Investigation and Characterisation of Highly Nitrogen Efficient Wine Yeast

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Declaration

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Finally, a special thank to my great partner Bo Xu for your love and encouragement to make me the achiever I am today, and your ideas, even when we have an argument.

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Abstract

Sufficient yeast assimilable nitrogen (YAN) is essential for wine yeast (*Saccharomyces cerevisiae*) to complete alcoholic fermentation. YAN largely consists of alpha-amino acids and ammonium ions. With adequate YAN (as well as other nutrients) in grape juice, yeast cellular processes such as protein synthesis, growth and proliferation occur, allowing alcoholic fermentation to proceed efficiently. By contrast, insufficient YAN may result in sluggish or stuck fermentation and is often coupled with the formation of undesirable aromas, such as hydrogen sulfide, which impact on wine quality. Employment of highly nitrogen efficient (HNE) wine yeast provides an alternative strategy to facilitate the completion of alcoholic fermentation under limited nitrogen conditions. In this study, a group of HNE candidate strains were investigated in both synthetic media and grape juice under different YAN conditions. A mutant with disruption of *ECM33* showed superior fermentation performance under various YAN conditions compared with the wild type. Accordingly, the Δ*ecm33* strain is defined as a HNE strain. The role of *ECM33* was further investigated using the loss-of-function Δ*ecm33* mutant. Growth on agar plates containing Calcofluor White (CFW) or Congo Red (CR) was limited, suggesting that Δ*ecm33* possesses a cell wall defect resulting in increased chitin (target of the antifungals CFW and CR). QRT-PCR results showed that the transcriptional abundance of a group of key genes involved in the cell wall integrity (CWI), high osmolarity glycerol (HOG) and central nitrogen metabolism (CNM) pathways were altered in Δ*ecm33* mutant. In order to understand the HNE mechanism in the Δ*ecm33* strain, two genes *PTP2* and *SLT2* were investigated by overexpression, and also evaluated for their fermentation performance in synthetic media. Results showed that the overexpression of *PTP2* improved yeast fermentation performance in the late stages of fermentation. *SLT2* overexpression was

not found to be helpful for fermentation. Metabolites examined in fermentation samples showed that a higher concentration of citric acid and ethanol were produced in *PTP2* overexpression (OEX) strains. Increased ethanol yield was also observed in the *SLT2* OEX strain. Less acetaldehyde was produced in the Δ*ecm33* background strains during alcoholic fermentation. Based on the observations in this study, it is suggested that the HNE phenotype of Δ*ecm33* might be triggered by activation of a metabolic network(s), including: one or more of the CWI, HOG or CNM pathways, resulting in a more robust yeast cell with good fermentative capability and adaption to the dynamic environment of alcoholic fermentation.

List of abbreviations

Abbreviation	Full term
Δ	Gene deletion
3'	Three prime, of nucleic acid sequence
5'	Five prime, of nucleic acid sequence
A	Adenine
AGRF	Australian Genome Research Facility
Ap^r	Ampicillin resistance
AWRI	Australian Wine Research Institute
bp	Base pairs, of nucleic acid
C	Cytosine
CaCl$_2$	Calcium chloride
Cat #	Catalogue number
CDGJM	Chemically defined grape juice medium
CDGJSM	Chemical defined grape juice starter medium
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
cfu	Colony forming units
CFW	Calcofluor white
Conc.	Concentration
CR	Congo red
CRS	Congo red sensitive
CWI	Cell wall integrity
D	Aspartic acid
DAP	Diammonium phosphate
DNA	Deoxyribonucleic acid
DO	Drop-out
E	Glutamic acid
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methanesulfonate
F/Fwd	Forward
G	Glycine
gDNA	Genomic deoxyribonucleic acid
GPI	Glycosyl-phosphatidylinositol
h	Hour
HNE	Highly nitrogen efficient
HOG	High osmolarity glycerol
I	Isoleucine
KO	Knock out
LWR	Length to width ratio
Lys	Lysine
MAPK	Mitogen-activated protein kinase
Mpa	Megapascal
N	Nitrogen group
N	Asparagine

NCR	Nitrogen catabolite repression
NF	Normalization factor
NREL	Normalized relative expression level
OD	Optical density
OEX	Overexpression
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PTP	Tyrosine phosphatase
Q	Glutamine
QRT-PCR	Real-time PCR/quantitative PCR (qPCR)
QTLs	Quantitative trait locus
R	Arginine
R/Rvs	Revers
RNA	Ribonucleic acid
S	Serine
SNPs	Single nucleotide polymorphisms
T	Thymine/threonine
TAE	Tris Acetate EDTA
TOR	Target of rapamycin
Tris	Tris(hydroxymethyl)aminomethane
UV/Vis	Ultraviolet visible
v/v	Volume per volume
WT	Wild type
YAN	Yeast assimilable nitrogen
YPD/YEPD	Yeast extract peptone dextrose

Chapter 1 Literature Review

1.1 Introduction

Yeast assimilable nitrogen (YAN) is an essential nutrient which is required in sufficient amounts by wine yeast to successfully complete alcoholic fermentation. With adequate nitrogen, yeast is able to grow, proliferate and subsequently catabolise available sugars (Bisson 1999). There is a natural variation in specific YAN requirements of commercial wine yeasts, which ranges from 300 ~ 400 mg L^{-1} (MAURIVIN^{TM}: http://www.maurivin.com). The nitrogen content in grapes is highly variable, ranging from 60 to 2,400 mg N L^{-1} (Ough and Amerine 1988). This variability is dependent upon factors such as climate, soil conditions, grape variety, timing of harvest and specific vineyard management (Bell and Henschke 2005). Grape musts with low YAN are often associated with problematic sluggish or stuck fermentations which are costly to rectify and may lead to reduced wine quality and significant financial losses for the winery. Undesirable flavours such as hydrogen sulfide (H_2S) and higher alcohols can form and may also be in combination with low yields of esters and long chain fatty acids due to the extended fermentation duration (Alexandre and Charpentier 1998; Bell and Henschke 2005; Bisson 1999; Blateyron and Sablayrolles 2001; Ingledew and Kunkee 1985; Jiménez-Martí et al. 2007; Jiranek et al. 1995b; Monteiro and Bisson 1991).

A common way to rectify low YAN issues is to supplement musts with ammonium salts, such as diammonium phosphate (DAP) (Henschke and Jiranek 1993), however, this supplementation strategy is not ideal, since it can be costly and difficult to appropriately evaluate the amount of additional nitrogen required. The winemaker also needs to take into account the grape-derived sugar present, desirable

fermentation duration and individual wine yeast strain used. If an inappropriate (over) addition is made, excess nitrogen can result in the formation of more unwanted compounds, such as ethyl carbamate or increased acetic acid due to microbial instability (Adams and van Vuuren 2010; Bell and Henschke 2005; Ugliano et al. 2007). Fundamental studies and previous reviews have well established the reasons and the potential remedial action for sluggish or stuck fermentation. In this review, the current understanding of nitrogen metabolism, influence of nitrogen on yeast and wine, including depletion prediction, are summarized. Moreover, as an alternative strategy for management of such difficult fermentations, selection and characterisation of nitrogen efficient yeast is introduced. To further understand nitrogen efficient yeast, functional systematic genetic analysis is also discussed.

1.2 Nitrogen composition of grapes

YAN in grapes consists of ammonium, amino acids, peptides and protein. Ammonium, which is one of the main YAN components can be utilised for vine growth, or accumulated and stored as nitrogenous compounds in grape berries during berry maturation. Its content in juices varies from 5 to 325 mg L^{-1} (Bell and Henschke 2005). Another main form of YAN are the amino acids whose accumulation in grapes is chiefly dependent upon the process of grape ripening, and is influenced by factors such as cultivar, rootstock, site, seasonal conditions and level of maturity. 22 amino acids have been reported to be present in grapes (Henschke and Jiranek 1993; Ough and Bell 1980; Spayd et al. 1994; Sponholz 1991) and specific composition can be affected by cultivation practices, including nitrogen application, trellis style, soil management techniques, fungal infection, and also grape processing method. Subsequently amino acid content ranges from 50% to 90% of total YAN at harvest (Bell and Henschke 2005). Among these amino acids, proline is the most predominant

one in grapes, however it is largely unavailable for yeast cells when under anaerobisis, and subsequently only small amounts are consumed at the beginning of alcoholic fermentation when oxygen is still present. Along with proline, large amount of preferable free alpha amino acids are consumed during the early stage of fermentation, such as: arginine, serine, glutamate, threonine, aspartate and lysine (Ingledew et al. 1987; Jiranek et al. 1995a; Ough et al. 1988; Ough and Stashak 1974; Poole et al. 2009).

1.3 Nitrogen utilisation by *Saccharomyces cerevisiae*

1.3.1 Central nitrogen metabolism

A completed oenological fermentation is defined as one where the residual sugar content is below 2-4 g L^{-1} for a dry style of wine (Alexandre and Charpentier 1998, Bisson 1999). When conditions are adequate, yeast is normally able to catabolise all sugars in a must with sufficient available nitrogen. Assimilated nitrogen is critical in the synthesis of sugar transporters for sugar transport and catabolism (Figure 1.1). Generally, yeast can utilise nitrogen sources directly or convert them to glutamate or glutamine in order to utilise them indirectly. The glutamate and glutamine can act as donors for the biosynthesis of all other nitrogenous compounds in yeast cells, a process known as central nitrogen metabolism (CNM; Figure 1.2) (Ljungdahl and Daignan-Fornier 2012; Magasanik and Kaiser 2002; ter Schure et al. 2000).
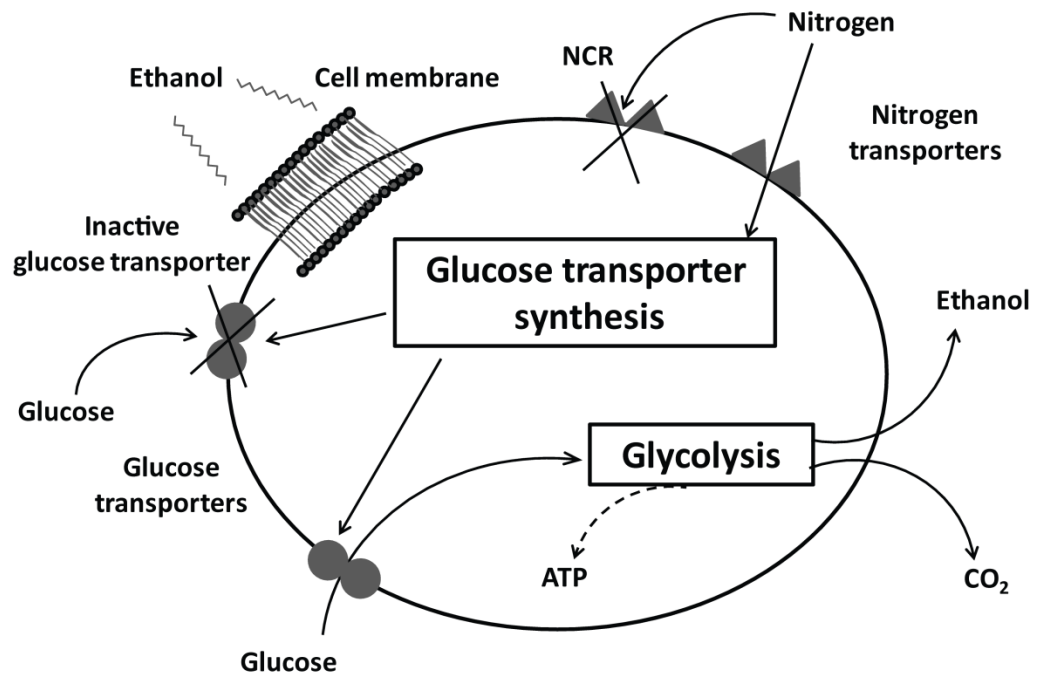
Figure 1.1 The action of nitrogen in yeast during alcoholic fermentation, adapted from Malherbe et al.

(2004)
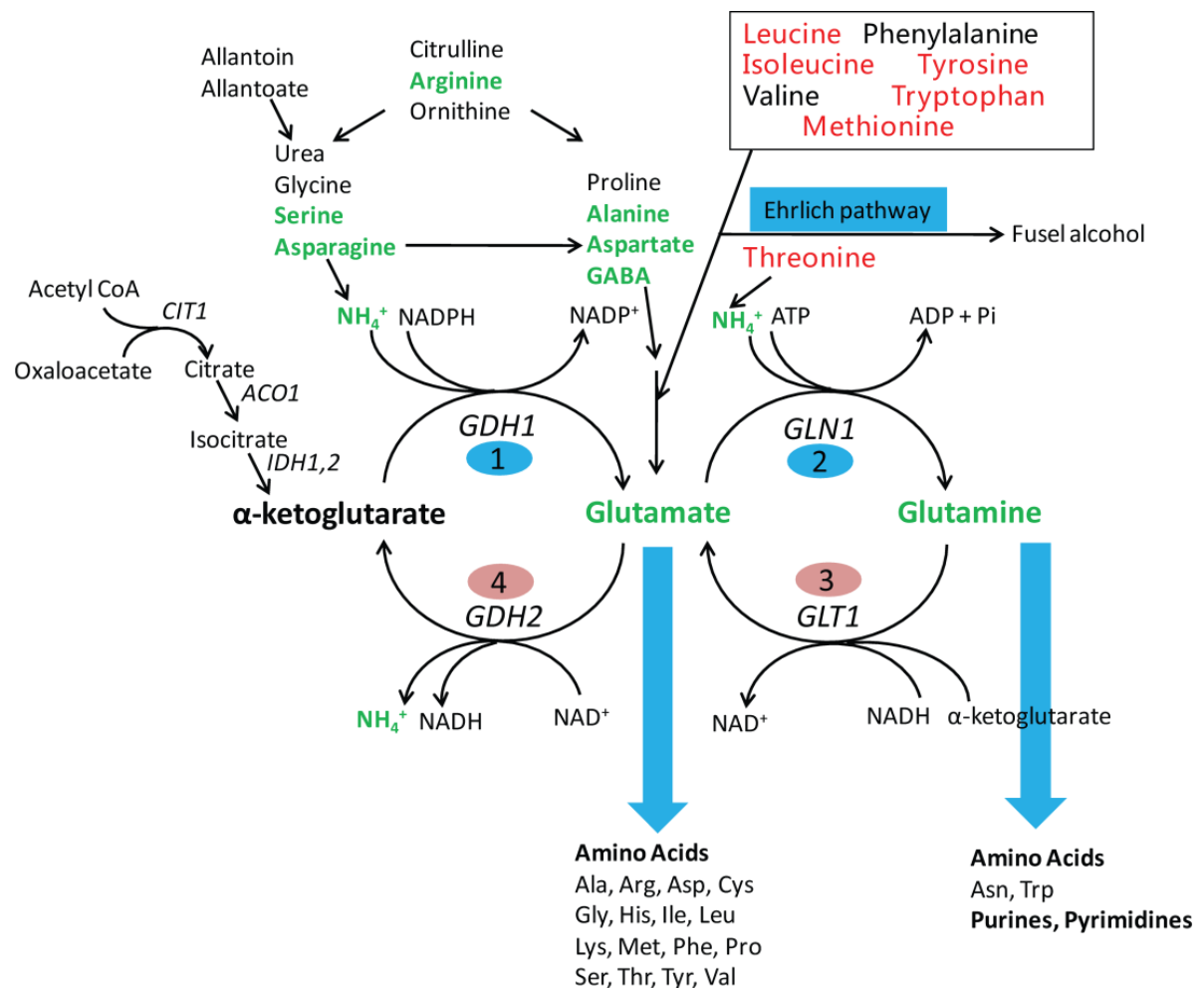
Figure 1.2 Schematic diagram of nitrogen assimilation pathways in *S. cerevisiae*: Glutamine is synthesised by combining glutamate produced from α-ketoglutarate (synthesized from acetyl CoA and oxaloacetate through an early step of the citric acid cycle, reaction 1) and ammonium (reaction 2). Other pathways of utilization of amino acids and production of compounds, such as amino acids and nucleotides are shown. Preferred nitrogen sources are indicated in green, while non-preferred nitrogen sources are indicated in red. The boxed amino acids can also be transaminated by aminotransferase to form glutamate. Different amino acids are synthesized from glutamate and glutamine (blue arrows). Reaction 1 and 2 (blue shade) require the catalysis of NADPH and ATP with NH_4^+, Gdh1p and Gln1p participation. Reaction 3 and 4 (pink shade) require NADH and NAD^+ respectively with enzyme participation of Glt1p and Gdh2p. Fusel alcohols are also formed via the Ehrlich pathway, adapted from Magasanik and Kaiser (2002), Hazelwood et al. (2008), Ljungdahl and Daignan-Fornier (2012).

1.3.2 Nitrogen metabolic pathways in S. cerevisiae

Yeast consumes nitrogen for its growth and metabolic activities, but different nitrogen sources do not support yeast growth equally (Magasanik 1992). For example, the presence of preferred nitrogen sources, such as ammonium, glutamate, and asparagine support maximal growth and suppress the transport of less preferable nitrogen compounds such as proline and urea (Cooper 1982; Magasanik 1992; ter Schure et al. 2000). The preferential assimilation of particular nitrogen sources and the blocking or down regulation of pathways for utilisation of non-preferred nitrogen sources are known as nitrogen catabolite repression (NCR) (Beltran et al. 2004; Magasanik 1992; Marks et al. 2003; Subileau et al. 2008; ter Schure et al. 2000). Yeast is also known to transport and utilise amino acids sequentially and in an ordered manner as a manifestation of this particular preference. For instance, ammonium ions and arginine are taken up first and then followed by glutamic acid, valine, isoleucine, leucine, histidine, aspartic acid, tryptophan, phenylalanine, and finally methionine (Henschke and Jiranek 1993). With the regulation of NCR, yeast is able to grow efficiently when presented with various nitrogen sources. If a preferable nitrogen source exists in the extracellular environment, it would inactive the enzymes that are responsible for utilization of poor nitrogen compounds (ter Schure et al. 2000). Often the inactivation process involves the phosphorylation state of the associated permease, which enables rapid adaption to changing environments. For example, *GAP1* encoding amino acid permease is regulated by preferable nitrogen presence in the medium (ter Schure et al. 2000; Beltran et al. 2004). The dephosphorylation of Gap1p can be activated by NCR and followed by Gap1p degradation via ubiquitylation of its N-terminal tails (Lys^9 or Lys^{16}) (Grenson 1983; Grenson 1992; Merhi et al. 2011; Soetens et al. 2001; Stanbrough and Magasanik 1995).

In central nitrogen metabolic pathways, ammonium has a crucial role in nitrogen catabolism and biosynthesis. Its transportation relies on three permeases, Mep1p, Mep2p and Mep3p, by which NH_4^+ is transferred from the extracellular environment into the yeast cell (Marini et al. 1994; Marini et al. 1997). Mep2p has the highest affinity for NH_4^+ (Km 1-2 μM), followed by Mep1p (Km 5-10 μM), and Mep3p (Km 1.4-2.1 mM). Triple deletion of the MEP genes in yeast leads to growth lethality in media with lower than 5 mM of ammonium as a single nitrogen supplement (Marini et al. 1997; ter Schure et al. 2000). Compared with *MEP1* and *MEP3*, *MEP2* expression is higher under low ammonium conditions. For higher expression of *MEP2*, at least one of the GATA family members (Gln3p and Nil1p) is needed as the activator. The expression of the MEP genes is repressed under high concentrations of ammonium (Marini et al. 1997). It has been noted that the presence of either one of the three NH_4^+ transporters is sufficient to allow growth on the same medium (Marini et al. 1997). When the amount of NH_4^+ is above 20 mM, none of the three transporters are necessary for yeast growth (Marini et al. 1997).

1.3.3 Nitrogen-related gene regulation in S. cerevisiae

Three enzymes, NADPH-glutamate dehydrogenase (NADPH-GDH), NAD-glutamate dehydrogenase (NAD-GDH) and glutamine synthetase (GS), are active in ammonium metabolic pathways (Courchesne and Magasanik 1988). Glutamate biosynthesis is catalysed by NADPH-GDH from alpha ketoglutarate and ammonium, whereas NAD-GDH degrades glutamate releasing ammonia. GS catalyses the biosynthesis of glutamine from glutamate and ammonia. Courchesne and colleagues (1988) have shown that *URE2* and *GLN3* regulate the biosynthesis of these enzymes, thus maintaining the balance of intracellular ammonia, glutamate, and glutamine required for optimal yeast growth. As mentioned, growth in the presence of sufficient

ammonium results in increased growth rates as compared with growth in the presence of proline or urea (Magasanik 1992; ter Schure et al. 2000). When ammonium is the sole nitrogen source in a medium, it is converted into glutamine and, in turn, repression of gene expression occurs through NCR. As the increase in ammonium concentration in the yeast cell causes an increase in the production of glutamine, nitrogen repression is indirectly mediated by ammonium (ter Schure et al. 1995; ter Schure et al. 2000).

In *S. cerevisiae*, the general amino acid permease (Gap1p) is a general amino acid transporter. Chiva and co-workers (2009) constructed a Δ*gap1* mutant and compared the fermentation kinetics, biomass production and nitrogen consumption to its parent strain during alcoholic fermentation under different concentrations of assimilable nitrogen. They found different consumption rates of amino acids between mutant and parent strain, especially in a high nitrogen content (HNC) fermentation. In the HNC fermentation, the Δ*gap1* mutant consumed 90 mg L^{-1} more YAN, and assimilated more arginine and alanine than the wild type. Interestingly, Δ*gap1* consumed twice as much glutamine as the wild type. This particularly does occur for amino acids influenced by NCR, such as aspartic acid, glutamic acid, and arginine. *GNP1* encoding a high-affinity glutamine permease, its expression was reduced in the Δ*gap1* mutant (Chiva et al. 2009). This suggests that glutamine consumption may not directly correlate with the *GNP1* transcription level, and its permease may be modified with high activity at post translational level.

1.3.4 Effects of YAN and its supplementation on wine yeast performance during alcoholic fermentation

Limiting YAN may result in problematic fermentation, for which increased probability of low yield of cell biomass, and reduced fermentation rates (Barbosa et al.

2012; Martínez-Moreno et al. 2012; Varela et al. 2004). Studies have showed that other factors, such as oxygen, temperature, as well as lipid, biotin, and grape solid content are correlated with YAN influences on yeast growth and fermentation performance (Aceituno et al. 2012; Beltran et al. 2007; Blateyron and Sablayrolles 2001; Bohlscheid et al. 2011; Casalta et al. 2013; Tesnière et al. 2013).

Varela and colleagues (2004) have reported that the amount of available nitrogen affects yeast biomass yield. Two levels of nitrogen, 50 mg L^{-1} (deficient YAN) and 300 mg L^{-1} (sufficient YAN), were used to determine the performance of the commercial *S. cerevisiae* strain EC1118 in alcoholic fermentation. Less biomass was formed in the nitrogen deficient medium as compared to where nitrogen was sufficient (i.e. 1.5 *vs*. 5.8 g L^{-1}). Insufficient nitrogen led to sluggish and stuck fermentation (29 days *vs*. 7 days), and with 15 g L^{-1} sugar remaining unutilised. This study backs up the theory that there is a positive correlation between assimilable nitrogen and yeast biomass formation (Varela et al. 2004). However, this correlation between cell growth and fermentative vigour and the effect of nitrogen source has been shown to behave in a strain dependent way (Barbosa et al. 2012). In Barbosa et al. (2012) 3 wine yeast strains were tested with different nitrogen sources (the total content was 267 mg L^{-1} YAN). The fermentation performance of 2 strains (PYCC4072 and EC1118) was influenced by different nitrogen sources. Nevertheless, strain UCD522 performed equally well with ~145 h fermentation duration under different nitrogen supplements (Barbosa et al. 2012).

