenol was dissolved in 3 mL of absolute ethanol overnight before adding to medium (if needed).

pH is adjusted to 3.5

Filter sterilized (0.22 µm)

Minimal Drop-out medium components (1 Litre)

Ammonium sulphate	5 g
Glucose	20 g
Salts stocks	1.7 g
Trade mineral stock	1 mL
100 x vitamins stock	10 mL
10 mM Inositol stock	6.5 mL
10 x drop-out amino acid stock	100 mL

Adjusted pH to 6.2

Autoclaved at 121°C for 20 min

Phosphate buffered saline (PBS)

0.2 g L^{-1} KCl

8 g L^{-1} NaCl

0.2 g L^{-1} KH_2PO_4

1.15 g L^{-1} Na_2HPO_4

Adjusted pH to 7.2

Filter sterilized (0.22 µm)

Tris EDTA buffer (TE)

10 mM Tris

0.1 mM EDTA

Adjusted pH to 8.0; make as 10 x stock

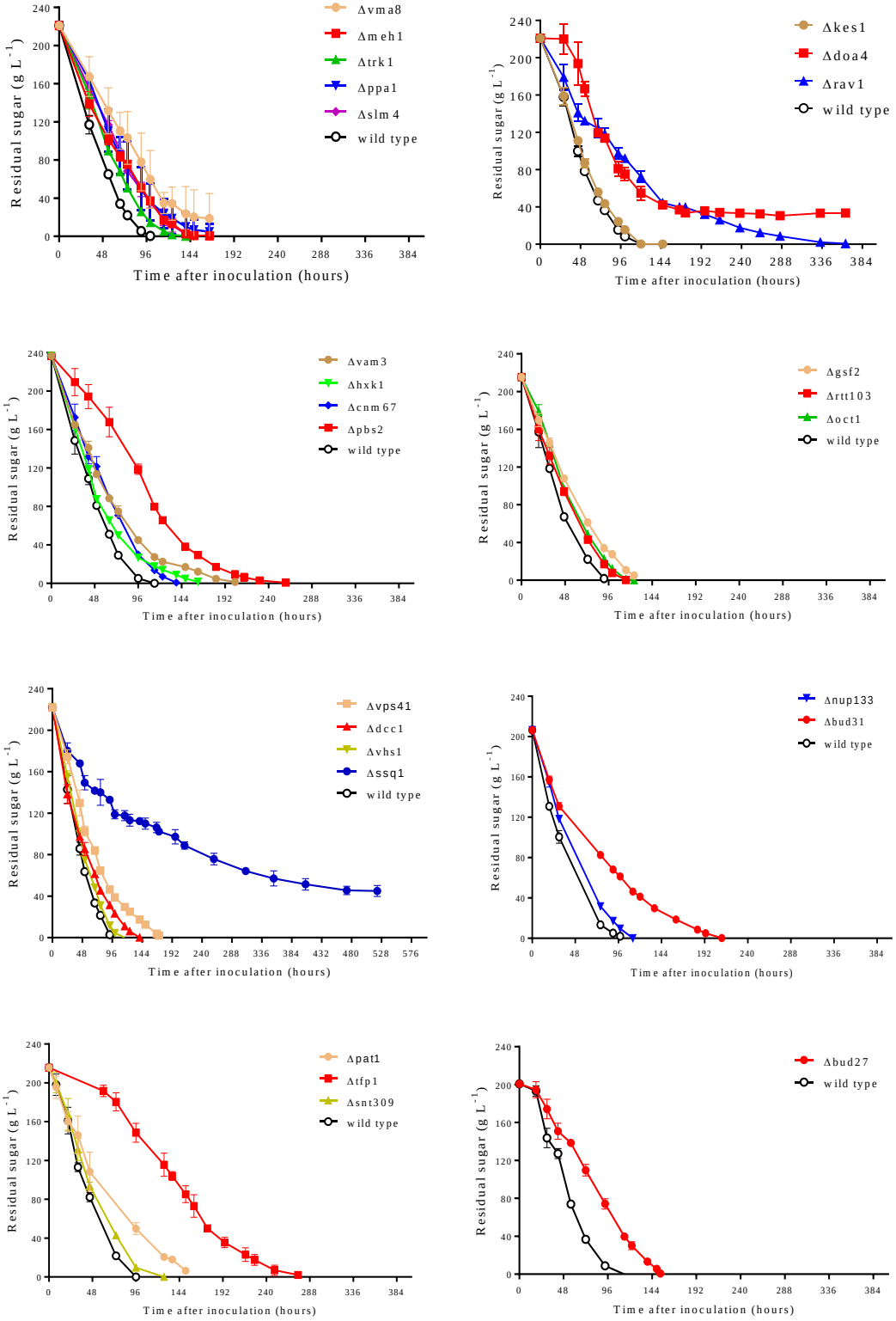
Glycerol stock of yeast cultures

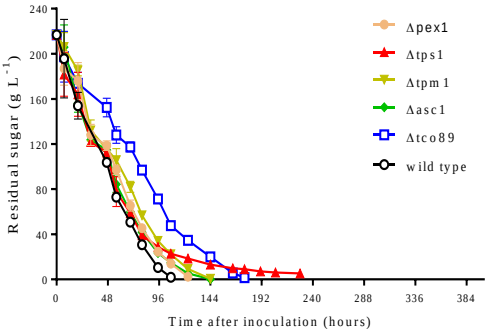
Yeast culture	1 mL
80% glycerol (autoclaved)	0.5 mL

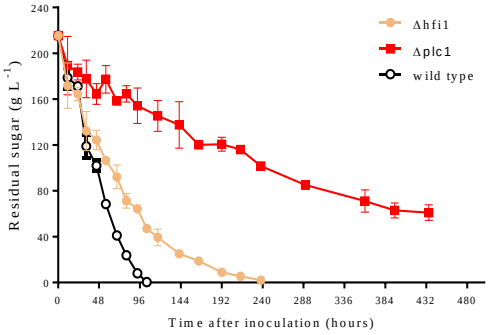
In 2 mL screw cap eppendorf tubes, mix well before putting in -80°C

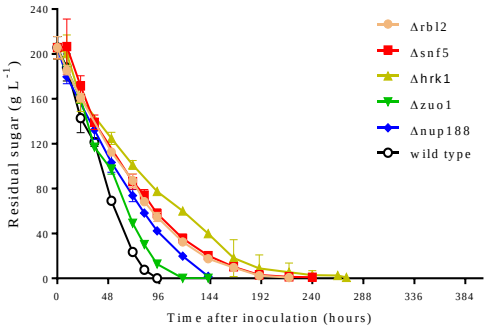
Appendix 2

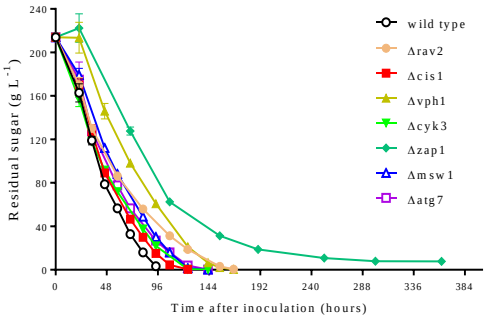
Figure 2.1 Fermentation charts of 93 gene deletants having protracted fermentation.

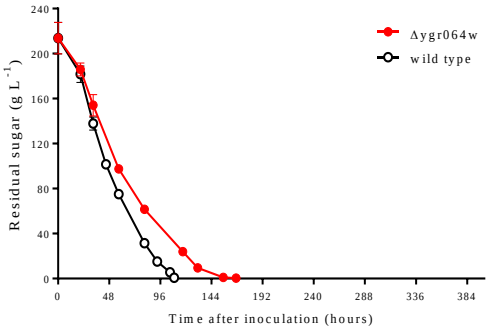


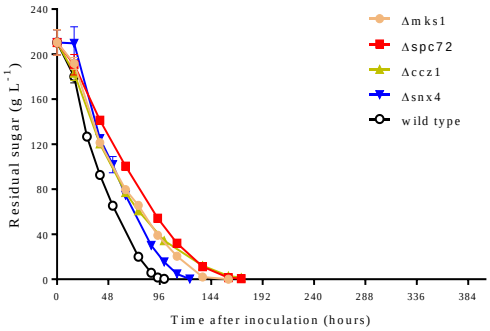

Residual sugar (g L-1)
Time after inoculation (hours)
Δpex1
Δtps1
Δtpm1
Δasc1
Δtco89
wild type

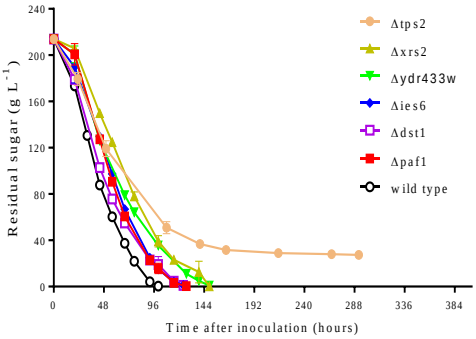

Residual sugar (g L-1)
Time after inoculation (hours)
Δhfi1
Δplc1
wild type

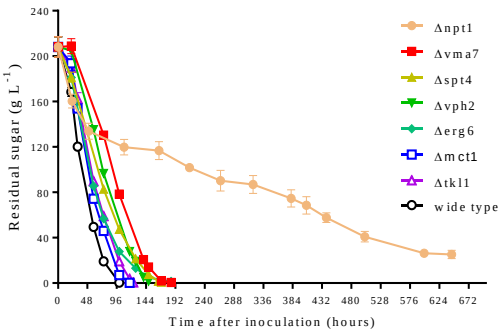

Residual sugar (g L-1)
Time after inoculation (hours)
Δrbl2
Δsnf5
Δhrk1
Δzuo1
Δnup188
wild type

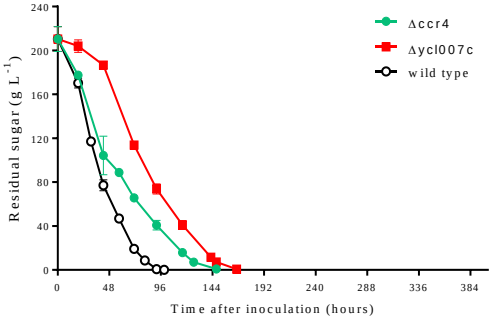

Residual sugar (g L-1)
Time after inoculation (hours)
wild type
Δrav2
Δcis1
Δvph1
Δcyk3
Δzap1
Δmsw1
Δatg7

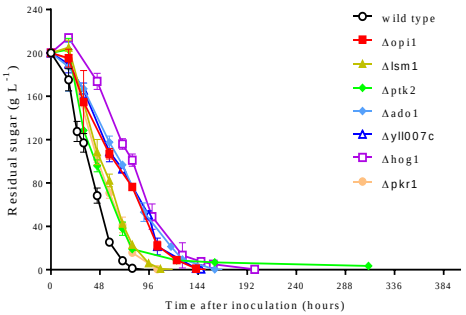

Residual sugar (g L-1)
Time after inoculation (hours)
Δygr064w
wild type

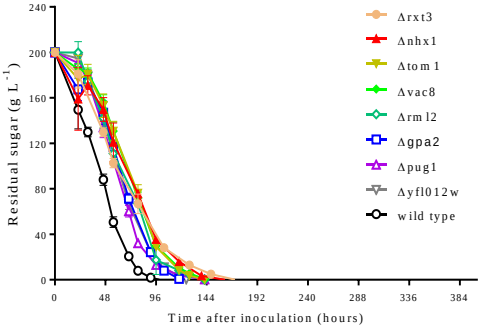

Residual sugar (g L-1)
Time after inoculation (hours)
Δmks1
Δspc72
Δccz1
Δsnx4
wild type

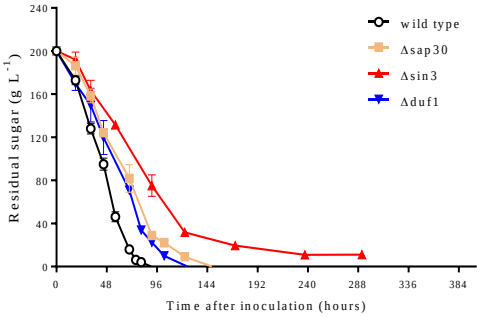

Residual sugar (g L-1)
Time after inoculation (hours)
Δtps2
Δxrs2
Δydr433w
Δies6
Δdst1
Δpaf1
wild type