The rate of yeast growth and fermentation is also reduced at lower temperatures (Beltran et al. 2007; Fleet and Heard 1993), implicating that nitrogen uptake may be linked to environmental temperature. Beltran and co-workers (2007) have shown that yeast nitrogen metabolic activities are affected by lower temperatures,

as less nitrogen is consumed at 13 ℃ than at 25 ℃ (Beltran et al. 2007). Another study by Abe and Horikoshi (2000) has demonstrated that under low temperatures (10~15 ℃), cell fluidity slows diminishing the transport ability of tryptophan permeases located on the cell membrane. Meanwhile, a study by Molina and co-workers (2007) reports no difference in biomass yield of strain EC1118 between high and low temperature conditions (28 and 15 ℃ for 5.2 vs. 5.7 g L^{-1}, respectively). Experimental differences here could simply be explained by differing YAN requirements and optimum fermentation temperatures between the different wine yeast strains examined. For example, QA23 catabolised all sugars (200 g L^{-1}) in the synthetic media (300 mg L^{-1} YAN in total) in 13 and 29 days, and consumed 146 and 118 mg L^{-1} YAN from media at 25 ℃ and 13 ℃, respectively, more biomass population was formed in the medium at 25 ℃ than at 13 ℃ (Beltran et al. 2007). On the other hand, Molina used EC1118 strain to ferment a synthetic medium with higher sugars (240 g L^{-1}) and a similar quantity of YAN supplement (300 mg L^{-1}). No measurable nitrogen was detected after 60 and 160 h at 28 and 15 ℃, respectively, and the sugar catabolism rate by EC1118 were 1.8-fold higher at 28 ℃ compared its rate at 15 ℃ (Molina et al. 2007). It is not surprising that EC1118 took longer time to form similar amounts of cell biomass under low temperature with the similar limited amount of available nitrogen.

To overcome sluggish or stuck fermentations caused by low initial YAN, nitrogen supplementation is widely employed during winemaking. The effectiveness of nitrogen addition has been investigated by numerous researchers (Bely et al. 1990; Blateyron and Sablayrolles 2001; Jiménez-Martí et al. 2007). An early study by Bely and co-workers (1990) has noted that nitrogen addition is effectively dependent on the initial nitrogen content. For example, if the initial nitrogen concentration is low at the

beginning of fermentation, adding ammonium salts can rapidly increase the rate of CO_2 production and shorten the fermentation process. However, if it is initially high ($\sim$ 300 mg L^{-1} N), the CO_2 production rate is not affected compared with the lower initial YAN must. Furthermore, the most effective point for the addition of assimilable nitrogen is before the half way stage of the fermentation (Bely et al. 1990). The reason could be due to arrest of sugar transporter protein synthesis at a later stage of fermentation after yeast cellular growth, which could not be corrected by addition of available nitrogen (Salmon 1989). Oxygen availability along with the assimilable nitrogen content is also a determining factor of the maximum fermentation rate (Blateyron and Sablayrolles 2001). The combination of these two nutrients in a supplementation strategy can be particularly effective, especially when additions are made at three stages (before yeast inoculation, at the half-way point of fermentation and in the stationary phase of cell growth with addition of oxygen) (Blateyron and Sablayrolles 2001). Nevertheless, the decision of nitrogen supplementation before or after yeast inoculation in must is still required to be made carefully. If available nitrogen is not severely limiting in musts, nitrogen supplementation would not be an ideal solution, as the addition may lead to unbalanced nutrients for yeast utilisation and growth (Bisson 1999), and may actually cause problematic fermentations. From a practical point of view, it is important to determine initial YAN in musts before fermentation. Also, the requirement for YAN for specific wine yeast strains needs to be determined correlated with YAN additions and their impact on wine flavour and style within a given oenological environment.

1.3.5 Effects of YAN supplementation on wine aroma and sensory characteristics

Various wine volatiles, by-products of wine yeasts formed during alcoholic fermentation, contribute to both wine aroma and the complex perception of 'mouth-

feel'. The formation of these yeast derived volatile compounds (such as: higher alcohols, organic acids and esters) is affected by the nitrogenous composition of the grape must, due to their direct involvement in the metabolic pathways of volatile formation in yeast (Henschke and Jiranek, 1993; Bell and Henschke 2005). As precursors of flavour and aroma compounds in wine, some amino acids form α-keto acids by deamination, which are then decarboxylated to produce the corresponding alcohols through the Ehrlich pathway (Hazelwood et al. 2008). In the reactions that follow, higher alcohols react with acetyl-CoA to generate esters (Bell and Henschke 2005; Hazelwood et al. 2008; Hernández-Orte et al. 2005; Nykänen 1986; Vilanova et al. 2007). Investigations of the relation between the YAN consumption and subsequent formation of by-products during alcoholic fermentation have found that the concentration of acetic acid, fatty acids and fusel alcohols is related to the particular yeast strain present, but also the concentration and type of nitrogen source in must (Hernández-Orte et al. 2005; Vilanova et al. 2007). Thus with addition of ammonium salts or amino acids, both fermentation rate and sensory profiles of wine can be affected.

A number of studies have investigated the influence of the addition of ammonium salts and/or amino acids on wine properties (Arias-Gil et al. 2007; Barbosa et al. 2009; Carrau et al. 2008; Garde-Cerdán and Ancín-Azpilicueta 2008; Hernández-Orte et al. 2005; Jiménez-Martí et al. 2007; Miller et al. 2007; Ugliano et al. 2011; Ugliano et al. 2008; Ugliano et al. 2010; Vilanova et al. 2007). Interestingly, aroma compound production is also found to be influenced by the timing of nitrogen supplementation (Barbosa et al. 2009). In Barbosa et al. study, three wine yeast strains, EC1118, PYCC4072 and UCD522, underwent fermentation in a synthetic medium with 200 g L^{-1} glucose and 67 mg L^{-1} initial YAN, consisting of DAP or an amino

acid mix. 200 mg L^{-1} DAP was supplied into the media initially or 72 h after inoculation. With the later addition of DAP, the concentration of some flavour compounds was altered. For example, there was an increase in 2-phenylethanol (honey and rose aroma), ethyl isobutyrate (sweet rubber), 2-phenylethyl acetate (fruity), ethyl 2-methylbutyrate (apple) and ethyl propionate (banana apple) detected, whilst ethanol and acetic acid were decreased in all three strains. An earlier study by Hernández-Orte and co-workers (2005) reported that adding any DAP or amino acid mix before yeast inoculation reduced 2-phenylethanol by 65% in an Airen grape must. This provides further evidence that both the time and context (environment and particular yeast(s) present) of nitrogen addition have the potential to influence wine flavours. Furthermore, another study by Webster and co-workers (1993) reported that increased nitrogen fertilizer supplementation in the vineyard decreased the concentration of 2-phenylethanol in the final wine. In any case, it seems clear that supplementation of ammonium salts is the most common way to adjust YAN content and alter aromas in winemaking. The effects of addition ammonium salts on volatile compounds at different stages of fermentation are summarised in Table 1.1.

Table 1.1 Effects of nitrogen addition on wine volatile formation

Volatile	Descriptors	Conc. of volatiles at different times		References
		Before yeast inoculation	**Stationary phase**	
Alcohols				
2-phenylethanol	honey and rose	–	–	Hernández-Orte et al. (2005); Barbosa et al. (2009)
methionol	boiled potatoes	–	NI	Hernández-Orte et al. (2005); Ugliano and Henschke (2009)
isoamyl alcohol/3-methylbutanol	alcohol, herbaceous	–	NI	Hernández-Orte et al. (2005); Ugliano and Henschke (2009)
Esters				
ethyl acetate	Fruity, solvent	+/–	+/–	Barbosa et al. (2009); Ugliano and Henschke (2009)
acetate esters	Flowery, fruity	+/–	+/–	Barbosa et al. (2009); Ugliano and Henschke (2009)
2-phenylethanol acetate	Fruity	+	++	Barbosa et al. (2009)
2-phenylethyl acetate	Fruity	+	++	Hernández-Orte et al. (2005)
isoamyl acetate	Banana, fruity	+	+	Hernández-Orte et al. (2005)
ethyl propionate	Banana apple	+	++	Barbosa et al. (2009)
ethyl isobutyrate	Sweet, rubber	+	++	Barbosa et al. (2009)
Acids				
propanoic acid	NI	+	NI	Hernández-Orte et al. (2005)

+, Increase; ++, Large increase; –, Decrease; +/-, Variable; NI, no information

Note that ammonium chloride was added in Jiménez-Martí et al. (2007), DAP was added in Barbosa et al. (2009), and for Hernández-Orte et al. (2005), both DAP and amino acids addition results are summarised here.

Wine yeast *S. cerevisiae* is also well known to have the potential to release H_2S during alcoholic fermentation. This is a constant issue in industrial winemaking since depletion of YAN in a fermenting must is identified as a frequent cause of increasing production of H_2S. However this is also dependent upon yeast strain, fermentative behaviour, availability of sulfur precursors, and the activity of sulfite reductive enzymes (Jiranek et al. 1995b). The over-production of H_2S can be rectified by nitrogen supplementation (ammonium or amino acids) at later stages of yeast growth, indicating that active sulphur metabolic pathways sequester H_2S thereby reducing its release into the medium (Jiranek et al. 1995b). Recently, Barbosa and co-workers (2012) have established that nitrogen composition affects H_2S release during alcoholic fermentation, as supplementation with methionine inhibited the formation of H_2S by wine yeast. It could be due to the suppression of sulfate reduction sequence pathway (Jiranek et al. 1995b). Also, use of DAP as a sole nitrogen source or as a component in nitrogen supplement (267 mg L^{-1} YAN in total) actually caused an increased H_2S observed in strain UCD522 and PYCC4072, whilst lower amounts of H_2S was released by EC1118 with DAP supplementation (Barbosa et al. 2012). Similar results have been reported by Butzke and Park (2011) that rectifying H_2S release by wine yeast with DAP supplementation is limited. A possible basis for the negligible effect of nitrogen addition on H_2S production may be linked to the genetic variation of key sulfur metabolic genes in different wine yeast strains. For example, based on genetic analysis on different commercial wine yeast progenies M2 and E4, the major sulfite reductive enzymes and corresponding genes, such as *MET2*, *MET5* and *MET10* have been identified to associate with low H_2S production in either M2 or E4 strain (Huang et al. 2013).

YAN supplementation also influences the formation of biogenic amines in wine, and the regulation of biogenetic amine production is currently attracting increased attention (Bach et al. 2011; Marco et al. 2006). During alcoholic and malolactic fermentation, amines often are present in other fermented foods and beverages generated by microorganisms via amino acid decarboxylation (Önal 2007; ten Brink et al. 1990). Toxic biogenic amines, histamine, tyramine, and β-phenyl-ethylamine (PHM) are regularly detected in wine at concentrations up to 4.15 mg L^{-1} (histamine), 7.6 mg L^{-1}(tyramine), and 1.7 mg L^{-1} (PHM). The toxic concentrations of these amines are reported as 20 mg L^{-1} for histamine, 25~40 mg L^{-1} for tyramine, and 3 mg L^{-1} for PHM (Soufleros et al. 1998). Non-toxic amine groups, putrescine and cadaverine, are suggested as precursors of nitrosamines regarded as carcinogens (Santos 1996). Marco and colleagues (2006) have studied the effects of the addition of yeast autolysate to Chardonnay must on amine production. Yeast autolysate supplies amino acids and fatty acids for yeast growth and metabolic activities. An increase of amine was found after malolactic but not alcoholic fermentation (Marco et al. 2006). The effects of DAP supplementation on biogenic amine formation has been investigated by Bach and co-workers (2011). Here primary and secondary fermentation of Grenache and Syrah grape musts were consecutively undertaken by *S. cerevisiae* and *Oenococcus oeni*. Both musts containing limiting nitrogen content (75 mg L^{-1}) were supplied with 75-385 mg L^{-1} YAN as DAP. After malolactic fermentation, histamine increased up to 10 mg L^{-1} in the wine received nitrogen supplementation (Bach et al. 2011).

1.3.6 YAN deficiency prediction during alcoholic fermentation

In view of the importance of YAN on yeast fermentation performance, the prediction of the likelihood and the time point at which nitrogen depletion occurs has

been targeted in several research studies. This information is expected to facilitate improved nutrient management in the winemaking process (Carrasco et al. 2003; Jiménez-Martí et al. 2007; Mendes-Ferreira et al. 2007a; Mendes-Ferreira et al. 2007b). Evidence has shown that amounts of specific chemicals, enzyme activity and gene expression can be used to predict nitrogen limitation (Table 1.2).

Some studies suggest that the expression of genes such as *CAR1* and *ACA1* are useful in revealing nitrogen depletion (Carrasco et al. 2003; Gasch et al. 2000; Jiménez-Martí et al. 2007). Arginase encoded by *CAR1* catalyses the degradation of arginine, which is diverted from the vacuole under nitrogen limitation (Sumrada and Cooper 1982). Arginase activity is determined by factors such as NCR, arginine concentration and transcriptional/post-translational modifications (Messenguy and Dubois 1983). Carrasco and colleagues (2003) have demonstrated that the activity of arginase is significantly increased under nitrogen starvation, although *CAR1* expression is independent upon the nitrogen sufficiency or insufficiency. The limitation of this study is that arginase activity has only been measured under ammonia exhaustion. However, as ammonium only makes up to 40% of the total YAN (with amino acids as the remainder) it would be worthwhile to examine the correlation of arginase activity and YAN (including amino acids) depletion. Jiménez-Martí and co-workers (2007) have performed a further study to determine arginase activity under limiting YAN (60 mg L^{-1}). A remarkable increase of arginase activity was detected ~72 h after inoculation, which was synchronized with nitrogen depletion. *ACA1* encoding an ATF/CREB family basic leucine zipper (bZIP) transcription factor involved in carbon source utilisation (Garcia-Gimeno and Struhl 2000) has also been investigated under laboratory conditions. Its transcriptional abundance was found to be induced by nitrogen starvation (Jiménez-Martí et al. 2007). In addition a sharp

increase of the *ACA1* mRNA occurred around 72 h consistent with nitrogen exhaustion (Jiménez-Martí et al. 2007).

A systematic genome-wide analysis of the transcriptional profile of wine yeast under a limited nitrogen with or without nitrogen refeeding has been investigated by Mendes-Ferreira and co-workers (2007a; 2007b), in order to identify nitrogen-responsive genes at transcriptional level to predict the timing of nitrogen starvation, the application of which aims to aid prevention of sluggish and stuck fermentations. Firstly, under limited nitrogen, the yeast global-wide transcriptional profile and reprogramming of gene expression after nitrogen supplementation have been examined. They have pooled out a list of genes whose expression was either up- or down-regulated by low nitrogen stress, compared to the control at normal nitrogen level (178 mg L^{-1} nitrogen after 24 h) (Mendes-Ferreira et al. 2007a). At early stage of nitrogen depletion (24 h ~ 48 h), approximately 2,400 out of 5,227 genes were upregulated, and 664 genes (13% of the total) were down-regulated. At later stage of fermentation (96 h ~ 144 h), 628 genes (12% of 5,284 genes) were determined with lower expression level compared to the control (24 h, 178 mg L^{-1} nitrogen). It suggests that the transcriptional response induced by low nitrogen stress is maintained till the later stage of fermentation (Mendes-Ferreira et al. 2007a). Another further comparison has revealed that under limited nitrogen conditions about 23% genes (1,328) were altered. In these variations, 826 genes correlated with metabolism, NCR, cell wall, and pH regulation were found to be down regulated. 207 genes associated with ribosomal synthesis and biogenesis were upregulated, suggesting their important function for cell viability under nitrogen depletion conditions (Mendes-Ferreira et al. 2007a). After nitrogen addition, the expression of 2,069 genes (40%) corresponding

with ribosomal synthesis and biogenesis, nitrogen catabolite repression (NCR) was observed to be altered (Mendes-Ferreira et al. 2007a).

Then Mendes-Ferreira and co-workers (2007b) have explored genes with vigorous changes response to nitrogen starvation in order to obtain useful genes (biomarkers) for monitoring nitrogen conditions during alcoholic fermentation. 36 genes have been identified down-regulated by $\geq$ 2-fold in the strain PYCC4072 after nitrogen refeeding (addition of 200 mg L^{-1} of nitrogen after 72 h when initially 66 mg L^{-1} of nitrogen exhausted), and with greatly up-regulation by $\geq$ 3-fold when nitrogen re-exhausted at 96 ~ 144 h (Mendes-Ferreira et al. 2007b). 4 candidate genes, *MSC1*, *XYL2*, *RTN2* and *ODC1*, are further validated by semi-quantitive PCR (for gene descriptions see Table 1.2). *MSC1*, *RTN2* and *ODC1* have been reported to be induced both under ammonium starvation and carbon limitation (Boer et al. 2003; Tai et al. 2005). *RTN2* and *MSC1* whose expression can also be changed under environmental stresses are suggested to be a part of the environmental stress response (ESR) in yeast (Gasch et al. 2000; Mendes-Ferreira et al. 2007b). *XYL2* encoding xylitol dehydrogenase, its expression has shown to be affected by glucose repression (Mendes-Ferreira et al. 2007b). The high transcript abundance of *XYL2* during diauxic growth hints carbon source limitation in media. In terms of applying these observations to aid in determination of the nitrogen depletion time point, Mendes-Ferreira et al. (2007b) validate the transcriptional profile of these 4 genes in another wine yeast strain ICV16 that displays a similar profile to PYCC4072.

Another approach to monitor nitrogen depletion is to determine NCR gene expression changes, as the nitrogen condition would shift from repression to de-repression during fermentation (Beltran et al. 2004; Gutiérrez et al. 2013). More recent research by Gutiérrez and co-workers (2013) has reported that 4 candidate

genes *DAL4, DAL5, DUR3* and *GAP1* involved in NCR regulation, were more induced under nitrogen limiting conditions (60 mg L^{-1}) in comparison to their repression statures under excess nitrogen (1200 mg L^{-1}, for selection NCR genes) in the wine yeast strain PDM. The authors propose the transcripts of *GAP1* and *DAL4* as good indicators due to their greatly increased transcripts (~100- to 400-fold) at nitrogen depletion than their suppression under excess nitrogen conditions. A fast and easy method to detect nitrogen depletion with the use of a green fluorescent protein (GFP) fusion construct under the control of the *GAP1* or *DAL4* promoter has also been developed (Gutiérrez et al. 2013). The fluorescence emission is observed to be strain dependent. For example, under limited nitrogen conditions, 10 h after inoculation, strain RVA and TTA were detected via emission of fluorescent signals, PDM and ARM were determined to have increased fluorescence after 14 h and 15 h, respectively. These suggest that the induction of genes and GFP signals correlate with nitrogen uptake rate of specific wine yeast strain, and it could be subsequently applied to determine the nitrogen requirements of commercial wine yeast strains, in order to prevent and rectify limiting YAN during fermentation (Gutiérrez et al. 2013).

Table 1.2 Genes investigated for prediction of nitrogen limitation during fermentation by Carrasco et al. (2003), Jiménez-Martí et al. (2007), Mendes-Ferreira et al. (2007b) and Gutiérrez et al. (2013)

Genes	Descriptions[a]	References
CAR1	Enzyme involved in arginine catabolism	Carrasco et al. (2003); Jiménez-Martí et al. (2007)
ACA1	Carbon source utilisation transcription factor	Gasch et al. (2000); Marks et al. (2003); Jiménez-Martí et al. (2007)
MSC1	Protein of unknown function involved in meiotic recombination	Mendes-Ferreira et al. (2007b)
XYL2	Xylitol dehydrogenase	Mendes-Ferreira et al. (2007b)
RTN2	Reticulon protein	Mendes-Ferreira et al. (2007b)
ODC1	Mitochondrial inner membrane transporter	Mendes-Ferreira et al. (2007b)
DAL4	Allantoin permease	Gutiérrez et al. (2013)
DAL5	Allantoin permease	Gutiérrez et al. (2013)
DUR3	Urea and polyamines plasma membrane transporter	Gutiérrez et al. (2013)
GAP1	General amino acids permease	Gutiérrez et al. (2013)

[a] information from SGD database (http://www.yeastgenome.org/)

1.3.7 Nitrogen efficient wine yeast

The minimum YAN in grape musts required by yeast for successful completion of alcoholic fermentation has been measured in many previous studies (Bely et al. 1990; Blateyron et al. 2003; Henschke and Jiranek 1993; Mendes-Ferreira et al. 2004). However, due to grape varieties, fermentation conditions and specific yeast strain requirements, this measurement is quite variable (Gardner et al. 2005; Gardner et al. 2002; Jiranek et al. 1991; Jiranek et al. 1995a; Manginot et al. 1998). Therefore, the nitrogen demand of yeast for the successful completion of fermentation is now considered as a criterion for industrial strain classification and selection (Jiranek et al. 1991).

Selection of nitrogen efficient wine yeast strains, regardless of the nitrogen conditions they are presented with, provides an alternative approach to overcome the issues caused by limited YAN. For later identification and characterisation of mutants, a definition of nitrogen efficiency is proposed here: nitrogen efficient wine yeast strains are those capable of efficient sugar catabolism (fermentation) under limiting nitrogen conditions, or else are able to catabolise a given amount of sugar whilst utilising less nitrogen. For example, nitrogen efficient yeast strains have a reduced nitrogen demand in the context of the fermentable sugars present.

Manginot and co-workers (1998) investigated the nitrogen demand of several commercial yeast strains during alcoholic fermentation of media with initial assimilable nitrogen set at 125 mg L^{-1} and supplied other nitrogen sources (ammonium phosphate, glycine, or methionine) ranging from 50 to 250 mg L^{-1}. This research showed that the nitrogen demand among yeast strains was different. Within this study a positive correlation between nitrogen utilisation and biomass formation was observed.

Yeasts require different YAN amounts also correlate with H_2S liberation. Gardner and co-workers (2002) reported that AWRI835 performed as a more nitrogen efficient strain than AWRI796 whose YAN requirement was 8% more, and its H_2S production was higher (120 *vs.* 87 μg) under the limited nitrogen condition (125 mg L^{-1}). Another study demonstrated that yeast strains with the absence of either one or both of the *NGR1* or *GID7* genes had an improved fermentation performance (Gardner et al. 2005). Notably, only the double-deletion strain completely catabolised 200 g L^{-1} glucose under limited ammonium supplement (75 mg L^{-1}). This double deletant was found to use 71% more glucose than the wild type, and 30 - 45% more glucose than either of the single deletants. Based on these results, *NGR1* and *GID7* are described as genes that affect nitrogen efficiency, i.e. deletion confers high nitrogen efficiency (HNE). Combined with other research evidence, one current hypothesis that might explain nitrogen efficiency caused by the absence of these two genes relates to redox balance; i.e. that the conversion of NAD$^+$/NADH and NADP$^+$/NADPH (van Dijken and Scheffers 1986) has been altered in these mutants. Yeast cells maintain vital metabolic activities with the aid of redox balance in the cytoplasm and mitochondria. During glycolysis, NAD$^+$ is regenerated through the conversion of pyruvate to ethanol under anaerobic conditions (alcoholic fermentation). If pyruvate is transferred into the mitochondria, it is respired to produce energy (e.g. ATP). NADH can also be transported through a porin channel into the mitochondria, which would affect the distribution of NADH across the inside or outside of mitochondria. Buu et al. (2004), showed that the level of mRNA for the mitochondrial porin protein was influenced by Ngr1p, which could begin to explain the basis for nitrogen efficiency. The other nitrogen efficiency candidate gene, *GID7*, when deleted affects the gene expression of *GDH2*, whose transcriptional regulation is influenced

by the nitrogen source (Gardner et al. 2005; Miller and Magasanik 1991). Gdh2p is a NAD-dependent glutamate dehydrogenase, and functions in degrading glutamate to ammonium and α-ketoglutarate. Taking this into consideration, deletion of *GID7* may cause the conversion between glutamate and ammonium and further result in nitrogen efficiency. Mechanisms for nitrogen efficiency hypothesised by Gardner and co-workers (2005) also include the following: (1) Efficient and maintained sugar transporter synthesis; and (2) Reduction of non-essential fermentation unrelated energy costs. Clearly, further characterisation is required.

In order to further investigate the mechanism of high nitrogen efficiency and gain a deeper understanding as to how HNE provides enhanced fermentation performance in wine yeast, several other HNE candidate genes isolated by Maria Astorga, a previous member of our group, remain uncharacterised. Identification and understanding of how a greater number of HNE genes contribute to nitrogen efficiency would greatly enhance understanding of the genetic determinants, and opportunity for manipulation of nitrogen efficiency. This has formed the basis of this study.

1.3.8 Improvement of industrial wine yeast

Commercial wine yeast strains have been selected and improved over hundreds of years and are widely regarded as a superior choice since these strains possess desirable fermentation characteristics such as stress tolerance, fermentative vigour and formation of desirable aromas and flavours in wine (Astorga et al. 2007; Bauer and Pretorius 2000; Donalies et al. 2008). This choice is also influenced by the convenience and reliability of readily available purified yeast strains. Generally, the development of industrial oenological yeast strains is dedicated to increasing the velocity and efficiency of wine fermentation and the quality of the final product

(Donalies et al. 2008). The continual occurrence of challenging fermentation conditions and the desire to produce novel wines for emerging markets merits further improvement of yeast strains to provide winemakers with a tool kit to meet changing consumer demands and production pressures.