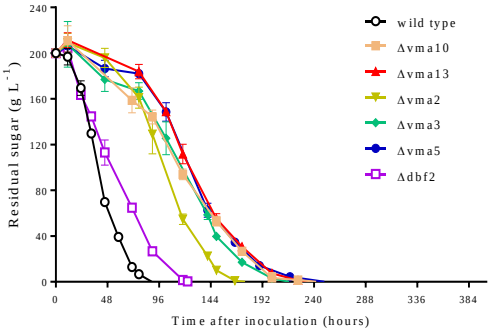

Residual sugar (g L-1)
Time after inoculation (hours)
Δnpt1
Δvma7
Δspt4
Δvph2
Δerg6
Δmct1
Δtkl1
wide type

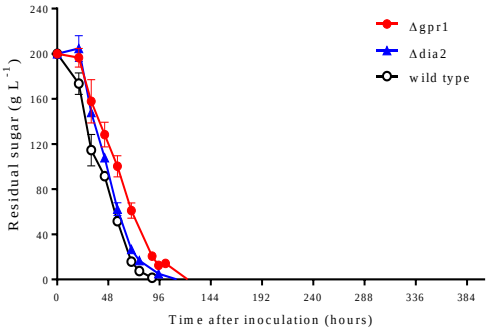

Δccr4
Δycl007c
wild type
Residual sugar (g L-1)
Time after inoculation (hours)


wild type
Δopi1
Δlsm1
Δptk2
Δado1
Δyll007c
Δhog1
Δpkr1
Residual sugar (g L-1)
Time after inoculation (hours)


Δrxt3
Δnhx1
Δtom1
Δvac8
Δrml2
Δgpa2
Δpug1
Δyfl012w
wild type
Residual sugar (g L-1)
Time after inoculation (hours)


wild type
Δsap30
Δsin3
Δduf1
Residual sugar (g L-1)
Time after inoculation (hours)


wild type
Δvma10
Δvma13
Δvma2
Δvma3
Δvma5
Δdbf2
Residual sugar (g L-1)
Time after inoculation (hours)


Δgpr1
Δdia2
wild type
Residual sugar (g L-1)
Time after inoculation (hours)

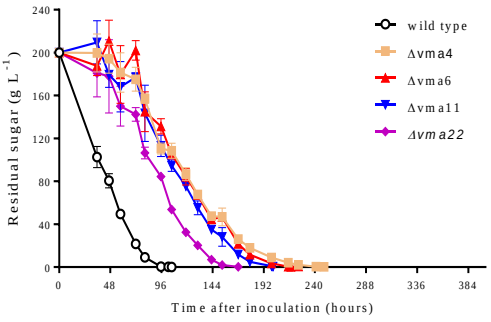

wild type
Δvma4
Δvma6
Δvma11
Δvma22
Residual sugar (g L-1)
Time after inoculation (hours)

Table 2.1 Primers used for PCR amplification and DNA sequencing of 44 fermentation essential genes

Primer name	Primer sequence (5'-3')
KanB	CTGCAGCGAGGAGCCGTAAT
KanC	TGATTTTGATGACGAGCGTAAT
VMA13-AF	CGCTGTGTTGTATATTGCTCTAAAA
CCR4-1-AF	TCAAAGGTAAAGTAGCCGTTTAGGCCT
CCR4-2-AF	TCGTGCTCTAGCACTGATAAGTAAC
VMA16-AF	CGTTAACGGTTATTATGCTGAGTCT
VAM3-AF	GACACAGGAAGAGCAAAACTAAGAT
PAF1-AF	ATATCGAAGTTGACCATACGAAAAG
YDR433W-AF	TGGAAGTTGTTTACTCTAAATTGCC
DBF2-AF	TCATGGTTAGGGCTCTTCTACATAC
BUD27-AF	AGTAACCTCTCACAGGTATTGCTTG
VMA2-AF	TATTGGACTTTTGGGATGTTGTACT
TFP1-AF	CGATTAGACTCCACTTTGCTTCTAT
PEX1-AF	ATGAGTCCATAATACTGTACTGGCG
VMA3-AF	GAGGAACTGAATTCTTTATCAGCAA
DIA2-AF	CTGCGTTCGTAGTAAAATCAGAAAT
IES6-AF	TGCATAGTTTATTAGTCTGTGCCTG
DCC1-AF	GGCAGCGTATAGTATAACCAGAAAA
VMA5-AF	AAAACAATTTTCCGTAAAGAGAAGC
VMA10-AF	TTGTATTTGAACTCTTTTATTCCGC
XRS2-AF	GTATTGAAGCAATTTGTAAGCTGGT
CYK3-AF	AACAAGAGAAAAGGCCAAAATTAAC
CNM67-AF	TGCCAAAATTAAAAAGAATAGATCG
VMA7-AF	TCACCCAGATACCTATTTCTTACCA
YCL007C-AF	TGTTTTGTGCAGTGTATTACCAACT
VPH2-AF	GGGGAACTTTTAGGATATTCTGTGT
ERG6-AF	CTGTTGCCGATAACTTCTTCATTGC
SPC72-AF	TGCTATCAACATAACAAGCAAGAAG
VPH1-AF	CTCAGATAAGTCGATATGGAAAACC
HOG1-AF	GAGATTATTCGGCATTTTGACATAC
PBS2-AF	AAGGATCTTTCTAACGTGTGTTGTC
VMA13-DR	GAGGAGGCGTCATGAAAAAG
CCR4-1-DR	AAAGAAACAGGACGAAAAAGATACGC
CCR4-2-DR	GGTTTGGTTAAATAATTTTGGCTCT
VMA16-DR	TTAAAGAAGAACCCTGACTATCGTG
DOA4-DR	CTCAAGAGGGATTGAAAGATGTTAG
VAM3-DR	AAGTAGAAAAAGGAAAAAGGGCATA
PAF1-DR	TATTTGTGTCAGTGTAACTGCTGGT
YDR433W-DR	AAGGATGTTAAATGTTATCATGGGA
DBF2-DR	CCCAACATGAATGCTTAAGTTTAGT
BUD27-DR	AAATCGGTAACTCTGGTATGTTCAG

Table 2.1 (Cont.)

Primer name	Primer sequence (5'-3')
VMA2-DR	CAAGTGTATTGCAATGTTCTTTGTC
TFP1-DR	GGGTGTAAACCATTGCCTACTATTT
PEX1-DR	TATATATGCAACTTCGGATTTCGTT
VMA3-DR	ATAGTGATCTCATCGTACCCATTGT
DIA2-DR	TGGAACAATTTCCTCTTATGATGTT
IES6-DR	GTGGCTATTTTGTCGATAGACACTT
DCC1-DR	TTGATTTCAACATCTTCCAGTTGTA
VMA5-DR	TAGTGCTAAATCCTGTAGATTTCCG
VMA10-DR	CGGCGTTTAGTATTTTTCGTTAATA
XRS2-DR	TCCAATTTTAAGATTTTCACTCTGC
CYK3-DR	CGTTCTGTTTGATAAGTAGGGGATA
CNM67-DR	AAAAGCTCATAGTAGCAGGTCTTCA
ZAP1-DR	CTTCGGTTACCTAGTTGTCACTCAT
VMA7-DR	TCTTTATTGAAGCATTAGCCAAATC
YCL007C	ACAGGAATACTGTGAGGAGATGAAC
VPH2-DR	TAGGCCATAATTAATTCCCATAACA
ERG6-DR	GGCCTGCTAGCAATGAACGTGCTA
SPC72-DR	GATGAGAAATCTCTTGGGATACTGA
VPH1-DR	AGGATTTGTCAACTGTTTAGACCTG
HOG1-DR	CTCTTCGGTAAAAGCACAGAATTAG
PBS2-DR	ACATCCCCTCAATACTCTGTCATAA

Appendix 3

Manuscripts were contributed by results of this PhD project

1. Nguyen, T. D., Walker, M. E., Gardner, J. M., Jiranek, V. (2013) 'Appropriate vacuolar acidification in *Saccharomyces cerevisiae* is required for efficient high sugar fermentation', *Journal of Biological Chemistry* (Submitted) MS ID JBC/2013/536961

2. Walker, M. E., Nguyen, T. D., Liccioli, T., Schmid, F., Kalatzis, N., Sundstrom, J. F., Gardner, J. M., and Jiranek, V. (2013) Genome-wide identification of the fermentome; genes required for successful and timely completion of fermentation by *Saccharomyces cerevisiae. BMC Genomics* (Submitted), MS ID 2039431431111758