So far, the technologies used for improving yeast strains include selection, classical traditional selection/non-recombinant methods and genetic metabolic engineering/recombinant methods. Traditional selection methods are more acceptable to consumers than genetically modified organism (GMO). McBryde and co-workers (2006) have isolated mutants via adaptive evolution. These selected mutants often possess different behaviours in metabolic productions, and shorter fermentation durations compared with the parent strain (McBryde et al. 2006). However, such strategies also have drawbacks such as time consuming, non-specific, and can potentially result in the loss of desirable characteristics (Liccioli 2010). Recombinant methods offer fast and specific development of yeast properties that can be utilised to explore and recognize the mechanism behind specific characteristics such as fermentation efficiency under limited nitrogen conditions. Although using genetically modified yeast in winemaking is illegal in Australia, these genetic approaches can assist traditional wine yeast breeding, and also help to explore the correlation between genes and corresponding traits.

1.4 Functional systematic genetic analysis of *S. cerevisiae* with the use of yeast mutant libraries

The ever-increasing improvement and accessibility of systems biology methods have allowed an ever expanding "top down" understanding of the metabolism of yeast. *S. cerevisiae* in particular has been used as a model organism for many years, now resulting in an array of research tools, such as systematic libraries,

available for this species. This has allowed the exploration of genetic interaction networks, and several recent investigations have focused on understanding how these networks result in operation of biological pathways (Boone et al. 2007; Breitkreutz et al. 2010; Costanzo et al. 2010; Mattiazzi et al. 2010; Tong et al. 2004).

Compared with laboratory strains, commercial wine strains that exhibit good sensory properties, show better performance in the industrial production environment of low pH, high sugar concentration (e.g. over 200 g L^{-1}), nutrient limitation, etc. (Astorga et al. 2007). Thus, the information on gene interactions in laboratory strains might fail to reflect the traits of an industrial wine strain (Borneman et al. 2011). Unfortunately there are not yet any commercialized resources for systematic genetic investigation of industrial wine yeast. For the moment, any efforts to use such tools are restricted to using those developed for and in the laboratory yeast background.

Based on the principles shown by recent studies, genetic interactions and networks are systematically being constructed in laboratory yeast background, and many tools and methods for large-scale functional analysis in laboratory yeast may be manipulated for application to industrial strain research (Boone et al. 2007; Costanzo et al. 2010). For example, many systematic laboratory yeast gene mutant libraries are currently available commercially, such as the yeast deletion library established by the University of Stanford (Giaever et al. 2002), by which target genes were disrupted using a PCR-based gene deletion strategy (Wach et al. 1994). Briefly, this set of libraries was made via constructing a kanamycin-resistance cassette, *KANMX*, including two unique 20 bp flanking barcodes, which replaced the open reading frame (ORF) of each target gene. 90% of the known ORFs are thought to have a representative clone in this library. Winzeler et al. (1999) studied the functional characteristic of 6926 ORFs by a high-throughput strategy where they found that 17%

of the genes are essential for the yeast cell life cycle. The phenotypes of mutants were also analysed through parallel assays with different growth conditions, where they also found that 40% of the analysed deletion strains displayed growth defects either in rich or minimal media (Winzeler et al. 1999). Libraries such as these can be screened for genes linked to desirable industrial traits and phenotypes can then be checked within an industrial yeast background.

To get comprehensive information about wine yeast genomics, a Wine Yeast Gene Deletion Library (WYGDL) has been constructed by The Australian Wine Research Institute (AWRI) (AWRI website: http://www.awri.com.au). The screening of this library is ongoing. The genetic information obtained under different conditions will be beneficial for the exploitation of specific yeast strains possessing desirable properties that can be used to improve the quality of wine. So far this library is not commercially available.

Additionally, a mutagenesis library created with a multipurpose transposon (mTn-3xHA/GFP/URA3) designed by Dr M Snyder (Yale University) has been obtained by this laboratory. This enabled construction of a random disruption library in a wine yeast by way of insertion of transposons across the genome. In a previous study within this laboratory, Maria Astorga initially extracted each plasmid in *E. coli* and transformed them into a haploid wine yeast strain (1B14). 3 stages of fermentation screening were performed: (1) 1 mL 48-well plate microfermentations (2) 10 mL mini-scale fermentations (3) 100 mL laboratory scale fermentations, each in chemically defined grape juice media with limiting nitrogen. Based on these screenings and selection work, 6 HNE candidate mutants were isolated and the genes identified. Together with other potential HNE candidates from data mining and other

current research from this laboratory, a group of candidate genes that may be involved in nitrogen efficiency was assembled for further analysis in this study.

In other recent genetic studies, over-expression libraries have also been a powerful tool for understanding of gene function. Two *S. cerevisiae* gene over-expression libraries are commercially available (Gelperin et al. 2005; Jones et al. 2008). One is a moveable open reading frame (MORF) library, containing 5854 yeast expression plasmids. Each plasmid has a single complete ORF driven by the *GAL1* promoter. The ORF cassettes are readily transferable into vectors for various purposes. The other is a systematic collection of the *S. cerevisiae* genome (as overlapping fragments) containing over 1,500 *E. coli* strains bearing the high copy vector pGP564. This library is thought to cover ~95% of yeast genome. This over-expression library can also be employed to screen the whole yeast genome at a functional level. Each of these overexpression libraries has disadvantages. For example, in the MORF library, target gene expression is driven by the *GAL1* promoter, which is regulated by the presence and quantity of galactose (Ramer et al. 1992). More importantly, this promoter is suppressed tightly and rapidly by trace amounts of glucose (Johnston et al. 1994), making it unusable in a grape like medium containing glucose. The other drawback of using *GAL1* promoter is that the amount of galactose decreases during the culture. As such, one faces the inconvenience of maintaining galactose concentration. Moreover, there are lethality and functionality issues in this form of over-expression library due to gene expression being driven by an exogenous promoter (Boone et al. 2007; Park and Kim 2006). In order for the library to be useful, a host yeast background in which galactose metabolism is not affected by glucose repression is required. On the other hand, in the Jones library, each ORF is driven by its endogenous promoter. However, each plasmid contains more than one ORF (of

which are not all functional), whose expression would be less in transcriptional abundance than the MORF library. Currently, there is no perfect overexpression system for industrial fermentation screens. The elimination or reduction of these limitations would be of great benefit for further research.

1.5 Thesis outline and concluding statement

In this review, the significance of nitrogen utilization for yeast during alcoholic fermentation has been discussed including its metabolism, effects on yeast fermentative behaviour, volatile production, and nitrogen management. Based on the review, the knowledge gaps are summarized as:

(1)   Only a few genes have been identified as HNE genes. The question remains whether there are any more genes that influence nitrogen efficiency and whose mutants result in enhanced fermentation performance.

(2)   What are the mechanisms of nitrogen efficiency in wine yeast alcoholic fermentation? Are HNE genes all involved in similar metabolic pathways?

In order to address these gaps, in brief, this research includes the following:

Recombinant methods and related genetic manipulation techniques were applied for quick and specific research study purposes. Through these, HNE candidates were selected and further investigated. Hypothesises are proposed to determine the HNE mechanism for each mutant at the genetic level (Chapter 2 and 3). The *ECM33* gene became of particular interest due to the especially robust and efficient fermentation phenotype of its deletion mutant. Δ*ecm33* analyses led to the hypothesis that alteration of cell wall chitin may improve yeast tolerance to alcoholic fermentation. This was analysed and reported in Chapter 4. Yeast as a single eukaryocyte cell evolves many approaches for adaption to stresses during alcoholic

fermentation. Two of the yeast signalling pathways associated with Ecm33p and yeast tolerance to high osmolarity are the cell integrity (CWI) pathway and high osmolarity glycerol (HOG) pathways. To understand the involvement of these two pathways and yeast nitrogen efficiency during fermentation behaviour, gene overexpression and OEX strain phenotypic analysis were undertaken (Chapter 5).

Meanwhile, the Yeast Genomic Tiling Collection (Jones et al. 2008) containing overexpression constructs was also constructed for yeast gene overexpression analysis on a genome-wide scale. In order to make this library suitable for screening wine yeast fermentative traits, the host *E. coli* library is purified and transformed into a wine yeast background strain (isoC9d Δ*leu2*, a derivative of the wine yeast strain L2056). This work was completed in collaboration with two other PhD students, Mr Trung Dung Nguyen and Ms Jade Joyce Haggerty. Unfortunately due to time constraints, this library has not been screened for HNE candidate genes.

In conclusion, the aims of this PhD project were to further identify and characterise HNE candidate wine yeast strains, genes and relative mechanisms, in terms of clarifying the influence of nitrogen efficiency on sugar catabolism. The knowledge gained from this research would be expected to provide some clues for the generation of more robust yeast.

Chapter 2 Identification and characterisation of HNE wine yeast candidates

2.1 Introduction

Grape sugars are catabolised by wine yeast and converted into alcohol, carbon dioxide, and other metabolites in an anaerobic process known as alcoholic fermentation. Whilst residual sugar is acceptable in sweet wine styles, to successfully make dry wines, inoculated yeast are required to consume nearly all sugars (residual less than 0.4%) (Bisson 1999). Many factors could cause yeast to fail in the completion of this task; a common one being the lack of yeast assimilable nitrogen (YAN). YAN as a major nutrient is indispensable for yeast growth, proliferation and physiological activities. Insufficient YAN in grapes increases the risk of sluggish or stuck fermentations for wine makers. To overcome this issue, nitrogen supplementation is a common practice, with additions being made at the beginning and during fermentation, often to within maximal legal limits, although they are not always effective. Also measurement of YAN is not done routinely, nor is this technique accessible to many winemakers. The use of high nitrogen efficient (HNE) wine yeast has the potential to provide winemakers with a sensible and cost effective alternative to fermentation management, as these yeast exhibit improved sugar consumption under a given and often limited amount of YAN (Adams and van Vuuren 2010; Gardner et al. 2005; Jiranek et al. 1991; Julien et al. 2000; Manginot et al. 1998; Ugliano et al. 2007).

To improve the metabolic performance of wine yeast with regard to nitrogen utilisation, high-throughput screening of haploid (recombinant) wine yeast under limiting nitrogen conditions is expected to unveil potential HNE-related candidates,

the genetic characterisation of which would help to better understand the associated cellular mechanisms at the molecular level.

A group of HNE candidate haploid wine yeast strains were previously isolated from a mutagenesis library (constructed by a former PhD candidate, Ms Maria A. Astorga). 1B14 was used as background strain (made by Dr Jennifer Gardner). Three stages of consecutive fermentation screening were undertaken under limited nitrogen conditions (1.3 mL 48-well plate fermentations, 10 mL fermentations and 100 mL lab scale fermentations). Inverse PCR and subsequent DNA sequencing were used to identify the location of Tn insertion within the genome for each strain of interest. In this study reported here, the corresponding gene deletion (ORF*::KANMX4*) for each identified gene was constructed in haploid wine yeast C911D (Walker et al. 2003). The equivalent laboratory yeast deletants were sourced from commercially available laboratory yeast deletion libraries (Invitrogen; Yeast Deletion Project, http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). These potential candidate HNE yeast were evaluated for fermentation performance under both limited and excess nitrogen conditions. The fastest strains selected for further investigation are to be described in Chapter 3. The information on the mutagenesis library and the low nitrogen fermentation trials are also shown in Chapter 3, whilst Chapter 2 reports on the results from the fermentations in excess nitrogen conditions. Investigations of candidate HNE mutants including cell growth, fermentation performance, cell morphology analysis, and growth response to specific chemicals, are discussed here.

2.2 Materials and Methods

2.2.1 Yeast strains

Based on Gardner et al. (2005), a few HNE candidate strains were constructed in the wine yeast background (C911D) by Maria A. Astorga. To understand more

about the function of each HNE gene, and phenotypic differences dependent on the genetic background (diversity), laboratory yeast strains were also included in this study. Haploid laboratory deletants were sourced from the haploid yeast deletion library (BY4741 background, Yeast Deletion Project, Invitrogen). Heterozygous diploid deletions in the BY4743 laboratory yeast background were ordered from Millenium Science.

Table 2.1 Yeast strains used

Yeast Strains	Relevant genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Yeast Deletion Project
BY4743	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0 /ura3Δ0*	Yeast Deletion Project
BY4741 Δ*psr1*	BY4741 Δ*psr1*::*KANMX4*	Winzeler et al. (1999)
BY4741 Δ*syn8*	BY4741 Δ*syn8*::*KANMX4*	Winzeler et al. (1999)
BY4741 Δ*slt2*	BY4741 Δ*slt2*::*KANMX4*	Winzeler et al. (1999)
BY4741 Δ*sps2*	BY4741 Δ*sps2*::*KANMX4*	Winzeler et al. (1999)
BY4741 Δ*ssn8*	BY4741 Δ*ssn8*::*KANMX4*	Winzeler et al. (1999)
BY4741 Δ*pst1*	BY4741 Δ*pst1*::*KANMX4*	Winzeler et al. (1999)
BY4741 Δ*ecm33*	BY4741 Δ*ecm33*::*KANMX4*	Winzeler et al. (1999)
BY4743 Δ*ssn8/SSN8*	BY4743 Δ*ssn8*::*KANMX4/SSN8*	Millenium Science
BY4743 Δ*ptp1/PTP1*	BY4743 Δ*ptp1*::*KANMX4/PTP1*	Millenium Science
BY4743 Δ*psr1/PSR1*	BY4743 Δ*psr1*::*KANMX4/PSR1*	Millenium Science
BY4743 Δ*ecm33/ECM33*	BY4743 Δ*ecm33*::*KANMX4/ECM33*	Millenium Science
BY4743 Δ*syn8/SYN8*	BY4743 Δ*syn8*::*KANMX4/SYN8*	Millenium Science
C911D	L2056 Δ*ho MAT*α	Walker et al. (2003)

C911D Δptp1	C911D Δptp1::KANMX4	This study
C911D Δssn8	C911D Δssn8::KANMX4	This study
C911D Δleu3	C911D Δleu3::KANMX4	This study
C911D Δecm33	C911D Δecm33::KANMX4	This study
C911D Δ134	C911D Δynl134C::KANMX4	This study

2.2.2 Media

All strains were initially grown in yeast peptone dextrose (YEPD or YPD) medium (1% yeast extract, 2% Bacto-peptone, 2% D-glucose) with addition of 2% agar when solid medium was required. Geneticin (200 mg L^{-1}, G418 sulfate, A.G. Scientific, Inc.; G-1033) was added for antibiotic selection.

Wine yeast and heterozygous laboratory yeast strains were tested in Chemically Defined Grape Juice Media (CDGJM) containing 200 g L^{-1} sugars (equimolar glucose and fructose) and different concentrations of nitrogen (amino acids and ammonia sulphate mix) based on initial work by Jiranek and co-workers (1995a). 150 mg L^{-1} of uracil (37 mg L^{-1} nitrogen), not usually present in the original recipe (Jiranek et al. 1995a) was included for heterozygous laboratory strain fermentations, as these laboratory strains have auxotrophic growth requirements for uracil, histidine, leucine, and methionine. Nitrogen concentrations for wine yeasts and heterozygous laboratory yeasts fermentations were 441 mg L^{-1} and 487 mg L^{-1}, respectively.

2.2.3 Lab scale (100 mL) fermentations

The fermentation performance of each deletion strain was evaluated in small-scale laboratory fermentations as described by Walker and colleagues (2003). Briefly, 100 mL fermentations were performed in CDGJM in 3-6 replicates under anaerobic

conditions. To perform the fermentation, 5×10^6 cells mL^{-1} of yeast cell from overnight YPD culture were inoculated into Chemically Defined Grape Juice Starter Medium (CDGJSM; Jiranek et al. 1995a). After growth overnight, 5×10^6 cells mL^{-1} were inoculated into CDGJM. Samples were taken regularly throughout fermentation, and supernatants frozen for later chemical analysis. Refractive index (RI) of samples was measured to monitor the fermentation progress, and Clinitest® test (Bayer® AG) was used to determine fermentation completion (< 2.5 g L^{-1}). Residual sugars (glucose and fructose) were measured using hexokinase and glucose-6-phosphate dehydrogenase (Megazyme; E-HKGDH) together with phosphoglucoisomerase (Megazyme; E-PGIEC) by enzymatic analysis (Henniger and Mascaro Jr 1985).

2.2.4 Cell growth assay

Cell growth of haploid laboratory deletion strains (BY4741 as background) was measured using UV/Vis spectrophotometer (μQuant Bio-Tek Instruments, Serial # 157952, USA), and the growth curve plotted against time. Each strain was streaked for single colonies onto an YPD plate. A single colony isolate was chosen and resuspended in YPD liquid at a final density of 2×10^7 cells mL^{-1}. 10 μL of diluted cell culture (1×10^6 cells mL^{-1}) were then inoculated into a 96-well plate with 190 μL of sterile YPD, using a liquid-handling robot (Corbett Robotics, Model # CAS3800). The 96-well plate was sealed with breathable membrane (Adelab Cat # AXY BF-400) to allow for gas exchange whilst preventing contamination. The plate was placed in the UV/Vis spectrophotometer and incubated for 133 h at 10 ℃. The experiment was conducted for 35 h at 29 ℃ and 35 ℃ respectively. The cell density, as estimated by optical density at 600 nm, was measured continually by the UV spectrophotometer. In exponential phase, the maximum cell growth rate, measured as cell density (OD$_{600}$) against time, is represented by the slope of the straight line (Figure 2.1). Four

neighbouring OD_{600nm} datasets for each strain along each side were used to generate the trend line, equation with slope (μ_{max}) and the correlation coefficient R^2 (Stanbury 1988; Toussaint et al. 2006).
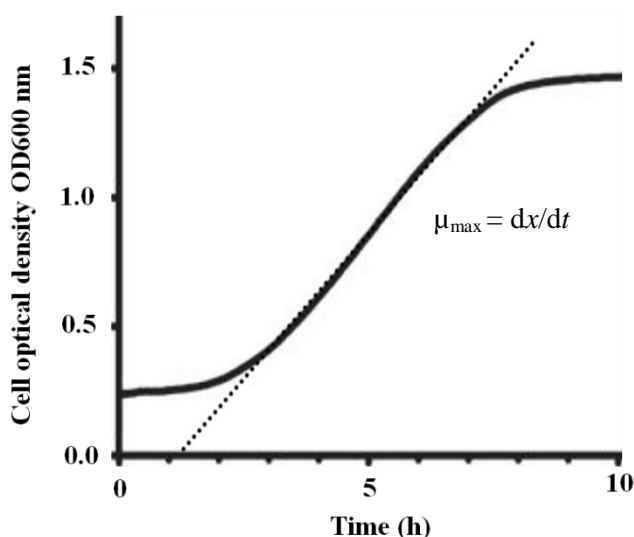

Figure 2.1 Cell growth curve ($OD_{600\ nm}$ versus time) and cell maximum growth rate μ_{max} (adapted from Stanbury 1988 and Tossaint et al. 2006)

2.2.5 Cell growth assay

A mutant lacking *ECM33* has been reported with certain phenotypes, including growth sensitivity to caffeine, Calcofluor White (CFW), Congo Red (CR), and $CaCl_2$ (Pardo et al. 2004, Takada et al. 2010). In order to investigate the role of *ECM33*, in this study, a serial dilution of overnight yeast cultures, grown in YPD were spotted onto YPD agar plates with different treatments. Phenotypic characteristics of Δ*ecm33* and other HNE candidates were measured with treatments of ethanol (7-13% v/v), pH (5-9), $CaCl_2$ (1.5 – 210 mM) and caffeine (12 mM). Overnight YPD cultures were re-inoculated into fresh YPD medium at 2×10^5 cells mL^{-1}. When the culture reached 1×10^7 cells mL^{-1}, 5 µL ten-fold serial dilutions were spotted onto solid YPD agar plates treated with different concentrations of chemicals. Growth was examined after 3 days incubation at 28 ºC.

2.2.6 Cell morphology examination

Cells were cultured in YPD overnight at 28 ºC with shaking (120 rpm), and then photographed via a Nikon microscope (ECLIPSE 50i) equipped with a Nikon (DS-2MBW) camera. 50 to 120 random selected cells were analysed by ImageJ (National Institutes of Health USA, http://rsbweb.nih.gov/ij/), for cell shape, length and width ratio.

2.2.7 Statistical methods

Excel 2007 (Microsoft® Office) and GraphPad Prism 6 (GraphPad Software Inc., La Jolla, C.A., USA) were applied for statistic analysis. Significant differences of specific data set were compared either using One-way ANOVA or Student t-test, both of which are integrated in the Prism 6.

2.3 Results

2.3.1 Fermentation trials

2.3.1.1 Haploid deletion wine yeast strain fermentation trial

Five HNE wine yeast candidates (in C911D, see Table 2.1) were employed for evaluation of their fermentation performance in CDGJM supplied with 200 g L^{-1} sugars and ~441 mg L^{-1} nitrogen. Four deletants exhibited increased sugar consumption when compared with the wild type strain: C911D Δ*ssn8*, C911D Δ*ecm33*, C911D Δ*ptp1* and C911D Δ*134*. C911D Δ*ecm33* displayed the fastest sugar consumption, with no residual sugar demonstrable after 105 ± 7 h. This was up to 30% faster than the wild-type C911D; the latter completing fermentation in 151 h. The other three strains, C911D Δ*ssn8*, C911D Δ*ptp1* and C911D Δ*134*, consumed sugar 14%, 8% and 6% faster, respectively, when compared to C911D (Figure 2.2 and Table 2.2). Nitrogen consumption by HNE candidates was also measured. No

significant difference was observed between the wild type C911D and the deletants, with the exception of C911D Δ*ssn8* (Figure 4 in Chapter 3), which showed increased nitrogen utilisation during fermentation compared with the wild type strain.

2.3.1.2 Heterozygous laboratory strain fermentation trial

To examine the effect of gene copy number on fermentation duration, five heterozygous diploid laboratory yeast strains with a single copy of the corresponding gene deleted were investigated using BY4743 as the background strain (Table 2.1). The measurement of sugar utilisation indicated that these five mutants fermented quicker than BY4743. Strain BY4743 Δ*syn8/SYN8* completed fermentation in the shortest time (116 h), which was approximately 21% faster than the wild type (146 h). The performance of the other four strains (Δ*ssn8*/*SSN8*, Δ*ecm33*/*ECM33*, Δ*ptp1*/*PTP1*, Δ*psr1*/*PSR1*) was close to wild-type BY4743 (146 h), with total sugar consumption occurring between 136-143 h (Figure 2.3 and Table 2.2).
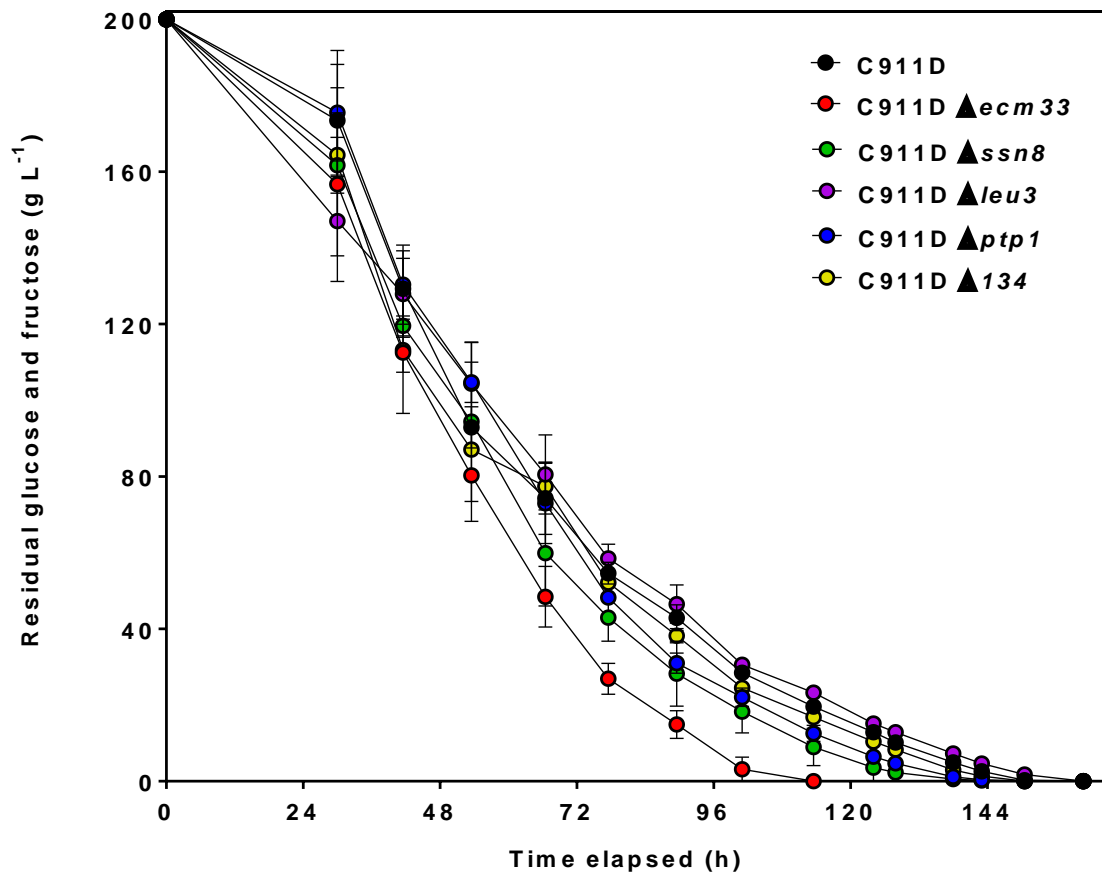
Figure 2.2 Sugar catabolised by C911D, C911D Δ*ptp1*, C911D Δ*leu3*, C911D Δ*ecm33*, C911D Δ*ssn8*, and C911D Δ*134* during fermentation in CDGJM with 440 mg L^{-1} nitrogen. Different strains are indicated with different colour circle symbols, data points are the mean (n = 3) ± SD.
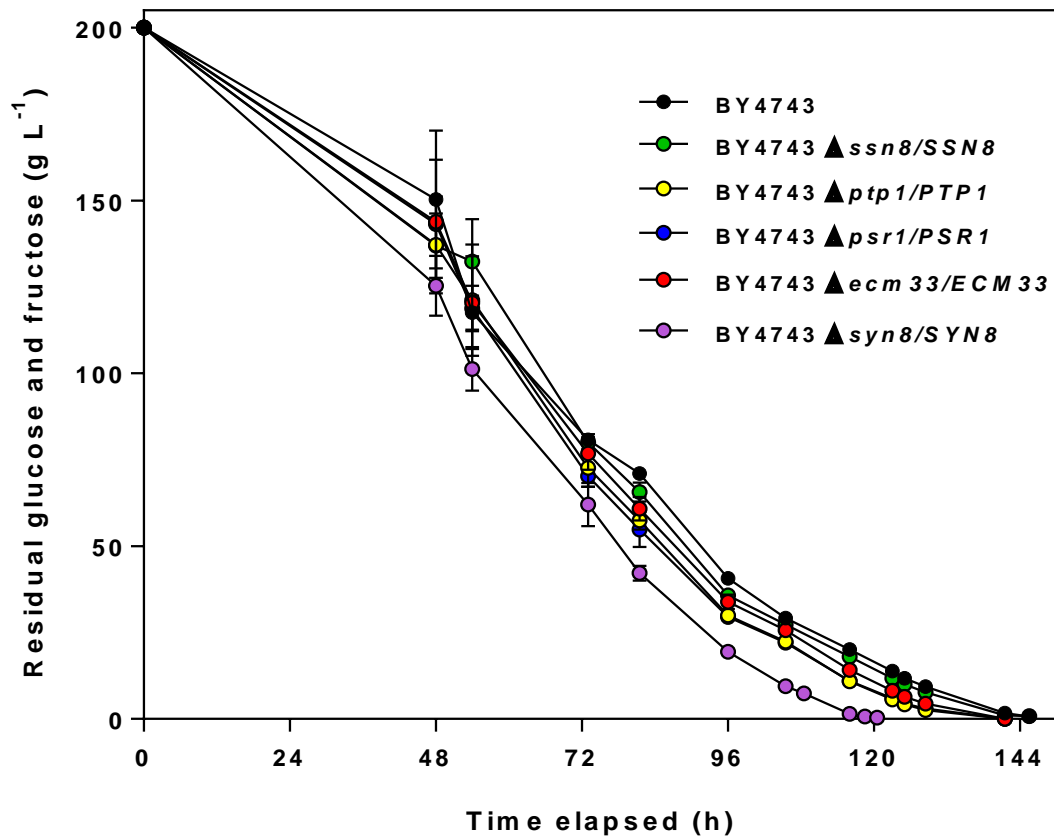
Figure 2.3 Sugar catabolised by BY4743, and heterozygous diploid deletants BY4743 Δ*ssn8/SSN8*, BY4743 Δ*ptp1/PTP2*, BY4743 Δ*psr1/PSR1*, BY4743 Δ*ecm33/ECM33*, and BY4743 Δ*syn8/SYN8* during fermentation in CDGJM with 200 mg L^{-1} sugars and 487 mg L^{-1} nitrogen. Individual strains are indicated with different coloured symbols. Data points are the mean (n = 3) ± SD.

Table 2.2 Fermentation durations of HNE mutants in high nitrogen (441 and 487 mg L^{-1} nitrogen, for later one it contains 150 mg L^{-1} uracil). Fermentation duration and percentage (%) of wild type are shown as a mean (n = 3) ± SD. One-way ANOVA was performed to compare differences of yeast fermentation duration with wild type controls (C911D or BY4743).

Yeast strain	Fermentation duration (h)	% of parent	Yeast strain	Fermentation duration (h)	% of parent
C911D	151 ± 0	100 ± 0	BY4743	146 ± 0	100 ± 0
C911D Δssn8	130 ± 7[b]	86 ± 5	BY4743 Δssn8/SSN8	143 ± 2	98 ± 2
C911D Δecm33	105 ± 7[c]	70 ± 5	BY4743 Δecm33/ECM33	142 ± 0	97 ± 0
C911D Δleu3	154 ± 6	102 ± 4	BY4743 Δsyn8/SYN8	116 ± 0[c]	79 ± 0
C911D Δptp1	138 ± 0[a]	92 ± 0	BY4743 Δptp1/PTP1	142 ± 0	97 ± 0
C911D Δ134	141 ± 3	94 ± 2	BY4743 Δpsr1/PSR1	137 ± 8[a]	94 ± 5

Significant differences were indicated by a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.0001$.

2.3.2 Determination of cell growth parameters under different temperature conditions

Since HNE mutants had varying fermentation durations and thus sugar catabolism (Figure 2.2 and Figure 2.3), it was hypothesized that other cellular phenotypes may also be altered by disruption of these genes, and this might indicate how disruptions of these genes confer a change in fermentation dynamics. To test this, initially, the growth of the haploid deletion laboratory yeast strains (BY4741 as background) was compared at three different temperatures 35 ºC, 29 ºC and 10 ºC. Mutant strains Δ*ecm33* and Δ*sps2* displayed slower growth at 35 ºC compared with wild type BY4741 (One-way ANOVA, **** $p < 0.0001$). However, no significant difference was observed between the other strains and the wild type (Table 2.3, Figure 2.4). At 29 ºC Δ*sps2*, Δ*ssn8* and Δ*ecm33* demonstrated slower growth than the wild-type strain (One-way ANOVA, **** $p_{\Delta sps2} < 0.0001$ and $p_{\Delta ssn8} < 0.0001$, *** $p_{\Delta ecm33} < 0.001$). For the rest of the strains growth was comparable with the wild type (Table 2.3, Figure 2.5). When the growth temperature was reduced to 10 ºC, all strains were similar with very slow growth. Six mutants grew better than wild type BY4741 at exponential stage, except BY4741 Δ*ssn8* at 10 ºC (Table 2.3, Figure 2.6).
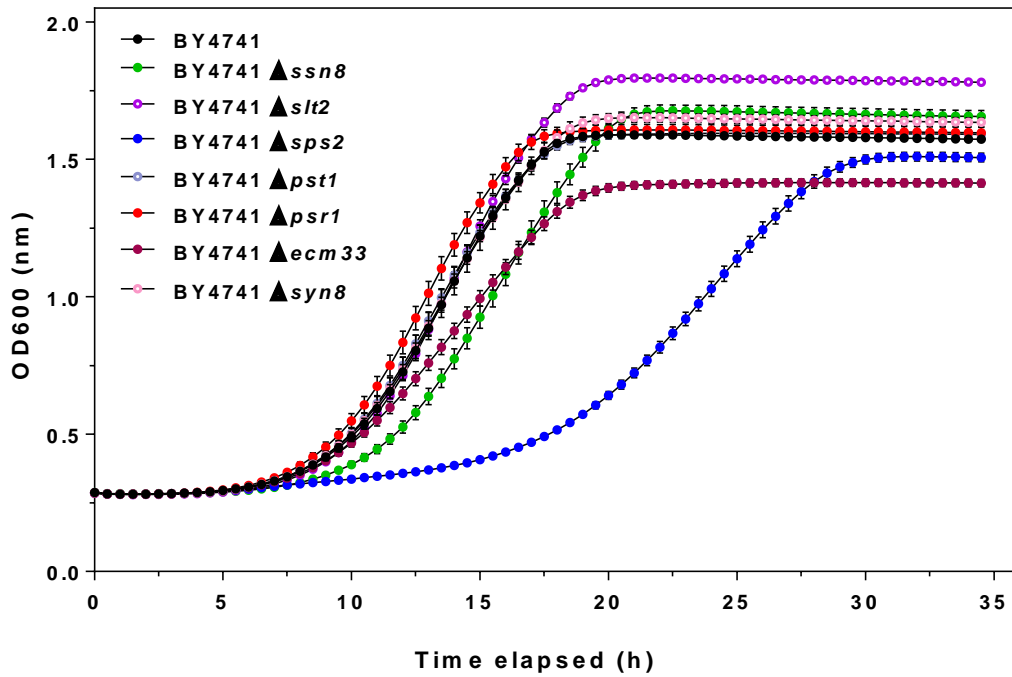
Figure 2.4 Cell growth curves of BY4741 and mutants at 35 ℃ in YPD liquid. Data points represent
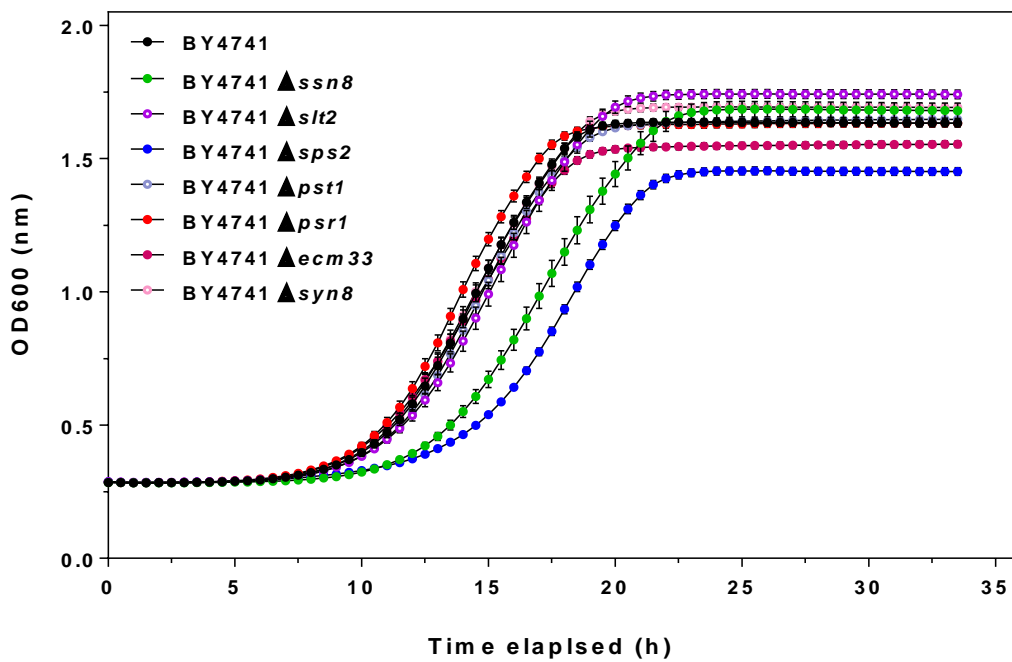
the mean (n = 11) ± SE.

Figure 2.5 Cell growth curves of BY4741 and mutants at 29 ℃ in YPD liquid. Data points are
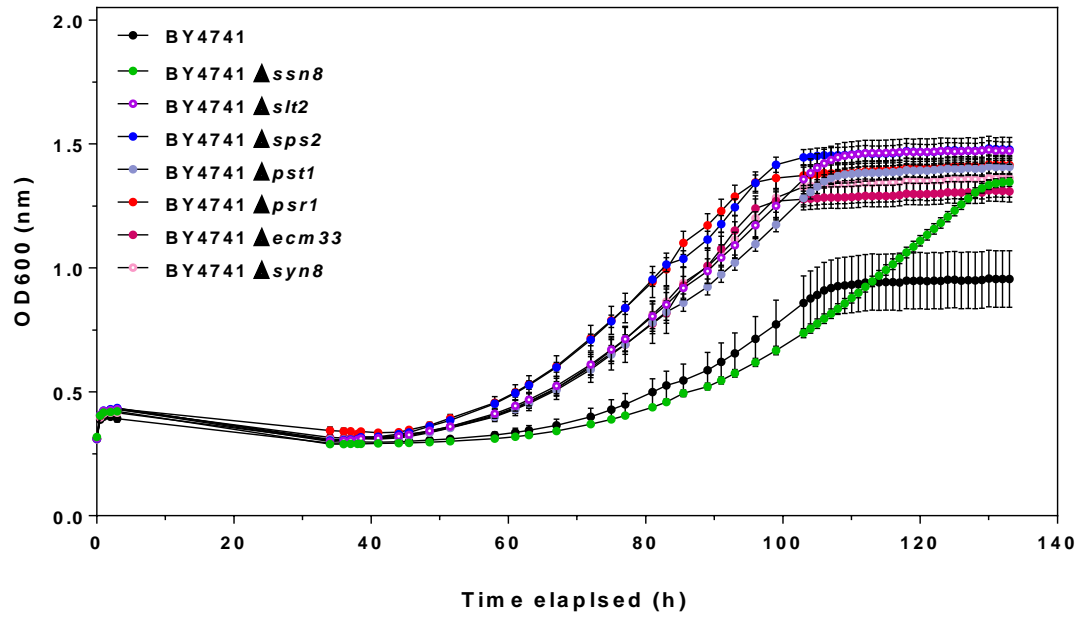
representative of the mean (n = 11) ± SE.

Figure 2.6 Cell growth curves of BY4741 and mutants at 10 ℃ in YPD liquid. Strains are indicated in different coloured symbols, data points are the mean (n = 11) ± SE.

Table 2.3 Cell growth rate of wild type and mutant yeast strains at three incubation temperatures (35 ℃, 29 ℃ and 10 ℃). One-way ANOVA was performed to compare differences of cell growth rate.

Strain	35 ℃		29 ℃		10 ℃	
	Maximum growth rate	R^2	Maximum growth rate	R^2	Maximum growth rate	R^2
BY4741	0.153 ± 0.012	0.998	0.181 ± 0.019	0.999	0.019 ± 0.010	0.996
Δ*sps2*	0.105 ± 0.003^a	1	0.141 ± 0.010^a	1	0.027 ± 0.002	0.998
Δ*ssn8*	0.156 ± 0.003	1	0.110 ± 0.010^a	0.996	0.015 ± 0.001	0.999
Δ*ecm33*	0.118 ± 0.004^a	1	0.152 ± 0.008^b	1	0.020 ± 0.009	0.979
Δ*slt2*	0.160 ± 0.014	0.998	0.172 ± 0.025	0.999	0.022 ± 0.007	0.998
Δ*pst1*	0.156 ± 0.013	0.999	0.176 ± 0.011	0.998	0.025 ± 0.002	0.999
Δ*syn8*	0.151 ± 0.026	0.998	0.174 ± 0.014	0.999	0.025 ± 0.008	0.997
Δ*psr1*	0.151 ± 0.024	0.997	0.180 ± 0.014	0.998	0.026 ± 0.006	0.999

Significant differences from BY4741 at each temperature indicated by a, $p < 0.0001$; b, $p < 0.001$.

2.3.3 Cell morphology

According to morphological screening results from Giaever et al. (2002) (Yeast Deletion Project), about 15% of deletants in screened homozygous strains (673 out of total 4401 strains) exhibit morphological differences from the typical elliptical shape of wild type yeast cells (Giaever et al. 2002). In this study, Δ*ecm33*, Δ*psr1*, Δ*ptp1*, Δ*syn8* haploids in BY4741 and as hemizgyotes in diploid BY4743, were compared with the corresponding wild-type strains. The appearance of Δ*ecm33* under the microscope differed from the elliptic form of wild type strains, BY4741 and C911D. The ratio of length to width was used to quantify the shape of yeast cells. The length-to-width ratio (LWR) of Δ*ecm33* was 1.102 (BY4741 background) and 1.160 (C911D background), whose shape appeared rounder than wild-type (about 1.208 LWR_{BY4741} and 1.246 LWR_{C911D}, respectively) (**** $p < 0.0001$, Student t-test). Δ*psr1*, Δ*ptp1* and Δ*syn8* in the haploid laboratory yeast background possessed elongated cells. Ratios of length and width were greater than for BY4741 (Student t-test, $LWR_{Δpsr1} = 1.426$, **** $p_{Δpsr1}$ value < 0.0001; $LWR_{Δptp1} = 1.373$, ** $p_{Δptp1}$ value < 0.01; $LWR_{Δsyn8} = 1.302$, ** $p_{Δsyn8}$ value < 0.01) (Figure 2.7, 2.9 and 2.10). However, when one copy of *ECM33*, *PSR1*, *PTP1*, or *SYN8* was deleted in the diploid laboratory yeast BY4743 background, the homozygous condition did not influence cell shape (Figures 2.8 and 2.10).
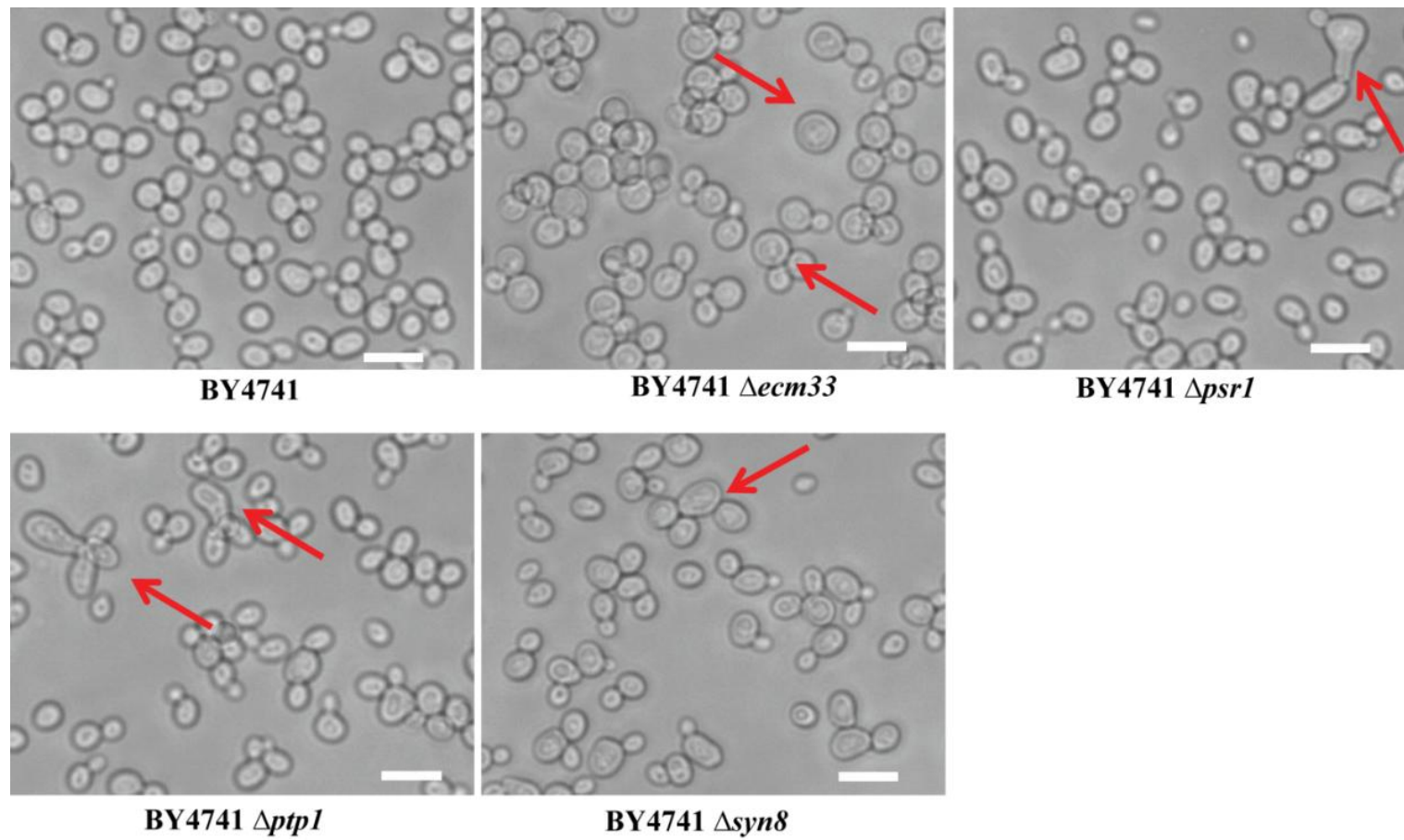
Figure 2.7 Morphology of haploid yeast deletion mutants compared with the wild type BY4741. Red arrows indicate some characteristic of cell shape changes. Scale bars equal 10 μm.
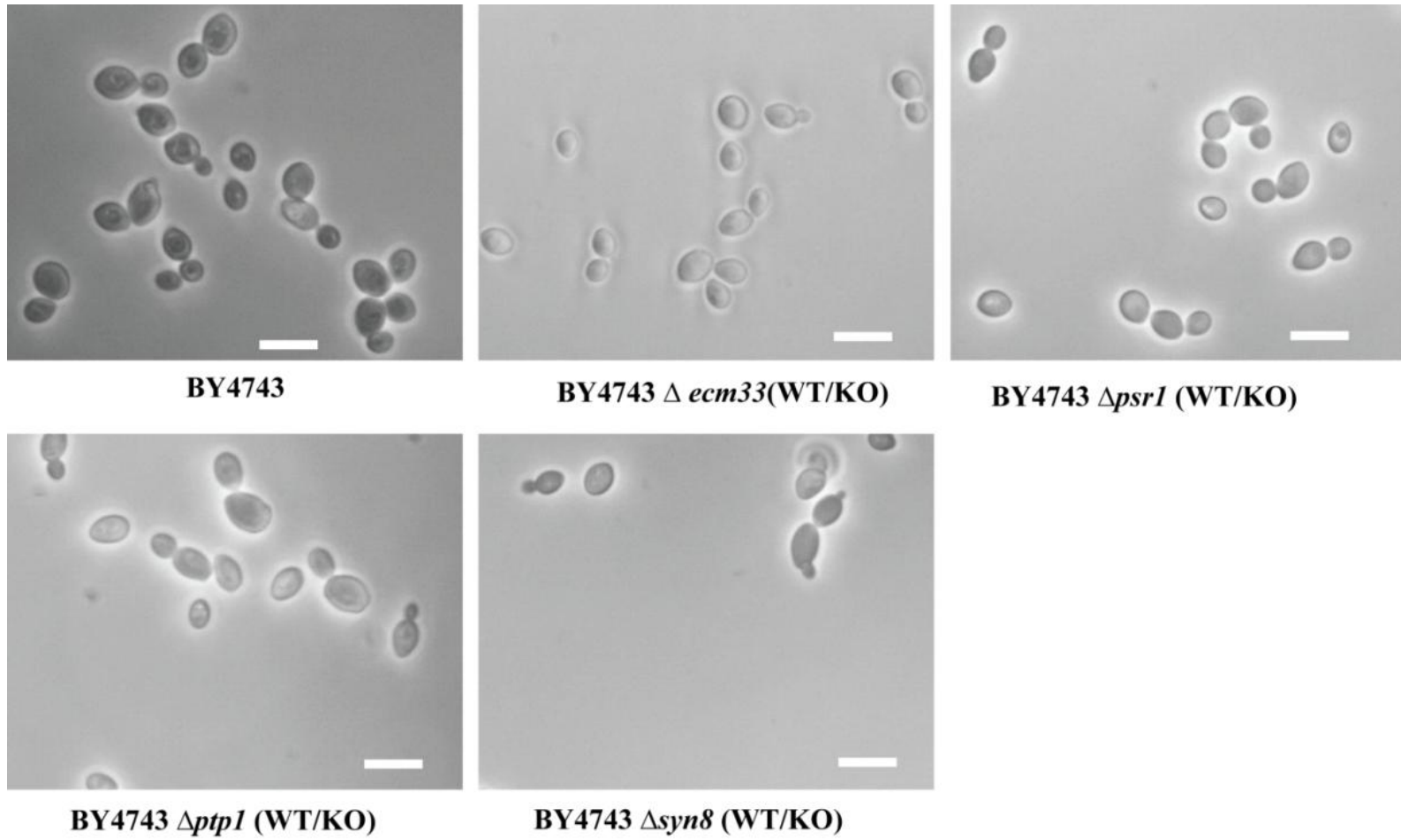
BY4743