Appendix 4

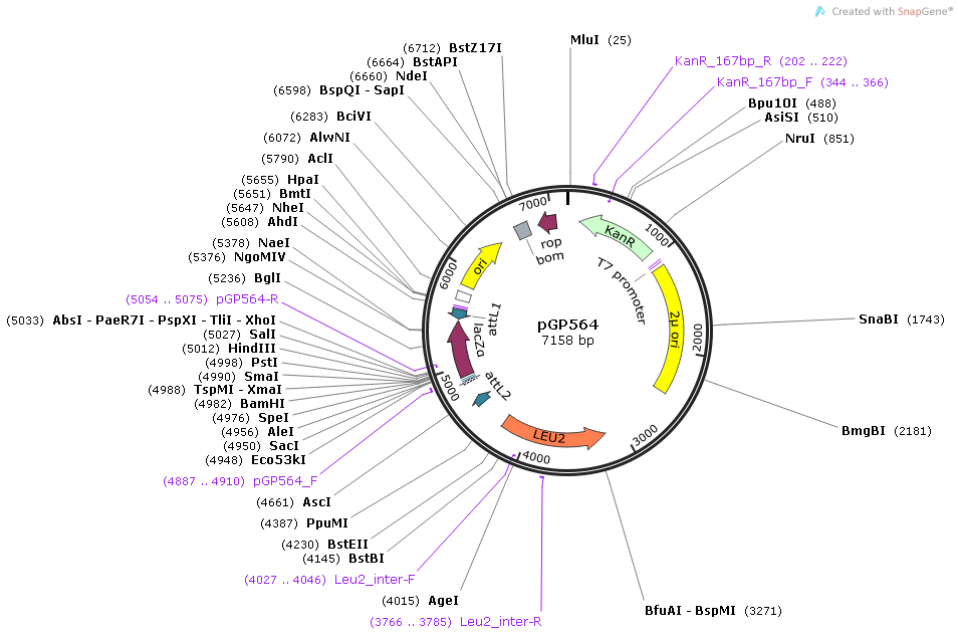


Figure 4.1 Map of pGP564 plasmid (adapted from Jones et al. 2008) with selectable marker (*LEU2* marker) and the restriction sites which can be used to insert genes or a segment of the yeast genome. Sequence position of primers used for PCR amplification and sequencing are shown in purple. Plasmid map constructed with SnapGene software (http://www.snapgene.com/).

Appendix 5

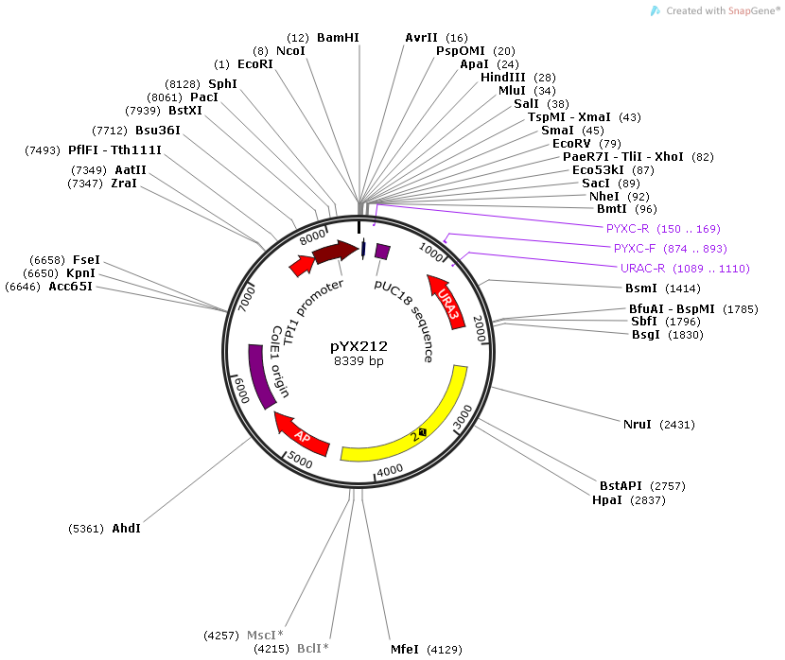


Figure 5.1 Map of pYX212 (Van Vaeck et al. 2001). Primers using for PCR sequencing are in purple.