BY4743 Δ *ecm33*(WT/KO)

BY4743 Δ*psr1* (WT/KO)

BY4743 Δ*ptp1* (WT/KO)

BY4743 Δ*syn8* (WT/KO)

Figure 2.8 Morphology of heterozygous laboratory yeast strains compared with the wild type BY4743. Scale bars equal 10 μm.
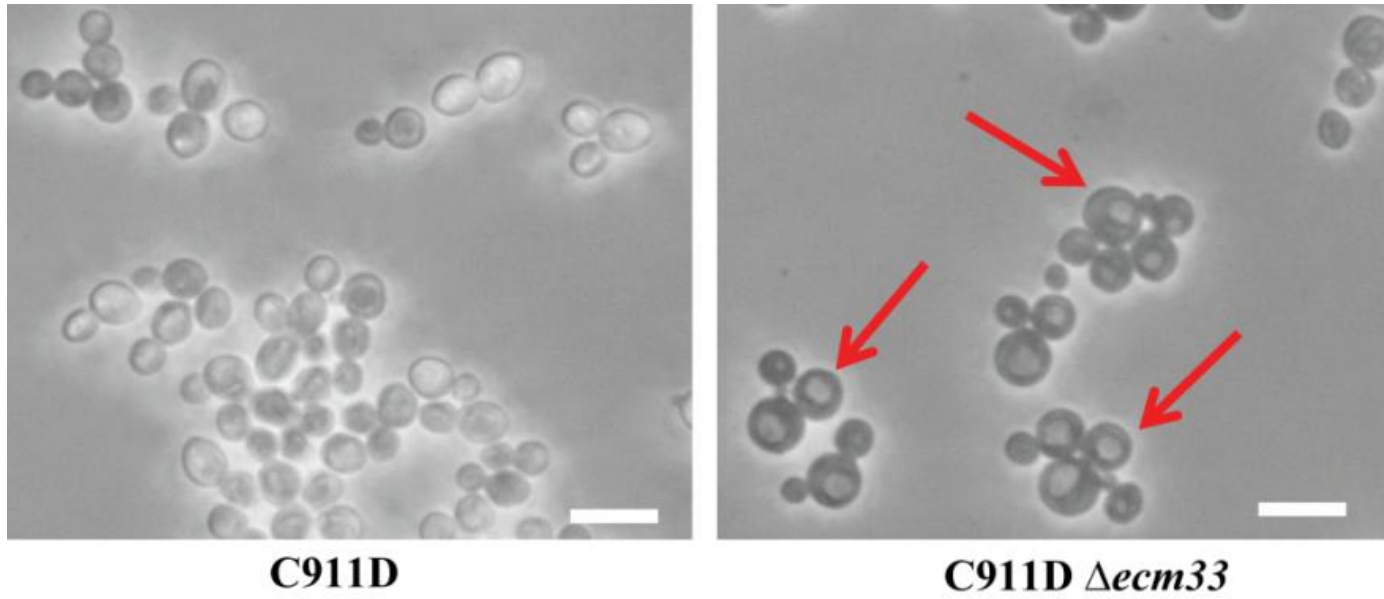
C911D

C911D Δ*ecm33*

Figure 2.9 Haploid wine yeast mutant Δ*ecm33* compared with the wild type strain C911D. Red arrows point out some examples of cell shape changes. Scale bars equal 10 μm.
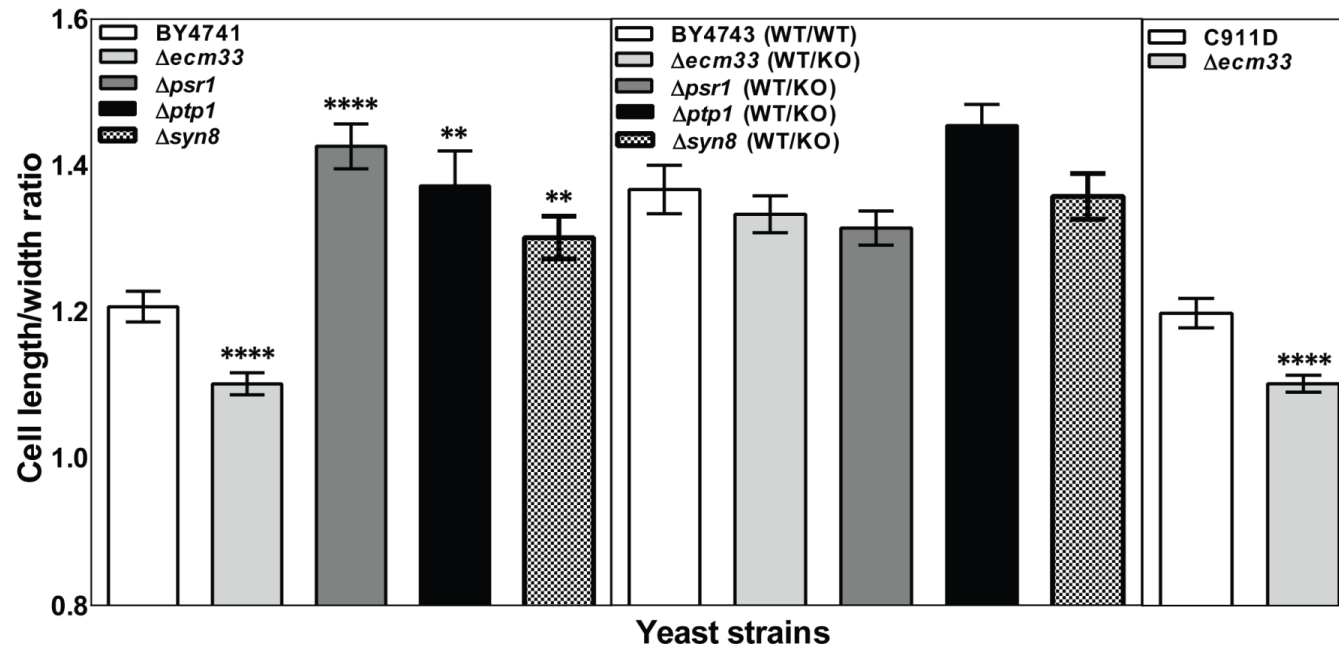
Figure 2.10 Cell length and width ratios of different yeast strains: haploid deletion laboratory yeasts, heterozygous diploid laboratory yeasts and haploid deletion wine yeasts are shown in different boxes. Data points are the mean of randomly selected 50-120 cells ± SE. Student t-test was performed and significant differences were indicated as ** p value < 0.01, **** p value < 0.0001.

2.3.4 The effect on yeast stress tolerance of deletion of ECM33 *and other selected genes*

2.3.4.1 Ethanol and pH tolerance of laboratory and wine yeast deletion strains

C911D Δ*ecm33* showed the best HNE performance in alcoholic fermentation (Figure 2.2, Table 2.2), so consideration was given to whether deletion of *ECM33* could result in increase ethanol tolerance and thus contributing to the HNE fermentation performance. Both laboratory and wine yeast Δ*ecm33* mutants were tested on YPD plates treated with different concentrations of ethanol (7 up to 13%, v/v) at 28 ºC. As expected, wine strains survived better than laboratory strains under all concentrations of alcohol. At high ethanol concentrations (10-13% (v/v)), the wine strain C911D Δ*ecm33* was observed to grow slightly better than wild type C911D. However, this difference was not observed in the laboratory yeast background (Figure 2.11). Such variation in ethanol tolerance is likely attributable to genetic diversity between yeast strains, adaptability of wine yeast to ethanol and possible differences in genetic control mechanisms between laboratory and wine yeast (Borneman et al. 2011; Mortimer and Johnston 1986).

Wild type laboratory and wine yeast were compared with their Δ*ecm33* derivatives for growth sensitivity under a range of pHs (5 to 9). Wine yeast again had better growth than laboratory yeast under both acidic and alkaline conditions. However, the disruption of *ECM33* did not impact on and improve the growth of either laboratory or wine yeast (Figure 2.11), suggesting that the gene *ECM33* might not appear to be involved in the cellular mechanism behind pH adaptation. Whilst wine yeasts were more tolerant to pH variation than laboratory yeast, both wild types had a wide range of pH from 5 to 9 for growth (Figure 2.11).

2.3.4.2 CaCl$_2$ tolerance on YPD or CDGJM agar

A previous study has reported that *ECM33* from *Schizosaccharomyces pombe* (fission yeast) might have a role in cellular calcium signalling, and that deletion of this gene results in growth hypersensitive to external calcium (Takada et al. 2010). In this study, both laboratory and wine yeast Δ*ecm33* were grown on CDGJM agar plates with a serial CaCl$_2$ supplementation from 1.5 mM to 210 mM. In all CaCl$_2$ treatments, wine yeast strains always grew better than laboratory yeast. With increasing CaCl$_2$ concentration, the colony spots were observed to be smaller, with a glistening surface appearance and white colouration. A similar growth phenotype was observed for Δ*ecm33* and the wild type of laboratory and wine yeast backgrounds (Figure 2.12). Since no difference in growth phenotypes were observed in the CDGJM, YPD was also used to supply the basic nutrients for yeast growth, to which additional CaCl$_2$ was supplied. Again subtle differences were noted between Δ*ecm33* and wild type strains both in the laboratory and the wine yeast backgrounds (Figure 2.13).
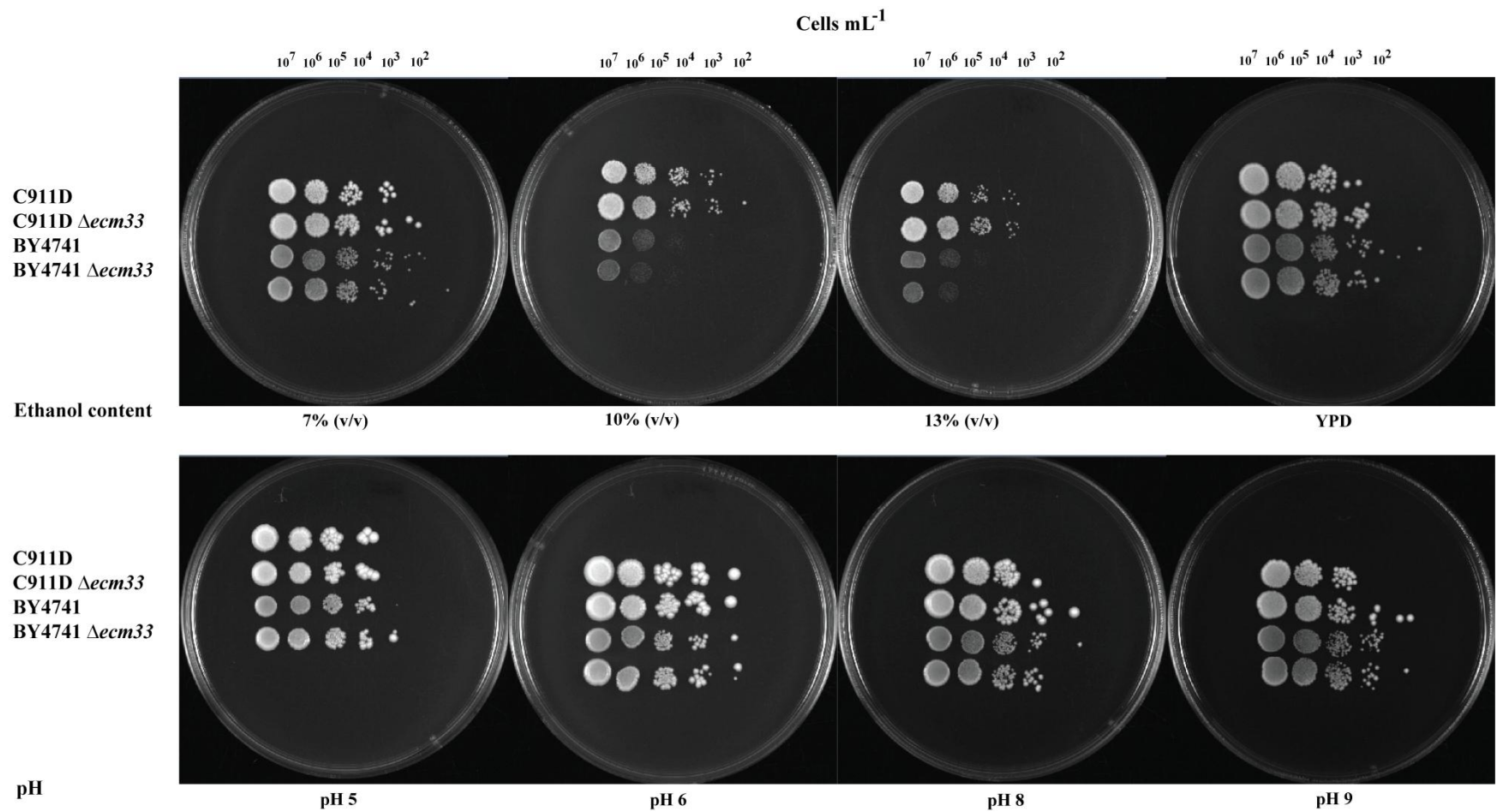
Figure 2.11 Ethanol and pH tolerance of haploid laboratory and wine *ECM33* deletants were compared with wild type parent strains. The first row represents different concentrations of ethanol plus a YPD plate as a control. The second row represents different pH treatments.
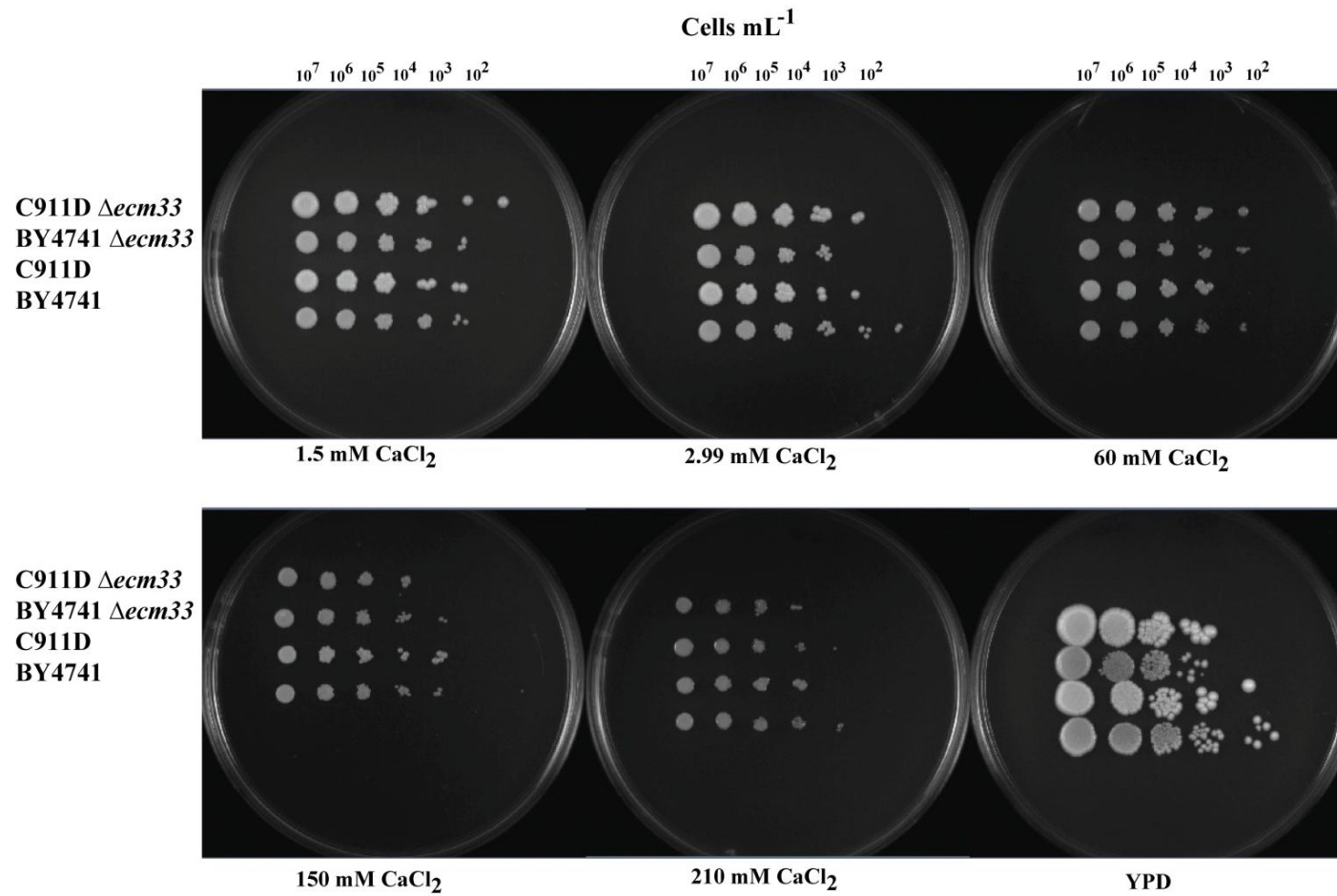
Figure 2.12 Tolerance to calcium (Ca^{2+}) by haploid laboratory and wine deletants Δ*ecm33* and wild type parent strains was compared on CDGJM agar based plates. Cells were tested on different concentrations of CaCl$_2$ ranging from 1.5 to 210 mM. YPD plate represented the control (with no addition of CaCl$_2$).
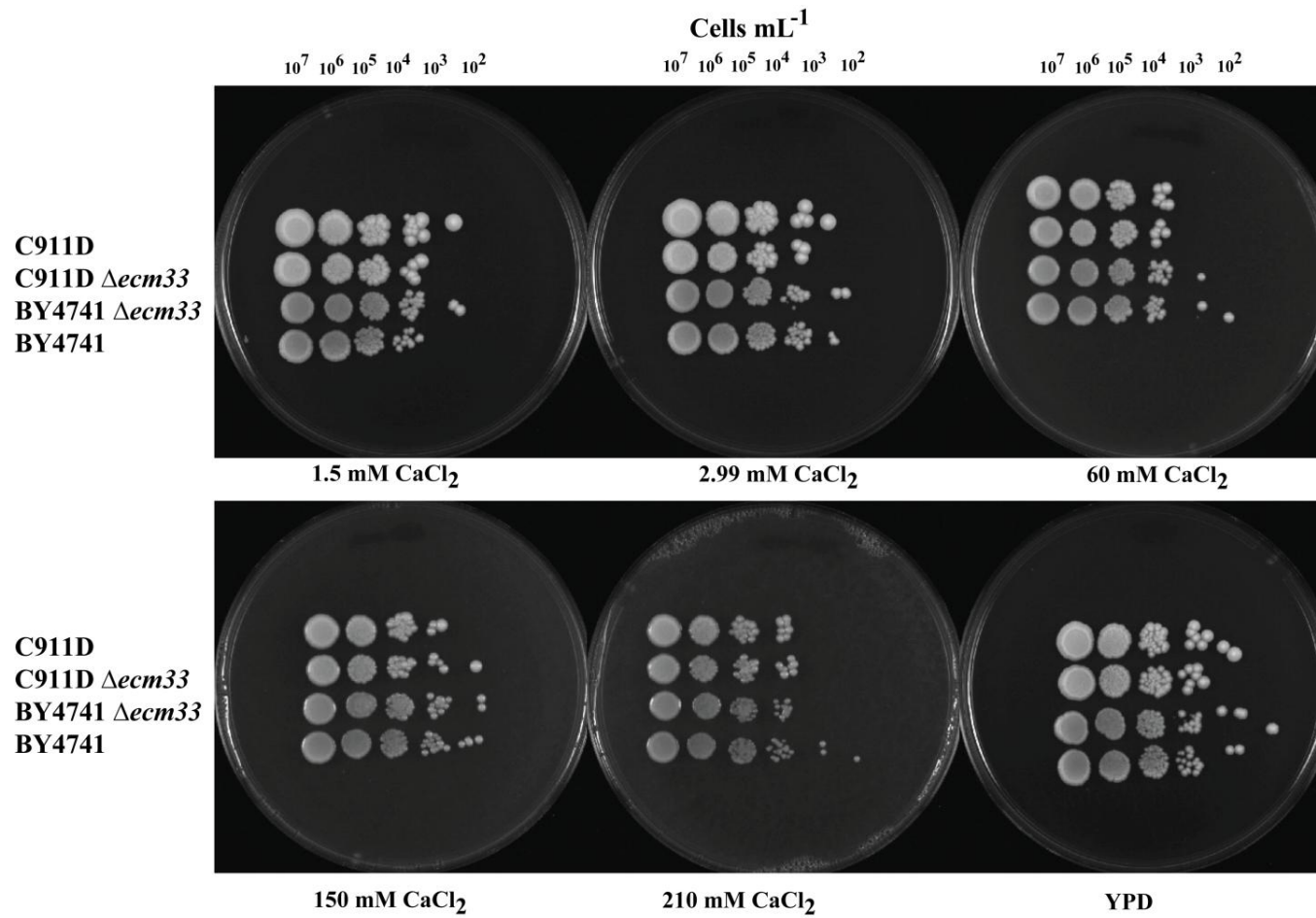
Figure 2.13 Calcium tolerance of haploid laboratory and wine deletant Δ*ecm33* were compared with control strains on YPD agar based plates. Cells were tested on different concentrations of CaCl$_2$ ranging from 1.5 to 210 mM. The YPD plate was set as control.

2.4 Discussion

Alcoholic fermentation is a key stage of the winemaking process. Commercial wine yeast strains (*S. cerevisiae*) adapt to the complex, dynamic and ever-changing environment of the grape must, and in the process convert sugars into alcohol efficiently, an important commercial standard in wine yeast strain improvement (Attfield 1997). In this study, HNE yeast candidates were initially screened and isolated from a mutagenesis library (Gardner et al. 2005). Five deletants were further investigated through 100 mL scale fermentations (in CDGJM, 200 g L^{-1} sugars, 441 mg L^{-1} nitrogen). Four out of five mutants were able to ferment all sugars within a significantly shorter duration than C911D, with the quickest mutant, Δ*ecm33*, only taking ~70% of the time (Table 2.2).

Gene copy dosage was also examined in this study to assess if only a single copy of *ECM33* could vary the fermentation performance of a diploid strain. For instance having one non-functional gene allele might cause a growth deficiency (haplo-insufficiency) or increased growth (haplo-proficiency) (Pir et al. 2012). Several laboratory yeast heterozygous for HNE candidate genes, were examined for their fermentation phenotypes. In the case of *ECM33*, the possession of only one copy of the gene in the diploid did not significantly improve fermentation, as fermentation completion was only 3% less than the parent (Table 2.2). The recovery of normal fermentation phenotype in yeast possessing one allele of *ECM33*, was indicative of enhanced fermentation being caused by complete absence of *ECM33* in the yeast genome. Delneri et al. (2007) investigated which genes were responsible for haplo-insufficiency and haploproficiency with regards to yeast growth, using continuous culture under 4 treatments: carbon source (glucose) limitation, nitrogen (ammonium) limitation, phosphate limitation and white grape juice. The heterozygous

ECM33/Δ*ecm33* (WT/KO) strain was shown to be haplo-insufficient in growth under nitrogen-limited and phosphate-limited cultures, however, growth was not affected when it grew in grape juice. In this study, deletion of one copy of *ECM33* in the same laboratory strain as used by Delneri did not alter yeast fermentation performance when grown in synthetic medium with sufficient carbon and nitrogen (Figure 2.3, Table 2.2). Additionally, the *SYN8*/Δ*syn8* (WT/KO) heterozygous strain, exhibited haplo-proficiency, fermenting 21% faster than the wild type BY4743 under nitrogen sufficient conditions (Figure 2.3, Table 2.2). Furthermore, when *SYN8* was completely deleted as in the haploid, fermentation duration was only reduced by 7% compared to the parent C911D, when grown under limited (55 mg L^{-1}) nitrogen (data presented in Chapter 3). Interestingly, Delneri et al. (2007) demonstrated that the *SYN8*/Δ*syn8* (WT/KO) hemizygote was haplo-proficient for growth in the nitrogen limited condition. Further investigation is needed to determine whether one copy of *SYN8* could similarly enhance alcoholic fermentation under limiting nitrogen, or whether complete deletion of *SYN8* in the haploid C911D Δ*syn8* strain would improve fermentation performance under high nitrogen conditions.

Cell growth was measured in YPD liquid cultures at 35, 29, and 10 ºC, respectively, to study gene deletion effects. YPD liquid instead of CDGJM was used to determine mutant growth under different temperatures, as YPD a complete medium for yeast growth minimised other effects in CDGJM to the cell. Haploid laboratory deletant Δ*ecm33* exhibited temperature sensitivity at 29 to 35 ºC (Figure 2.4, 2.5, Table 2.3). Toh-e and Oguchi (1999) reported similar results. This might imply that reduced GPI protein disrupts cell wall integrity, and triggers temperature sensitivity of the deletant Δ*ecm33*. Additionally, six mutants, Δ*slt2,* Δ*sps2,* Δ*pst1*, Δ*psr1*, Δ*ecm33* and Δ*syn8,* (except mutant Δ*ssn8*), grew better than the wild type at 10 ºC, suggesting

that these genes correlate with cell fitness at cold temperatures. This correlation is worth further investigation.

The effect of specific gene deletions on cell shape was determined in different backgrounds, such as haploid laboratory and wine strains and diploid heterozygous laboratory strains. In haploid yeast, cell shape was observed to be changed in absence of *ECM33*, *PSR1*, *PTP1* or *SYN8*. Mutant Δ*ecm33* showed rounder cells (smaller length and width ratio) compared with wild type (laboratory and wine yeast backgrounds) (Figure 2.7, 2.9 and 2.10). Similar observations have been made by Pardo et al. (2004), whilst in the fission yeast *S. pombe*, Δ*ecm33* presents with an irregular expanded size (Takada et al. 2010). These results suggest that Ecm33p is involved in cell wall structure and whose gene disruption causes cell shape alteration and may also indicate that other cell compensation mechanisms are functioning to maintain yeast viability.

Yeast typically prefers an acidic rather than alkaline environment, as alkalinity is believed to be a stress parameter with respect to yeast growth (Causton et al. 2001; Gasch and Werner-Washburne 2002). In tolerance to alkalinity, the cell maintenance pathway (CWI) is required for yeast survival under high pH conditions (Giaever et al. 2002). Deletion of *BCK1* and *SLT2*, two CWI pathway components, led yeast to alkaline-hypersensitivity (Giaever et al. 2002). Later Serrano et al. (2006) suggest that high pH-associated signalling may involve the *SLT2* mitogen-activated protein kinase (MAPK) pathway, as alkaline stress triggers phosphorylation of Slt2p and activates the Slt2 MAPK signalling pathway by Wsc1p (Serrano et al. 2006). Furthermore, Slt2p phosphorylation is also negatively regulated by *ECM33* expression (Pardo et al. 2004). *ECM33* has been reported to encode a GPI anchored protein that is localized at the plasma membrane, however, the role of Ecm33p on the plasma membrane is

unknown (Pardo et al. 2004; Terashima et al. 2003). In order to know whether the role of *ECM33* in *SLT2*-mediated CWI pathway is high pH dependent or not, cell growth sensitivity to high pHs (5-9) were determined. In this study, as depicted in Figure 2.11, these results suggest that there is no direct correlation between Ecm33p function and alkaline signalling, or that Ecm33p does not have a direct role in alkaline tolerance; or rather *ECM33* is not involved in the Slt2-mediated alkaline-tolerant signalling pathway.

ECM33 has been proposed to be associated with Ca^{2+} signalling in *S. pombe* (Takada et al. 2010). The deletion of *ECM33* in fission yeast (Δ*ecm33*) results in hypersensitivity to 150 mM $CaCl_2$, but no sensitivity to 300 mM NaCl, indicating the Δ*ecm33* sensitivity growth phenotype is related to the divalent Ca^{2+} ion instead of the chloride (Cl^-) ion. Such a high external Ca^{2+} concentration presumably perturbs the intracellular Ca^{2+} balance (Takada et al. 2010). Mutant Δ*ecm33* in *S. cerevisiae* was therefore tested on CDGJM agar plates supplied with concentrations of calcium chloride ranging from 1.5 to 210 mM $CaCl_2$. The lack of growth sensitivity to $CaCl_2$ as observed in comparing Δ*ecm33* and wild type (Figure 2.12) indicates that the presence of salts, high osmotic and/or acidic conditions in CDGJM may overcome the sensitive growth phenotype of the mutant towards calcium. However, when these strains were spotted onto YPD plates treated with the same concentrations of $CaCl_2$ (Figure 2.13), the Δ*ecm33* mutants still showed resistance to high concentrations of calcium. This might be because in *S. cerevisiae*, unlike fission yeast, *ECM33* has no direct link with the calcium signalling network, or might indeed require two or more components to initiate the appropriate adaptive response.

Laboratory yeast has been reported to be derived from industrial yeast, which is mainly diploid, aneuploid or even polyploid (Akada 2002). The generation of

laboratory yeast has proven advantageous to research, given the useful characteristics such as having stable haploid or diploid populations; good sporulation capability as a diploid; and presence of multiple auxotrophic markers (Akada 2002). Accordingly, laboratory yeast strains are suitable for molecular genetic modification, and other specific gene function analysis. On the other hand, with those genetic markers, laboratory yeast strain, e.g. BY4741 is auxotrophic for some genes responsible to nitrogen metabolism, such as: *HIS3*, *LEU2* and *MET15*. These features lead to inappropriateness of the laboratory strains for HNE strain selection.

By contrast, industrial yeast is more complex, exhibiting genetic heterogeneity, poor rate of sporulation and spore survival. However, industrial yeast strains are considered to be more tolerant to high ethanol, low pH and able to generate more desirable flavour/aroma compounds during fermentation (Akada 2002; Pretorius 2000; Schuller and Casal 2005). So, C911D a haploid derivative of the commercial wine yeast Lalvin L2056 has been constructed (Walker et al. 2003). The phenotypic plate analysis using C911D showed that this wine yeast is able to grow better than a laboratory yeast (BY4741) under a range of stress treatments including ethanol, acidity, alkali and calcium chloride as well as in un-supplemented YPD agar plates, regardless of the presence of a deletion (Figures 2.11, 2.12, 2.13). These findings would implicit that the haploid wine yeast derivative, C911D, may have a broader growth range than laboratory yeast strains, which perhaps is attributable to the diversity in genetic background or transcriptome and proteome. Whilst techniques such as whole genome DNA sequencing (Borneman et al. 2011), deep sequencing of transcriptome (Wang et al. 2009) and LC-MS/MS analysis of tryptic digested peptide fragments (Picotti et al. 2013) are beyond the scope of this PhD study, they are very

useful tools in uncovering the mechanisms behind the adaptability of this stress tolerant strain.

STATEMENT OF AUTHORSHIP

Disruption of the cell wall integrity gene, *ECM33*, results in improved fermentation

efficiency of wine yeast

Zhang, J. (Candidate)

Performed strain deletion, fermentations, cell morphology analysis, qPCR experiments, and wrote the manuscript

I hereby certify that the statement of contribution is accurate.

Signed ………………………………………………………..Date ……………………………

Gardner, J.M.

Conceived the project and planned experiments, supervised the research and revised the manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed…………………………………………………….Date……………………………

Walker, M.E.

Conceived the project and planned experiments, supervised the research and revised the manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed…………………………………………………….Date……………………………

Astorga, M. A.

Performed strain deletion

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

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Grbin, P.R.

Conceived the project and planned experiments, supervised the research and revised the manuscript.

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Signed………………………………………………….Date……………………………

Jiranek, V.

Conceived the project and planned experiments, supervised the research and revised the manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed………………………………………………….Date……………………………

Chapter 3 Paper manuscript

Disruption of the cell wall integrity gene *ECM33* results in improved fermentation efficiency of wine yeast

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Key words

wine yeast; high nitrogen efficiency (HNE); fermentation; gene expression; industrial biotechnology

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Abstract

Limiting assimilable nitrogen restricts yeasts ability to successfully undertake fermentation. Utilizing a library screening approach, a group of yeast single-gene deletants were identified as being better able to complete a model oenological fermentation with limited nitrogen availability. Analogous single gene disruptions were then constructed in a wine yeast strain background and the performance of these during fermentation was analysed. Deletion of *ECM33* resulted in the most efficient fermentation phenotype (fermentation duration being decreased by 15 to 31%) in either synthetic medium or grape juice of all deletants examined. Interestingly, no significant differences were found in nitrogen utilization, cell viability or biomass yield between Δ*ecm33* and the wild type. Δ*ecm33* did, however, display growth hypersensitivity to Calcofluor White and Congo Red suggesting a link to cell wall integrity. Transcriptional profiling of Δ*ecm33* in fermentation revealed up-regulation of *SLT2* and *HOG1*, encoding mitogen activated protein kinases involved in the cell wall integrity (CWI) and high osmolarity glycerol (HOG) pathways. *CHS3* a major chitin synthase gene was also found to be upregulated, and transcript abundance of key genes of central nitrogen metabolism, *GLN1*, *GLT1*, *GDH1* and *GDH2* in mutant Δ*ecm33* were altered.

1 Introduction

Yeast assimilable nitrogen (YAN) is one of the major limiting factors that influences wine yeast performance during alcoholic fermentation (Alexandre and Charpentier 1998; Bely et al. 1990; Blateyron and Sablayrolles 2001). With adequate YAN (as well as other nutrients) in grape juice or must, nitrogen-requiring metabolic reactions, such as protein synthesis, can occur and subsequently sugar catabolism would proceed efficiently. Conversely, insufficient YAN may result in sluggish or

stuck fermentation, which is often coupled with the production of undesirable metabolites such as hydrogen sulfide (Jiranek *et al.* 1995b). Addition of nitrogen supplements, such as diammonium phosphate (DAP) is a common remedy, however, these additions are not always ideal as they are time and energy consuming, and could also impact wine flavor profile (Ugliano *et al.* 2007). The use of wine yeast of high nitrogen efficiency (HNE) able to complete fermentation despite low YAN availability (i.e. $< \sim 150$ mg L^{-1}) provides an alternative strategy to address this issue (Gardner et al. 2005; Julien et al. 2000; Manginot et al. 1998).

Wine strains of *Saccharomyces cerevisiae* are exposed to a multitude of severe conditions during industrial fermentations. These include an anaerobic environment, rapid temperature shifts, hyper-osmotic conditions because of high grape juice sugar concentration, hypo-osmotic conditions (during rehydration), nutrient starvation, and ethanol toxicity later in fermentation (Bauer and Pretorius 2000; Querol *et al.* 2003). Such stresses elicit a systematic cellular signaling activation and transduction; perhaps from the cell surface to the nucleus in response to external environment stimuli (Igual and Estruch 2000). Translation of these signals is achieved through the modulation of gene expression and/or protein post-translational regulation, ultimately leading to alteration of cellular pathways, improvement of cell health and survival under stress (Bauer and Pretorius 2000; Querol *et al.* 2003). Current commercially used wine yeasts have been selected as best able to cope with these stresses whilst maintaining other desirable winemaking attributes. But a requirement for strain improvement still remains.

This research extends upon a previous study which sought to develop yeast strains able to utilize limited nitrogen more efficiently (Gardner *et al.* 2005). In the former study, transposon mutagenesis of laboratory yeast strains was used combined

with high-throughput analysis of fermentation efficiency to identify nitrogen efficient mutants (defined as HNE mutants), which catabolized more sugar for a given limited amount of nitrogen. Here we report on a screen performed in a wine yeast background and detail the analysis of the most promising candidate Δ*ecm33*. *ECM33* has previously been reported to encode for a cell surface glycosylphosphatidylinositol (GPI)-anchored protein of unknown function (Pardo *et al*. 2004; Terashima *et al*. 2003). The localization of the Ecm33p to the plasma membrane appears crucial to its function in cell wall integrity, including ultra-structural organization and proper assembly of mannoprotein outer layers and N-glycosylation of proteins (Martinez-Lopez *et al.* 2004; Pardo *et al.* 2004; Takada *et al.* 2010). In a Δ*ecm33* deletant, increased levels of activated Slt2p, a MAP kinase of the cell wall integrity pathway, have been reported (Pardo *et al*. 2004). de Groot *et al.* (2001) suggested that *ECM33* might act as a negative regulator of *SLT2* in the cell wall integrity pathway, or its deletion may activate the *SLT2*-related rescue mechanism. For instance, when compared with wild type, Δ*ecm33* shows greatly increased dual phosphorylation of Slt2p under 24ºC (de Groot *et al.* 2001). Also an upregulated Slt2p-mediated cell integrity pathway enables sustained cell viability of Δ*ecm33* (Pardo *et al.* 2004). In the fission yeast, *Schizosaccharomyces pombe*, Ecm33p has been suggested to be involved in the negative regulation of Pmk1p, a homolog of Slt2p and similarly involved in the cell wall integrity signal transduction pathway (Takada *et al.* 2010). There are no studies published of *ECM33* in wine yeast or high sugar fermentation. Here we report that deletion of *ECM33* significantly improves fermentation performance of wine yeast. We also report on how the loss of *ECM33* may confer high nitrogen efficiency. This could involve changes to chitin in the cell wall, activation of both the *SLT2*-mediated cell wall integrity pathway and the HOG

pathway. It is proposed that these processes contribute to the ability of $\Delta ecm33$ mutant to maintain efficient fermentation and/or adapt to the dynamic environment of oenological fermentation.

2 Materials and Methods

2.1 Media and strains

S. cerevisiae strains BY4741, C911D and their deletion strains were used for this study (see Table 1). BY4741, a common laboratory derivative was used as a reference. C911D (Walker *et al.* 2003), was derived from wine yeast strain L2056 (Lallemand, Montréal, Canada).

All strains were initially grown in yeast peptone dextrose (YPD) medium (1% yeast extract, 2% Bacto-peptone, 2% D-glucose) with addition of 2% agar when solid medium was required. Geneticin or G418 sulfate (200 mg L^{-1}, A.G. Scientific, Inc.; G-1033) was added for antibiotic selection. Fermentations were conducted in Chemically Defined Grape Juice Medium (CDGJM). The media were supplemented with either high or low nitrogen (total nitrogen amount: 450 or 55 mg L^{-1}, respectively) as a mixture of amino acids and ammonium sulfate (Jiranek *et al.* 1995a). In order to mimic industrial fermentation conditions more closely, yeast fermentation performance was also analysed in two white grape juices, Chardonnay (2010 Adelaide Hills, 242 g L^{-1} sugar, 172 mg L^{-1} YAN) and Riesling (2011 Barossa Valley, 245 g L^{-1} sugar, 64 mg L^{-1} YAN).

Table 1 Yeast strains used in this study

Yeast Strains	Genotype	Source
BY4741	*MAT*a *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0	Yeast Deletion Project

S288C	Fully sequenced reference strain	Mortimer (1993); Mortimer and Johnston (1986)
BY4741 Δ*ptp1*	BY4741 Δ*ptp1*::*KANMX4*	Winzeler *et al.* (1999)
BY4741 Δ*ssn8*	BY4741 Δ*ssn8*::*KANMX4*	Winzeler *et al.* (1999)
BY4741 Δ*leu3*	BY4741 Δ*leu3*::*KANMX4*	Winzeler *et al.* (1999)
BY4741 Δ*syn8*	BY4741 Δ*syn8*::*KANMX4*	Winzeler *et al.* (1999)
BY4741 Δ*slt2*	BY4741 Δ*slt2*::*KANMX4*	Winzeler *et al.* (1999)
BY4741 Δ*sps2*	BY4741 Δ*sps2*::*KAMMX4*	Winzeler *et al.* (1999)
BY4741 Δ*psr1*	BY4741 Δ*psr1*::*KANMX4*	Winzeler *et al.* (1999)
BY4741 Δ*ecm33*	BY4741 Δ*ecm33*::*KANMX4*	Winzeler *et al.* (1999)
1B14	MATa Δ*ura3*Δ*ho*::*KANMX4*	Gardner (2005)
C911D	MATα L-2056 Δ*ho*	Walker *et al.* (2003)
C911D Δ*ptp1*	C911D Δ*ptp1*::*KANMX4*	This study
C911D Δ*ssn8*	C911D Δ*ssn8*::*KANMX4*	This study
C911D Δ*leu3*	C911D Δ*leu3::KANMX4*	This study
C911D Δ*ecm33*	C911D Δ*ecm33::KANMX4*	This study
C911D Δ*psr1*	C911D Δ*psr1::KANMX4*	This study
C911D Δ*syn8*	C911D Δ*syn8::KANMX4*	This study
C911D Δ*slt2*	C911D Δ*slt2::KANMX4*	This study
C911D Δ*sps2*	C911D Δ*sps2::KANMX4*	This study

2.2 Identification of nitrogen efficient strains

A transposon mutagenized library for investigation of yeast gene function was provided by Professor M Snyder (Department of Biology, Yale University, New Haven, USA). Each plasmid of this library has a mini-transposon, mTn-3xHA/GFP/URA3, flanked by yeast genomic DNA (Ross-Macdonald *et al.* 1997). Generation of yeast mutants using this library and subsequent screening for nitrogen efficient strains was essentially performed as reported in Gardner (2005), and the haploid wine yeast strain 1B14 was utilized as a background strain. The location of the transposon and thus the identity of the affected gene were determined using Inverse PCR and Southern blot analysis (Gardner *et al.* 2005). Based on the previous work, eight candidate genes, *ECM33*, *SLT2*, *SPS2*, *SSN8*, *LEU3*, *PTP2*, *PSR1* and *SYN8* were selected, and their potential HNE function were investigated by examination of their deletion strains in another wine yeast strain C911D (as background) during fermentation.

2.3 Small-scale fermentations

The fermentation performance of each deletion strain was evaluated in small-scale laboratory fermentations as described by Walker *et al.* (2003). Briefly, 100 mL fermentations were conducted in CDGJM in 3-6 replicates under oxygen-limited conditions. To perform the fermentation, 5×10^6 cells mL^{-1} of yeast from overnight YPD cultures were inoculated into Chemically Defined Grape Juice Starter Medium (CDGJSM; Jiranek *et al.* 1995a) or diluted grape juice starter medium (grape juice:sterile MilliQ water in a 1:1 ratio). After growth overnight, 5×10^6 cells mL^{-1} were inoculated into CDGJM or juice. Samples were taken regularly throughout fermentation, and supernatants were frozen for later metabolite analysis. Refractive

index (RI) of samples was measured to monitor the fermentation progress, and Clinitest® tablets (Bayer® AG) used to determine fermentation completion (< 2.5 g L^{-1}). Residual sugars (glucose and fructose) were measured by enzymatic analysis (Henniger and Mascaro Jr 1985) using hexokinase and glucose-6-phosphate dehydrogenase (Megazyme; E-HKGDH) together with phosphoglucoisomerase (Megazyme; E-PGIEC). Residual ammonium was measured by enzymatic analysis using glutamate dehydrogenase (Roche; 10197734001) (Bergmeyer and Beutler 1985), whilst residual free amino acids were detected by the NOPA (o-phthaldialdehyde/N-acetyl-L-cysteine) method (Dukes and Butzke 1998).

Cell growth was monitored by optical density (OD) at 600 nm with appropriately diluted samples. Biomass yield was determined as dry cell weight of 5 mL culture samples. Cell viability within the fermentation was determined by yeast population scored as colony forming units (cfu) following spread plating on YPD agar plate and 3 days incubation at 28 ºC.

2.4 Identification of cell wall mutants

The extent of growth of cell wall mutants was determined on solid media supplied with Calcofluor White (CFW) or Congo Red (CR). These two anionic dyes interfere with the construction of the cell wall by binding with chitin chains and are widely used for detecting cell wall mutants (Ram and Klis 2006). Overnight YPD cultures were re-inoculated into fresh YPD medium at 2×10^5 cells mL^{-1}. When the culture subsequently grew up to 1×10^7 cells mL^{-1}, ten-fold serial dilutions were spotted onto solid YPD with different concentrations of CFW (5 to 250 µg mL^{-1}) and CR (10 µg mL^{-1} to 1 mg mL^{-1}). Growth was examined after 3 days incubation at 28 ºC.

2.5 Analysis of cell wall mutants by microscopy

Overnight YPD cultures with 100 μg mL^{-1} of CR were washed three times in phosphate buffered saline (PBS) at room temperature. The CR distribution in stained cells was then detected using a Nikon fluorescent microscope (ECLIPSE 50i, Japan) and a Nikon camera (DS-2MBW, Japan).

2.6 Wine yeast strain deletion strategy

Genomic DNA was isolated from overnight YPD cultures of appropriate commercial yeast deletion clones (from BY4741) using the method of Adams *et al*. (1997). *PTP1*, *SSN8*, *LEU3*, *ECM33*, *PSR1*, *SPS2*, *SLT2* and *SYN8* were disrupted in the haploid wine yeast derivative C911D (Walker *et al*. 2003), using the PCR-based gene deletion strategy (Baudin *et al.* 1993; Wach *et al.* 1994). Genomic DNA was isolated from an individual yeast clone of the Yeast Deletion Project (Invitrogen; 95401.H2P) and used as the template to amplify PCR products containing the *KANMX4* cassette flanked with the gene of interest (http://www-sequence.stanford.edu/group/yeast_deletion_project/). PCR was performed with gene specific primer pairs, e.g. *SLT2*_Del_A and *SLT2*_Del_D using VELOCITY DNA polymerase (Bioline; 21098), according to the manufacturer's instructions. PCR products were confirmed by DNA sequencing before being transformed individually into the C911D, transformants being selected on YPD agar with geneticin (0.2 g L^{-1}) (Daniel Gietz and Woods 2002). The identity of the transformants was verified by sequencing of the PCR amplified (ORF::*KANMX4*) products using gene specific primers. Southern blot hybridization (van Miltenburg *et al*. 1995) was used to confirm the single insertion at the target locus. PCR confirmation was also performed with forward and reverse primers as listed in Table 2.

2.7 RNA isolation and cDNA synthesis

2 to 5 mL of culture samples were collected and washed immediately in ice cold PBS. Total RNA was isolated according to Chomczynski and Sacchi (2006) and resuspended in 60 μL of diethyl pyrocarbonate-treated MilliQ water. Genomic DNA was removed using an Ambion TURBO DNA-*free*™ Kit (AM1907). RNA quality and quantity were determined by both gel electrophoresis and spectrophometric measurements at 230, 260 and 280 nm (Nanodrop ND 1000, Thermo Scientific). cDNA was synthesized from RNA templates using iScript™ cDNA synthesis kit (BioRad 170-8897) according to the manufacturer's instructions.

2.8 Quantitative gene expression analysis

Quantitative gene expression analysis was performed by real-time (RT)-PCR with a Bio-Rad Thermal Cycler (Model # CFX96™ Optics Module, Serial # 785BR04233). Reactions (10 μL) were prepared with 5 μL SsoFast EvaGreen supermix (Bio-Rad; 172-5203), gene specific forward and reverse primers at 5 pmol each (Table 2) and 200 ng of cDNA. Six biological replicates and two technical replicates were performed in each experiment and included three reference genes (*TAF10*, *UBC6* and *ALG9*) that were chosen as internal controls to allow calculation of a normalization factor (NF) (Teste *et al.* 2009). Excel 2007 (Microsoft® Office), geNorm (Vandesompele *et al.* 2002) and GraphPad Prism 6 (GraphPad Software Inc., La Jolla, C.A., USA) software were used for data analysis. The RT-PCR cycling protocol was as follows: 95 ℃, 30 seconds, 95 ℃, 5 seconds and 60 ℃, 5 seconds for 40 cycles followed by a melt curve (65 − 95 ℃, 5 seconds with a 0.5 ℃ increment per cycle) to check PCR product integrity. PCR products were also run on a 1.5% agarose gel in Tris Acetate EDTA (TAE) buffer to check for the correct size of a single product.

2.9 HPLC analysis

Glycerol and ethanol from terminal fermentation samples (Fig. 5) were determined by HPLC analysis. 1 mL of each sample was filtered (0.45 µm filter, Phenex^{TM}), and 20 µL of each sample was injected into an Agilent Series 1100 HPLC with a HPX-87H # 125-0140 column (Aminex) at 60 ℃ for chemical separation. 2.5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.5 mL min^{-1}. Chemical peaks were detected by a Refractive Index Detector (RID, Agilent) and Diode Array and Multiple Wavelength Detector (DAD, Agilent). The quantity and identity of each peak within the samples was determined by comparison with standard curves. Data was analysed using Agilent ChemStation software (version # 3.0.1 B). Statistical analysis was performed by Prism 6 using multiple Student t-test.