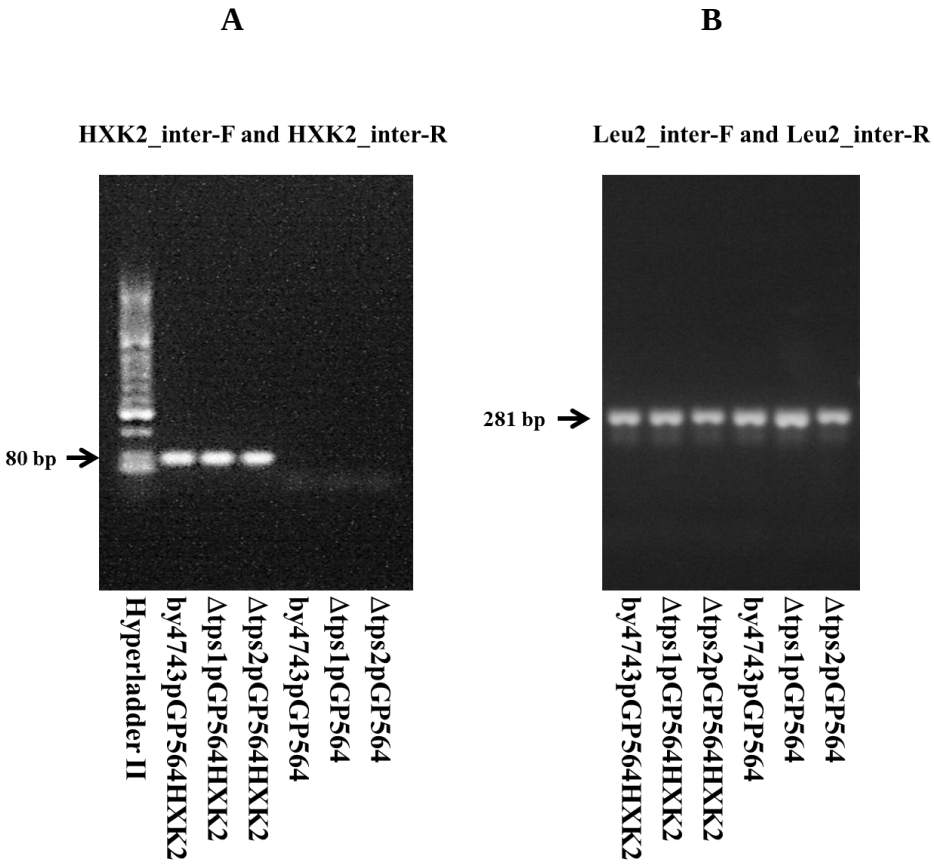


Figure 5.2 PCR confirmation of the correct transformation of Δtps1, Δtps2 and the parent strains with plasmids pGP564 (empty plasmid) and pGP654HXK2 (overexpression plasmid) using HXK2_inter primers (**A**) and Leu2_inter primers (**B**).

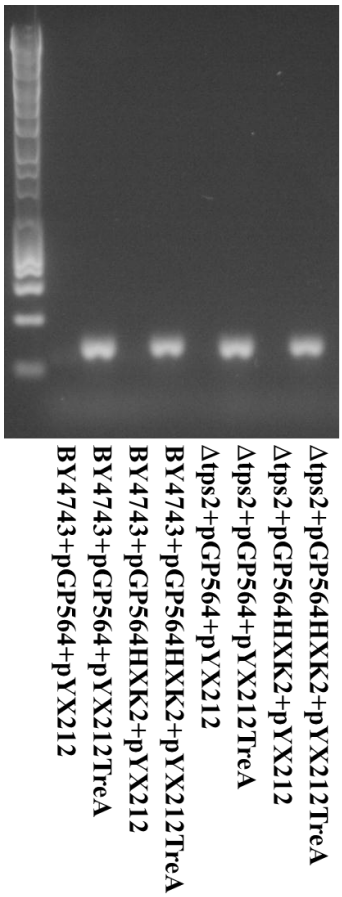


Figure 5.3 PCR confirmation of the transformation of Hexokinase gene *HXK2* and *B. subtilis* phosphotrehalase gene TreA in △tps2 and BY4743 strains using TreAF and pYXCR primers. TreAF and pYXCR were designed to sequences within the TreA gene (http://www.genome.jp/dbget-bin/www_bget?bsu:BSU07810) and pYX212 vector (Van Vaerch et al. 2011).