2.10 Statistical methods

Excel 2007 (Microsoft$^{®}$ Office) and GraphPad Prism 6 (GraphPad Software Inc., La Jolla, C.A., USA) were used in this study for statistical analysis. According to the specific comparison, significant difference was determined either using One-way ANOVA or multiple Student t-test, which are integrated in Prism 6 software.

Table 2 Oligonucleotides used for PCR and sequencing

Primer	Sequence (5'-3')
*SLT2*_Del_A	GCGGCGATTTTTATATTATCTTTTT
*SLT2*_Del_D	TAAAGGGCTTCTCAGTGAATACATC
*SPS2*_Del_A	TAAAGCAATTCATTAAAACTGAGGC
*SPS2*_Del_D	GAGTAAGCAAAATGTGAGTGGTTTT
*SYN8*_Del_A	AAAATTTCCCAGCACGACTTTGAT
*SYN8*_Del_D	TCAAAACCAAACTTCGTATTCGAGC
*PSR1*_Del_A	TCACGTGACACAAACCTAATAACTT
*PSR1*_Del_D	AAGGAAGATGACAAACGTGATACTC

*ECM33*_Del_A	AGCCGGTATAAATATTCAATGTCAA
*ECM33*_Del_D	AGAAGAGCAGTAAAGATGGCAGTTA
*SSN8*_Del_A	GACCACTATTTAGTTCTTTACCGCA
*SSN8*_Del_D	TTGAATGTGTGTTATAAGTGGGCTA
*PTP1*_Del_A	CTTTCATTTCCAGATTTTGTTCACT
*PTP1*_Del_D	GAGTACTACAAAAGAAACGTCGAGC
*LEU3*_Del_A	TAAGAAGTTGATCAAAATGACTCGC
*LEU3*_Del_D	ATTTCCAATCCTGAGATGTGACTAA
*ECM33*_qPCR_F1	TTTGAAGTCTGTTAGAGGTGGTG
*ECM33*_qPCR_R1	GAAGAAGCGGAAGATGTAGCA
*PST1*_qPCR_F2	GCCAAGGTTTCCAAGTCATC
*PST1*_qPCR_R2	GTCAAAGACCGACCAAAGG
*SLT2*_qPCR_F4	AGCAGCAACAGCAACAGCA
*SLT2*_qPCR_R4	GGCAGGTCTCATCTCCATCA
*HOG1*_qPCR_F1	CCTTATTCGGCTCCTTACCA
*HOG1*_qPCR_R1	TCCATCACTGCCACCAATC
*NGR1*_qPCR_F	AGTACCATACGGAATGGGAAACG
*NGR1*_qPCR_R	CATCATCCTGGACACGTCATACAT
*GID7*_qPCR_F1	CGCTAAATACCACGCATCAA
*GID7*_qPCR_R1	ACCGTAGGCGAAGCAAGA
*PTP2*_qPCR_F1	GGTGTTTTCGTTACCTTGGA
*PTP2*_qPCR_R1	GCGTTCTTTATCTGCTTTTGC
*CHS3*_qPCR_F3	CCTATGGTGGATGTGCGTAT
*CHS3*_qPCR_R3	CCTTCATTCTCGTCTTGTGC
*GLT1*_qPCR_F	GGTGTTCCAACCAAGTTC
*GLT1*_qPCR_R	GGTAAGCCACGATCCAAAGTA
*UBC6*_qPCR_F	GATACTTGGAATCCTGGCTGGTCTGT
*UBC6*_qPCR_R	AAAGGGTCTTCTGTTTCATCACCTGT
*TAF10*_qPCR_F	CCAGGATCAGGTCTTCCGTA
*TAF10*_qPCR_R	CAACAGCGCTACTGAGATCG
*ALG9*_qPCR_F	CACGGATAGTGGCTTTGGTGAACAAT
*ALG9*_qPCR_R	TATGATTATCTGGCAGCAGGAAAGAA
*GDH1*_qPCR_F	CATGAGAGAATTGAGCAGACA
*GDH1*_qPCR_R	GTAAGCACCGAACAGGTAA
*GDH2*_qPCR_F	CCCAGGATTGGTAGAACA

GDH2_qPCR_R	ATGGATCGTTGATTAGAATG

3 Results

Identification of genes affecting high nitrogen efficiency of wine yeast

Single gene deletions in the haploid wine yeast C911D were constructed with the eight affected genes (*ECM33*, *SLT2*, *SPS2*, *SSN8*, *LEU3*, *PTP2*, *PSR1* and *SYN8*) from the best performing candidate HNE mutants in the initial screening of a transposon library under limited nitrogen conditions. Successful deletions were confirmed by PCR and subsequent DNA sequencing. The fermentation performance of the eight yeast deletant strains was evaluated in laboratory scale (100 mL) fermentations in nitrogen-limiting CDGJM (with 55 mg L^{-1} nitrogen, proline included, 200 g L^{-1} sugars containing 100 g L^{-1} glucose and 100 g L^{-1} fructose). Five mutants (deletants of *ECM33*, *LEU3*, *SYN8*, *PSR1* and *SPS2*) had shorter fermentation duration (Table 3) than the wild type (C911D). C911D Δ*ecm33* had the shortest fermentation duration consuming all sugars in 69 ± 2% of the time of the wild type (404 *vs.* 582 h, ****, p < 0.0001). The other four strains, Δ*leu3*, Δ*syn8*, Δ*psr1* and Δ*sps2*, performed marginally quicker than the wild type, completing fermentation in 96 ± 2%, 93 ± 2%, 93 ± 2% and 97 ± 2%, respectively, of the time taken by the wild type (Table 3).

Deletion of ECM33 in a wine yeast decreases fermentation duration in grape juices

The fermentation performance of C911D Δ*ecm33* was further evaluated in two grape juices (Chardonnay and Riesling) so as to more closely represent an industrial situation. In each of these juices the use of C911D Δ*ecm33* shortened fermentation duration to 84 ± 6% (Chardonnay, ** p < 0.01) and 81 ± 2% (Riesling, ** p < 0.01) of the wild type (Figure 1 and 2; Table 3).
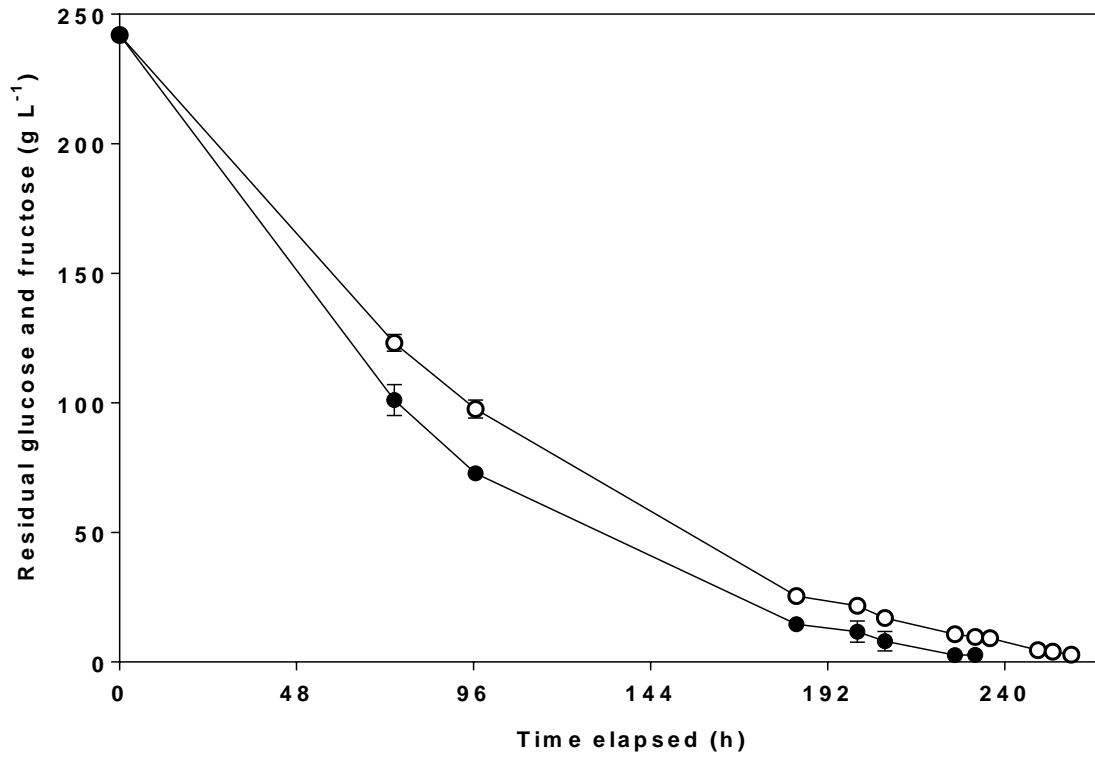
Figure 1 Sugar catabolized by C911D and C911D Δ*ecm33* during fermentation in a Chardonnay juice (Adelaide Hills, 2010) with 242 g L^{-1} sugars and 172 mg L^{-1} of YAN. C911D and C911D Δ*ecm33* are represented by empty and solid circles, respectively. Data points are the mean of triplicate fermentations ± SD.
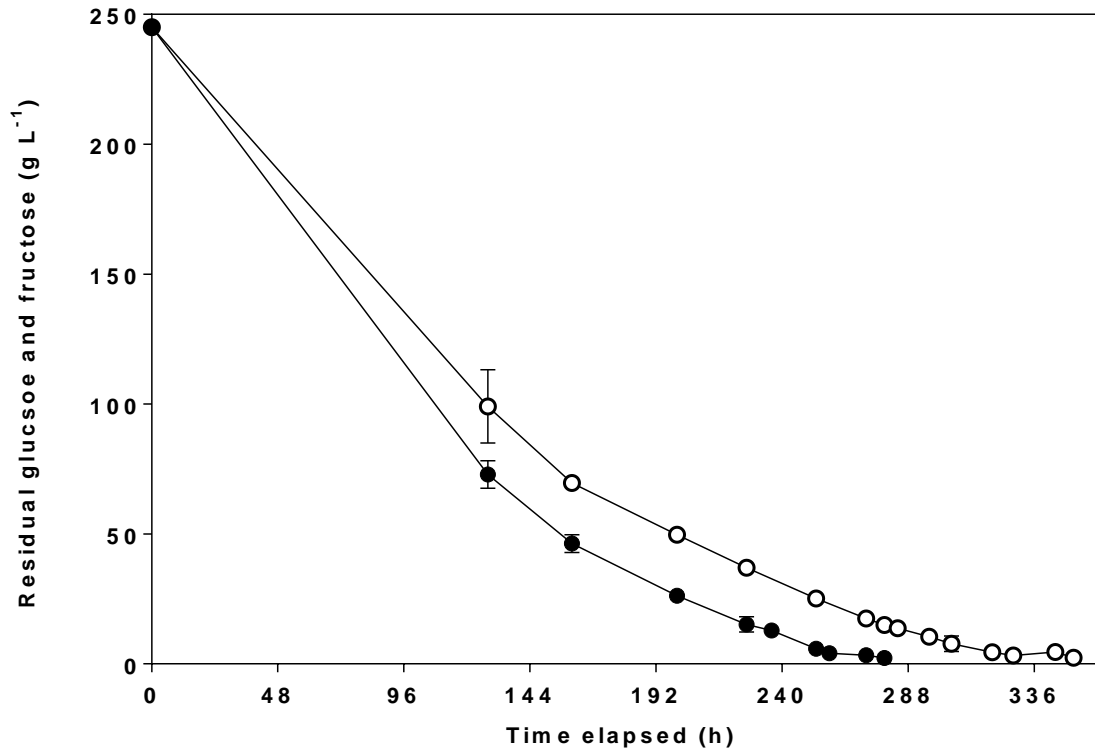
Figure 2 Sugar catabolized by C911D and C911D Δ*ecm33* during fermentation of a Barossa Valley Riesling (2011) with 235 g L^{-1} sugars and 64 mg L^{-1} of YAN. C911D and C911D Δ*ecm33* are represented by empty and solid circles, respectively. Data points are the mean from triplicate fermentations ± SD.

We also examined the nitrogen utilization of HNE candidates when supplied with both limiting and excess nitrogen (total nitrogen: 55 and 441 mg L^{-1}) in CDGJM and in two white juices. Nitrogen consumption of yeast strain was calculated from the total YAN minus residual YAN at the end of fermentation. In each medium, YAN was consumed mainly in the first 48 h in all of tested cultures, and no detectable residual nitrogen in CDGJM with limiting nitrogen supplement (55 mg L^{-1} nitrogen, data not shown). However, when nitrogen was supplied in excess (441 mg L^{-1}), residual YAN was detectable. Only mutant C911D Δ*ssn8* showed increased YAN usage compared to the wild type (* $p < 0.05$, Figure 3). No significant differences existed between the wild type and the other mutants. In this high nitrogen

fermentation Δ*ecm33* still performed 70 ± 5% quicker than C911D (supplied with 441 mg L^{-1} nitrogen, sugar consumption data not shown).
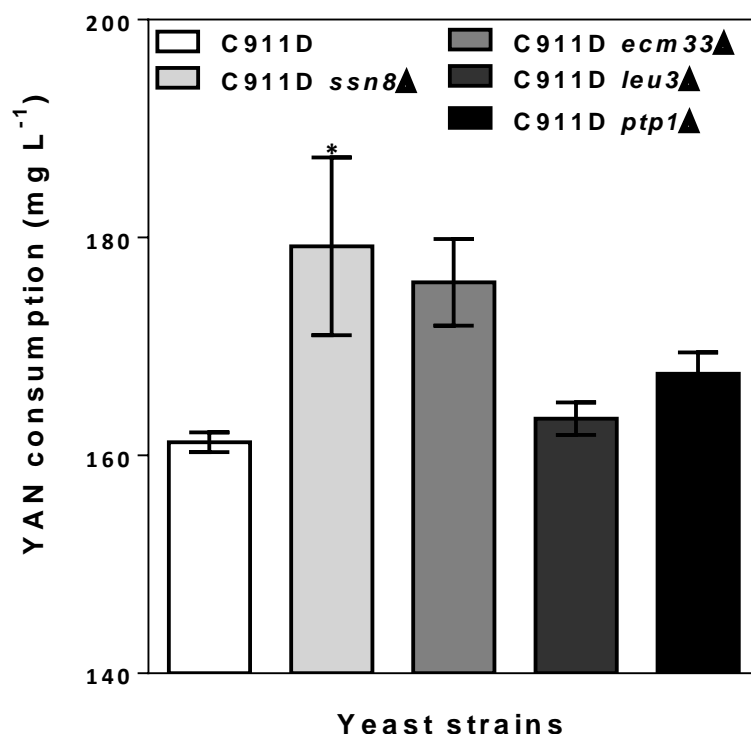

Figure 3 YAN utilized by wine yeast strains C911D, C911D Δ*ssn8*, C911D Δ*ecm33*, C911D Δ*leu3* and C911D Δ*ptp1* at the end of fermentation in CDGJM supplied with 200 g L^{-1} sugars and 441 mg L^{-1} nitrogen (total nitrogen). Data points are the mean from triplicate fermentations ± standard error (SE). One-way ANOVA was performed and significant difference was indicated with *, $p < 0.05$.

Basic parameters relating to cell health were examined, namely cell number and viability over the course of fermentation and final biomass. No significant differences were found for C911D Δ*ecm33* and wild type (Table 3). Some slight variations in cell viability between other mutants were found, yet there was no clear correlation between these and fermentation performance in limiting nitrogen. Cell biomass was analysed in the grape juice fermentations and no significant difference was determined (Table 3). No detectable nitrogen (YAN) was observed either at the end of two grape juice fermentations.

Table 3 Fermentation duration, cell weight and viability% of HNE mutants in CDGJM (55 mg L^{-1} nitrogen proline included) or grape juice. Cell viability% presents percentage culture viability that was calculated by colony forming number dividing the expected cell number spread on the plate. Data are the mean of triplicate fermentations ± SD. Fermentation duration is additionally expressed as a percentage of the wild type ± SD.

		Yeast strains (CDGJM, 55 mg L^{-1} nitrogen)								
		C911D	Δecm33	Δslt2	Δsps2	Δssn8	Δleu3	Δptp1	Δpsr1	Δsyn8
Duration	h	582 ± 14	404[a]	592 ± 40	563 ± 13	616 ± 17	560 ± 37	610 ± 27	543 ± 10	543 ± 10
	% of C911D	100	69 ± 2	102 ± 2	97 ± 2	106 ± 2	96 ± 2	105 ± 2	93 ± 2	93 ± 2
Biomass (10^{-11} g/cell)		1.5 ± 0.3	1.4 ± 0.1	1.6 ± 0.5	1.5 ± 0.1	1.5 ± 0.3	1.7 ± 0.2	1.2 ± 0.1	1.3 ± 0.2	1.6 ± 0.2
h		Cell viability% (CDJGM, 55 mg L^{-1} nitrogen)								
48.5		63 ± 7	67 ± 19	74 ± 10	65 ± 9	63 ± 15	81 ± 6	65 ± 10	75 ± 9	81 ± 6
119		70 ± 12	50 ± 6	76 ± 14	70 ± 6	57 ± 16	66 ± 8	56 ± 13	75 ± 11	56 ± 12
212		63 ± 13	62 ± 19	72 ± 5	65 ± 4	61 ± 12	61 ± 15	72 ± 7	70 ± 13	64 ± 10
308		47 ± 8	48 ± 5	60 ± 3	65 ± 4	50 ± 2	59 ± 10	58 ± 6	56 ± 7	62 ± 9

	Chardonnay			Riesling		
	Duration		Biomass (10^{-11} g/cell)	Duration		Biomass (10^{-11} g/cell)
	h	% of C911D		h	% of C911D	
C911D	267 ± 8	100	1.9 ± 0.1	341 ± 12	100	1.5 ± 0.3
Δecm33	225 ± 15[b]	84 ± 6	2.0 ± 0.3	276 ± 7[b]	81 ± 2	1.7 ± 0.4

Note: a: ****, p < 0.0001 (One-way ANOVA); b: **, p < 0.01 (Student t-test).

Deletion of ECM33 induces increased chitin synthesis in a wine yeast cell wall

In this study we stained Δ*ecm33* deletants in laboratory (BY4741) and wine (C911D) yeast backgrounds with CFW and CR, both of which bind with chitin in the cell wall. Laboratory and wine yeast Δ*ecm33* deletants were each hypersensitive to CFW and CR (Figure 4a). In order to characterize the occurrence of chitin in C911D Δ*ecm33*, CR stained cells were imaged by fluorescence microscopy (Figure 4b). In the wine yeast C911D, chitin was mainly detected around the bud neck between mother and daughter cells. However, in C911D Δ*ecm33* increased chitin was dispersed throughout the whole cell (Figure 4b).
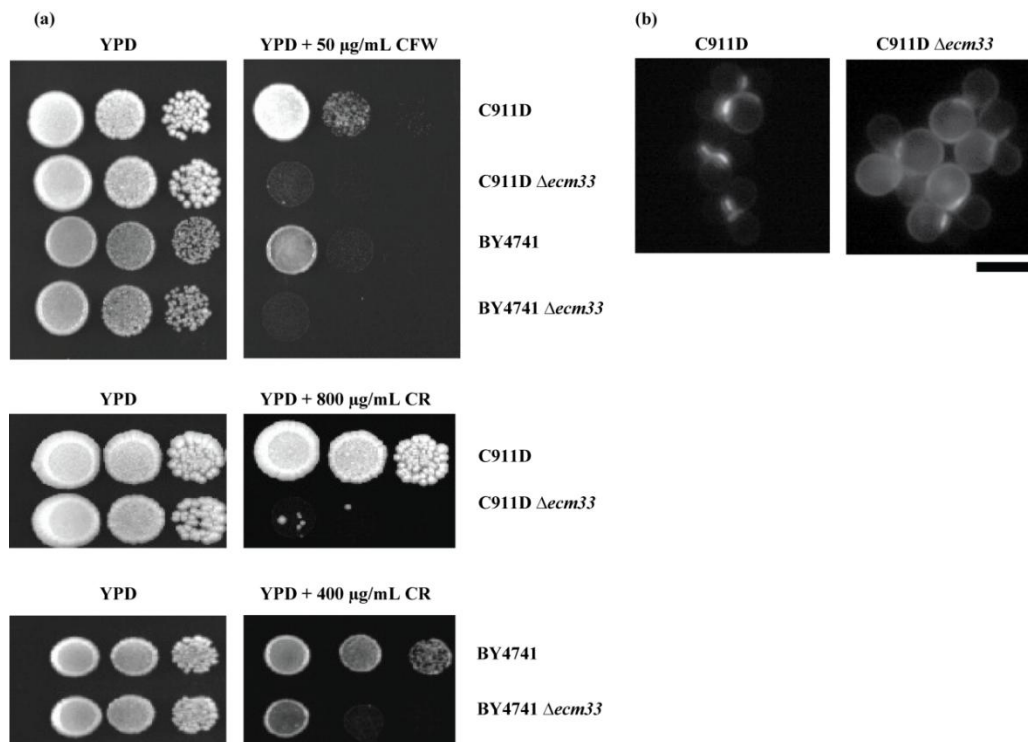
Figure 4 The effect of Calcofluor White or Congo Red on *ECM33* deletants: (a) Cells were grown to 1×10^7 cells mL^{-1}, serially diluted and spotted onto YPD in the presence or absence of CFW (50 μg mL^{-1}) or CR (400 or 800 μg mL^{-1}) (b) Fluorescence microscopy of CR (100 μg mL^{-1}) stained cells, C911D and C911D Δ*ecm33*. Scale bar equals 10 μm.

Expression profile of genes of interest in C911D Δecm33

In order to understand how the HNE phenotype is conferred, the expression of genes known to be involved with the normal function of *ECM33* was measured in C911D Δ*ecm33* throughout fermentation by Quantitative Real Time PCR (QRT-PCR). As we were interested in the putative relation of *ECM33* to nitrogen metabolism, the gene expression of key genes of central nitrogen metabolism was also examined. Four time points were chosen throughout the fermentation that were representative of when the culture reached maximum growth rate, maximum fermentation rate, fermentation rate decline and close to fermentation completion, corresponding to residual sugar concentrations of 130, 85, 15 and < 4 g L^{-1}, respectively (Figure 5).
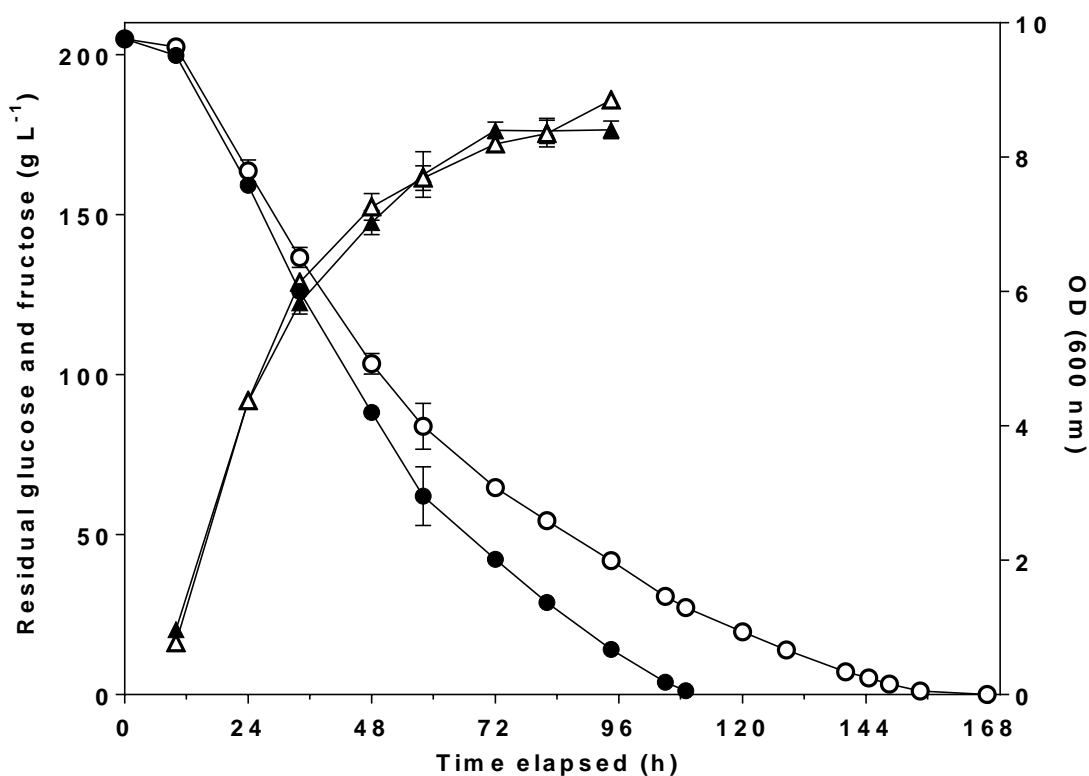
Figure 5 Sugar catabolism of C911D (open symbols) and C911D Δ*ecm33* (solid symbols) and optical density (OD 600 nm, triangles), fermentations were carried out in CDGJM supplied with 200 g L^{-1} sugars and 450 mg L^{-1} nitrogen. Data points are the mean of six replicate fermentations ± SD.

The expression of *ECM33* was evaluated in C911D to determine when this gene was normally active during fermentation. In this medium *ECM33* expression was maximal at the beginning and then declined over the course of the fermentation (Figure 6).

Genes related to SLT2-mediated cell wall integrity (CWI) pathway

Transcripts of *SLT2* were significantly increased at all four fermentation time points in the C911D Δ*ecm33*. *PTP2* was upregulated in the C911D Δ*ecm33* at a later stage of fermentation. Ptp2p has also been reported to be a negative regulator of the HOG pathway (Jacoby *et al*. 1997). However, the increase in *PTP2* transcripts at the later stages did not translate to decreased *HOG1* transcripts. Instead we found that *HOG1* transcripts were more abundant throughout fermentation in the C911D Δ*ecm33* compared with the wild type C911D (Figure 6).

Chitin Synthase III (CSIII), encoded by *CHS3* is required for the bulk of cell wall chitin synthesis (Bulik *et al.* 2003). Due to the increased chitin measured in the C911D Δ*ecm33*, the transcript level of *CHS3* was determined. *CHS3* expression decreased in the wild type C911D, however remained high in C911D Δ*ecm33* in the later stages of fermentation (Figure 6). As well as being a paralog of *ECM33*, *PST1* is also a member of the *SPS2* protein family (Caro *et al.* 1997). In this study, the expression of *PST1* was relatively unaffected in the Δ*ecm33* mutant. A slight increase in the transcription of *PST1* was only observed at 85 g L^{-1} sugars.

Genes of central nitrogen metabolism

In order to investigate the correlation between Ecm33p and nitrogen metabolism, the transcript profile of some central nitrogen metabolism genes were examined in Δ*ecm33* during fermentations. *GLN1* was upregulated in the C911D

Δecm33 at all three later stages of fermentation. Interestingly normally *GLN1* transcripts decreased during fermentation, but with the loss of *ECM33*, this trend was reversed, with the largest expression present at the end of fermentation. *GLT1* encoding glutamate synthase was also upregulated in the C911D *Δecm33*, but only at 85 g L^{-1} residual sugar. The transcripts of *GDH1* and *GDH2*, encoding two glutamate dehydrogenases, were down regulated at 15 g L^{-1} residual sugar by the deletion of *ECM33*, and no significant differences were observed at other time points.

Towards the end of fermentation *NGR1* deletants have significantly increased transcript abundance of both *GLN1* and *GLN1* (Gardner *et al.* 2005; J. Gardner pers. comm.). Interestingly, the transcriptional abundance of *NGR1* was much lower in the C911D *Δecm33* at the later stages of fermentation, and corresponded with the time points where increased abundance of *GLN1* transcript was observed.

Glycerol and ethanol contents

Glycerol and ethanol contents in terminal ferment samples (residual sugars < 2 g L^{-1}, dry) were determined via HPLC analysis to compare whether deletion of *ECM33* could affect final metabolites. Glycerol concentrations were decreased in *Δecm33* compared with wild type, 4.81± 0.04 *vs.* 5.07 ± 0.01 (g L^{-1}, p <0.001, multiple Student t-test). Deletion of *ECM33* did not affect the ethanol concentrations in final samples: 126.0 ± 1.0 g L^{-1} (*Δecm33*) *vs.* 125.8 ± 1.2 g L^{-1} (C911D).
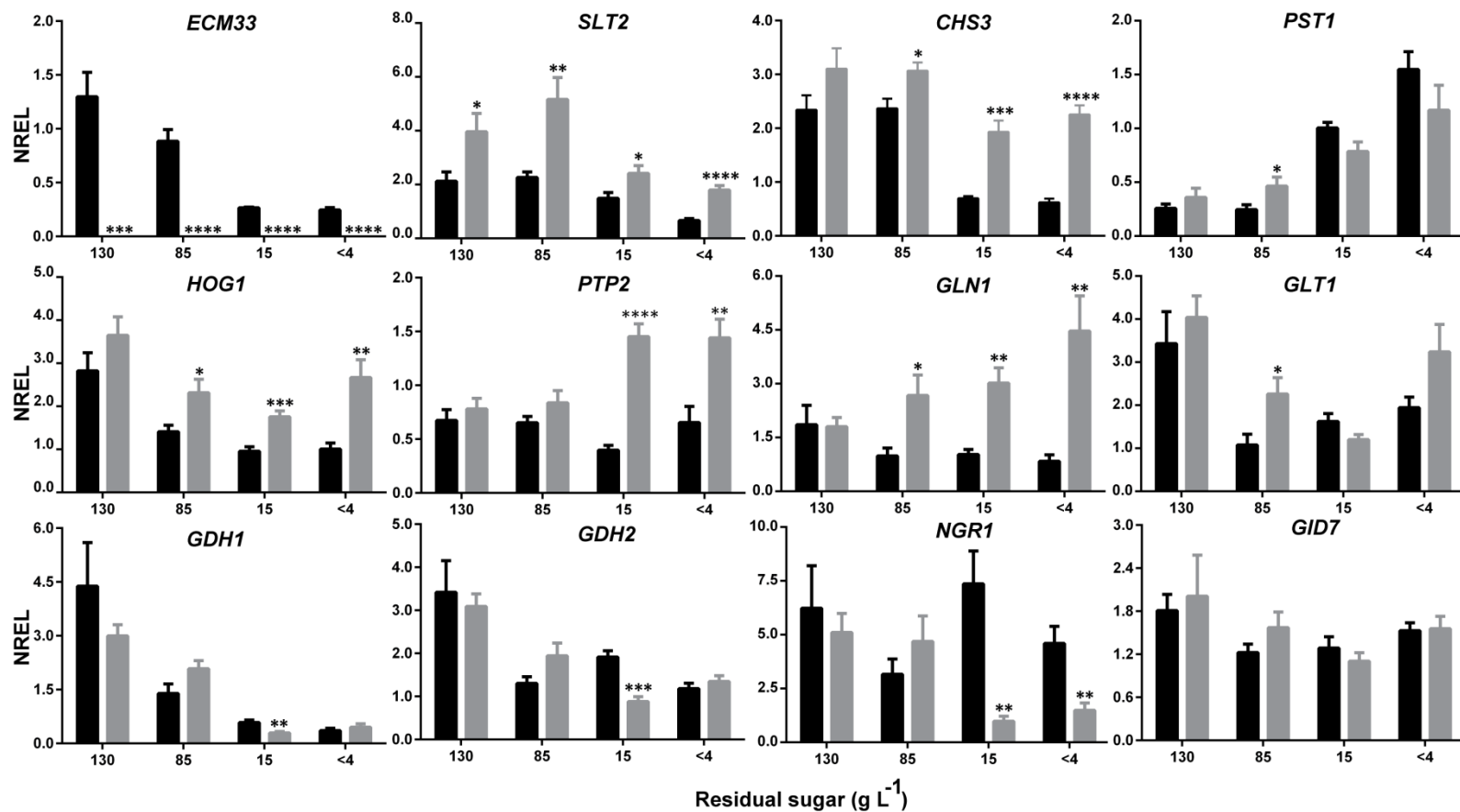
Figure 6 Normalized relative expression of key genes in C911D (■) and C911D Δecm33 (▩). Normalized relative expression level (NREL) is shown at selected residual sugar levels (g L^{-1}). Statistical analysis was performed via Prism 6 using multiple t tests. Significant difference of relative expression of specific genes is indicated with: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$.

4 Discussion

Insufficient yeast assimilable nitrogen is known to be a major causative factor of fermentation difficulties often resulting in sluggish or stuck fermentation (Alexandre and Charpentier 1998; Ingledew and Kunkee 1985). Selection of high nitrogen efficient (HNE) yeast is an alternative approach to address fermentation issues with low YAN juices.

Nitrogen efficient yeast are those which can efficiently catabolise sugar with a given amount of nitrogen, especially below 150 mg L^{-1} YAN. Gardner *et al.* (2005) sought to investigate the primary basis of variation in nitrogen efficiency. Transposon mutagenesis was applied to characterize yeast sequences related to nitrogen efficiency. Similarly in this study, eight HNE candidate strains were characterized. Single gene deletants in a wine yeast background were constructed since wine yeast, as opposed to laboratory yeast, possess desirable fermentation attributes, such as tolerance to high sugars, ethanol and desirable aroma production. These eight deletants were initially evaluated when supplied with limiting nitrogen (total nitrogen 55 mg L^{-1}). A mutant lacking the entire open reading frame of *ECM33* was selected due to its outstanding HNE phenotype, i.e. fermentation duration was decreased by 31% compared to wild type (Table 3).

Chitin as key component of cell wall structure shows its importance in fermentation

ECM33 encodes a GPI-anchored cell wall protein involved in cell wall architecture. Loss of Ecm33p affects cell morphology and triggers the activation of the CWI MAPK pathway in *S. cerevisiae* and *S. pombe* (Pardo *et al.* 2004; Takada *et al.* 2010). Some GPI-anchored proteins have been identified to connect covalently with β-1,6-glucan with the other end attaching to β-1,3-glucan (Kollár *et al.* 1997).

Some others are hypothesized to attach with β-1,3-glucan directly (Klis *et al.* 2002). Yeast cell wall consists of mannoproteins, *β*-(1, 3 / 1, 6)-glucan and chitin and is responsible for cellular shape and protection from environmental changes (Klis *et al.* 2002). *β*-glucan and chitin present in the inner layer mainly contribute to the mechanical strength and framework of the cell wall (Klis et al. 2002; Kollár et al. 1995). The termini of chitin chains are attached to *β*-(1, 3)-glucan by a *β* (1→4) linkage, and this synthesis process is catalyzed by chitin synthase 3 (Chs3p) (Kollár *et al.* 1995). Additionally, Chs3p is also responsible for 90% of cellular chitin synthesis (Bulawa 1993). In spite of only making up 2% of cell wall dry weight, chitin is suggested to be an essential component involved in cell wall integrity (Levin 2005). In response to cell wall damage, chitin synthesis in mutants such as Δ*cwh53* and Δ*mnn9* can be activated and may increase up to 20% of cell wall content, acting as a branch of cell compensatory mechanism involved in cell integrity pathway (Dallies *et al.* 1998; Kapteyn *et al.* 1999). This increase in chitin can be detected by Calcofluor White (CFW) and Congo Red (CR) (Ram and Klis 2006); whereby the affected mutants exhibit growth sensitivity in the presence of these dyes.

Previous studies have shown that deletion of *ECM33* from *S. cerevisiae* or *Candida albicans* results in a hypersensitive growth phenotype in the presence of Calcofluor white (CFW) and Congo Red (CR) (Martinez-Lopez *et al.* 2004; Pardo *et al.* 2004). Since CFW and CR stain chitin in fungal cell walls, they are thought to interfere with the assembly of chitin and *β*-glucan (Ram and Klis 2006). In addition, under cell wall stress, such as when stained with CFW and CR, it is proposed that the cell initiates a response including the activation of genes encoding proteins to maintain/minimize damage to the cell. Increased chitin synthesis of cell wall is also one of the repair mechanisms (Boorsma *et al.* 2004; Ram and Klis 2006). As expected,

increased chitin was observed in Δ*ecm33* and was dispersed throughout the cell wall (Figure 4). It is speculated that increased chitin deposition leads to a strengthened cell wall enabling Δ*ecm33* to resist the harsh environment, especially towards the end of fermentation.

Currently there is no obvious link between increased chitin in the yeast cell wall and fermentation performance. In order to disclose how genetic networks act in Δ*ecm33* to display such highlighted HNE phenotype, the transcripts of some key genes involved in CWI pathways during fermentation were determined. *CHS3* transcript levels were observed to decline in the wild type as sugars were consumed. No noticeable decline was observed in mutant Δ*ecm33*, which correlated with cell susceptibility in the presence of CFW and CR as well as increased and dispersed chitin. The chitin synthetic process is very complex, involving seven chitin genes and a number of other intermediate factors. Ketela *et al.* (1999) suggested that overexpression of *SLT2* caused Chs3p activation resulting in hypersensitivity to CFW. Slt2p a serine/threonine MAP kinase is involved in cell wall integrity pathway (CWI) that mainly contributes to cell wall maintenance (Levin 2005). Previous work by Pardo *et al.* (2004) has shown that Slt2p is activated in an Δ*ecm33* mutant. There could be a link between Ecm33p and chitin synthesis through Slt2p in the maintenance of CWI amongst other processes. The observed synthetic lethality of mutants with double deletions of *ECM33* and *SLT2*, *SLT2* and either *CHS3*, *CHS5*, *CHS6* or *CHS7* (Lesage et al. 2005; Santos and Snyder 2000; Tong et al. 2004), further indicates that *ECM33*, *SLT2* and these four chitin genes may share some common pathways.

Importance of CWI pathway and HOG pathway in fermentation

The yeast cell wall communicates with the nucleus by transmission of signals from cell surface sensors to transcriptional responses, which are known as MAPK cascades (Levin and Errede 1995). Both Slt2p and Hog1p are important mitogen activated protein kinases (MAPKs) involved in CWI and HOG pathways, respectively. Ptp2p and Ptp3p, two tyrosine phosphatases (PTPs), inactivate Slt2p and Hog1 in *S. cerevisiae* (Mattison et al. 1999). Ptp2p has been shown as a more abundant and efficient transactivator, thus the transcript level of *PTP2* was determined (Mattison *et al.* 1999). The upregulation of *PTP2* observed in Δ*ecm33* (Figure 6) may reduce cell sensitivity to osmotic stress during fermentation as suggested by Jacoby *et al*. (1997).

During fermentation, yeast cells are immediately challenged with osmotic stress when inoculated into grape must (usually in excess of 200 g L^{-1} sugars). This induces yeast to synthesize glycerol, reduce glycerol permeability, and consequently buffer the osmotic gradient between the inside and outside of the cell (Brewster *et al.* 1993). As fermentation proceeds, the sugar content of must decreases, the concentration of which becomes lower than inside the yeast cell. This hypo-osmotic stress is known to activate CWI pathway. Davenport *et al.* (1995) reported that when osmotic conditions decreased from 1 M sorbitol to 0.2 M, no tyrosine phosphorylation was detected in mutant Δ*slt2*. By comparison, the wild type strain had an increased Slt2p tyrosine phosphorylation band. This suggested that the phosphorylation of Slt2p was caused by hypo-osmotic shock (Davenport *et al.* 1995). To date, the mechanism of the *SLT2* signaling pathway during fermentation in a wine yeast cell wall mutant has not been well demonstrated.

In this study, *SLT2* and *CHS3* were up-regulated in Δ*ecm33*, suggesting the CWI pathway might be activated during fermentation (Figure 6). It may also suggest that under hypotonic stress, cell swell or burst is inhibited by increased chitin in cell wall protecting cell integrity and shape. However, even though *HOG1* was up-regulated in Δ*ecm33,* glycerol was only slightly decreased in terminal fermentation samples according to HPLC analysis. This may be because post-transcriptional and/or translational regulation with HOG possibly interacts with other pathways, which operate to maintain the physiological status. Or it could be re-catabolized during fermentation. Further work is needed to confirm these.

The correlation between nitrogen utilization and fermentation

Since deletion of *ECM33* enabled shortened fermentation duration when either limiting or excess nitrogen was supplied, we hypothesized that in the absence of *ECM33* yeast may alter nitrogen utilization to catabolise sugars more efficiently. Thus the expression of a set of key central nitrogen metabolism genes; *GLN1*, *GLT1*, *GDH1*, *GDH2* (Cooper 1982; Magasanik and Kaiser 2002) were evaluated in C911D Δ*ecm33* (Figure 6).

GLN1 and *GLT1* encoding glutamine synthetase and glutamate synthase, respectively, are responsible for glutamine and glutamate exchange (Magasanik and Kaiser 2002). The QRT-PCR data showed that in absence of *ECM33*, *GLN1* and *GLT1* were up-regulated during fermentation (being responsible for synthesis of glutamine and glutamate, respectively), whereas another pair of glutamate dehydrogenase encoding genes, *GDH1* and *GDH2* were down regulated at 15 g L^{-1} (Figure 6). Metabolite analysis of terminal fermentation samples demonstrated decreases of glycerol in the Δ*ecm33*. Up-regulation of *GLN1* and *GLT1* in C911D Δ*ecm33* would be expected to result in the conversion of NADH to NAD^{+} for

glutamate synthesis from 2-oxoglutarate and ammonium, with the reduction of NADH potentially resulting in a lower glycerol and increased ethanol yield as described by Nissen and co-workers (2000). However, no significant increases of ethanol in mutant final samples were determined. Further investigations are required to verify the role of *ECM33* in nitrogen metabolism during fermentations.

Through mutagenesis and single gene deletion strategies, Δ*ecm33* was identified as displaying a high nitrogen efficiency phenotype and this was further characterized. Our findings draw a connection between efficient fermentation and nitrogen utilization, and also indicate links between the MAPK pathways (CWI and HOG) in osmo-adaption during fermentation. Whilst Δ*ecm33* would not be suitable for industry use, there is potential for such strains to be constructed whereby no extraneous genetic material is present (Horecka and Davis 2014), which may in the future be acceptable to the wine industry. This work makes a significant contribution to the current knowledge on how one of the several mechanisms by which wine yeast adapts and grow in high sugar conditions such as found during wine production.

Chapter 4 Investigation whether chitin content influences the HNE fermentation phenotype

4.1 Introduction

S. cerevisiae survival is tightly linked with the ability of the cell wall, which protects the cellular contents, to maintain cell shape and prevent cell lysis when under stress, such as when exposed to hyper-/hypo - osmotic shock, or rapid temperature shift during alcoholic fermentation (Cid et al. 1995; Hohmann 2002; Levin 2005; 2011; Smits et al. 1999). The osmotic pressure across the cell wall can reach up to 3 Mpa in some fungi (Griffin 1981). The yeast cell wall consists of β-glucan, chitin, mannoproteins and lipids (Klis et al. 2002). The proportion of chitin within the cell wall varies and can determine cell wall strength. For instance, chitin normally represents 1 to 2% of cell wall dry weight but can be increased up to 2 to 3 times after treatment with Calcofluor White (CFW), a similar agent to Congo Red (CR), both of which have an antifungal effect, interfering with cell wall structure through binding to chitin (García-Rodriguez et al. 2000; Klis et al. 2002).

In previous chapters, C911D Δ*ecm33* has been shown to have an HNE phenotype, and simultaneously increased chitin (based on greater sensitivity to CFW and CR). This overproduction of chitin may contribute to yeast tolerance to stresses during fermentation and may somehow correlate with the nitrogen efficient utilisation that observed in Δ*ecm33*. Therefore, it is hypothesised that this increase in chitin corresponds with cell-wall firmness, and this thicker or firmer cell wall facilitates enhanced cell survival and may also link with optimized nitrogen catabolism during fermentation. Based on this, it is proposed that other mutants with more chitin in the cell wall may also display an HNE phenotype. To test this notion, Congo Red

<u>Sensitive</u> (CRS) mutants were isolated from a population of mutants generated by chemical mutagenesis and their fermentation performance was investigated.

4.2 Materials and Methods

4.2.1 Yeast strains

Two commercial wine yeasts were used to generate CRS mutants. EC-1118, *S. cerevisiae* (Lallemand), is one of the most widely used commercial wine yeasts, possessing a number of desirable properties, such as the ability to undertake fermentation under a wide range of temperatures (10 to 30 ºC), low H_2S production and high osmolarity and ethanol tolerance (www.lalvinwine.com). Distinction (DST), *S. cerevisiae* (Maurivin), a relative newcomer to the commercial yeast market, is known to produce quite small amounts of H_2S even in low YAN grape juice (www.maurivin.com).

4.2.2 Media

YPD liquid/agar was used for yeast propagation with or without the addition of Congo Red (1 mg mL^{-1}). Inoculated plates were incubated at 28 ºC until colonies appeared typically after 2 days. CDGJM (200 g L^{-1} sugars (glucose and fructose ratio 1:1), 450 mg L^{-1} nitrogen, Appendix 1) was used to culture CRS strains in micro and 100 mL scale liquid fermentations.

4.2.3 EMS mutagenesis and subsequent selection of CRS mutants

Yeast mutants were generated by ethyl methanesulfonate (EMS) mutagenesis (Sigma-Aldrich Cat. # M0880-10G). This was performed by Miss Sijing Li, an Honours student in this lab. Briefly, 1 mL of yeast culture was treated with 45 µL EMS for up to 2 h, with time points taken every 10 minutes. The treated cells, following washing with 5% (w/v) sodium thiosulfate (made fresh), were plated out on

YPD agar, and then incubated at 28 ºC for 2 days to determine the survival rate (%). Cells from the exposure time that yielded 50% survival were then plated out onto YPD agar to give ~ 200 or 500 colonies on small and large plates, respectively. Single colonies from each strain were re-streaked onto YPD agar plates in the presence and absence of Congo Red. EMS mutants that had limited or no growth on CR plates were picked from the corresponding YPD plates (Figure 4.1) and re-streaked for single colonies onto fresh YPD plates. Three colonies of each mutant were rechecked for sensitivity to CR on solid media. Following this, glycerol stocks of CR sensitive (CRS) isolates were made in a 96-well plate (Costar® Crown Cat # CORN3596), which was convenient for micro-fermentation screening.
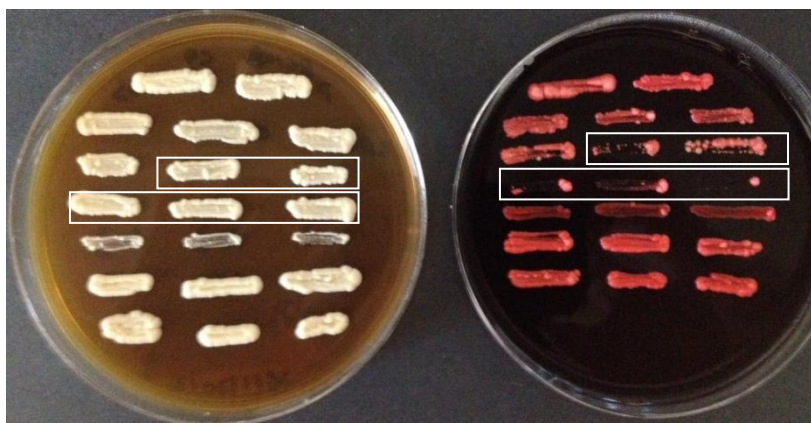
Figure 4.1 Selection of CRS mutants on a CR plate containing 1 mg mL^{-1} Congo Red. White boxes highlight strains that failed to grow on CR plates (right panel), but grew well on YPD (left panel).

4.2.4 Micro-fermentations (Adapted from Gardner et al. (2005))

Yeast plate cultures of CRS mutants were prepared from glycerol stocks by growth on YPD agar for 2 days at 28 ºC. Single colonies from each mutant were inoculated into 1 mL YPD liquid in 96 deep-well plates (QUASAR Instruments Cat # 3845-43001-0020) and sealed with a cloth membrane (Adelab Cat # 601-03-051). The plate was shaken at approximately 160 rpm overnight. 5 µL of YPD overnight culture

were then used to inoculate 200 µL of CDGJSM and incubated with shaking. 800 µL of sterile CDGJSM was then added to 200 µL overnight culture ($\sim 10^8$ cells mL^{-1}) and fully mixed on a shaker, which represented the completion of the source plate set up. An example of the source plate layout is shown in Figure 4.2.

Sacrificial micro-fermentations were carried out as described by Liccioli et al. (2011). Briefly, 20 experimental fermentation plates were prepared, ie, 10 time points for each of two source plates. 10 µL of diluted cultures from source plates were added into 190 µL of CDGJM resulting at a cell density of 1×10^6 cells mL^{-1}. Liquid handling was performed by a Corbett liquid handling robot (Model # CAS3800). Inoculated plates were sealed with breathable membrane (Diversified Biotech Cat No BEM-1), and placed in a incubator with a beaker filled with water at room temperature ($\sim$21 ℃). Nitrogen gas was pumped through a plastic tube into the beaker at a rate of 3 cm^3 s^{-1} to maintain humidity and low oxygen conditions. At different time points of interest, 2 sets of plates (from 2 source plates) were taken out, sealed with plastic titre tops (Molecular Solutions, Cat # T-TOPS-100), with a brief centrifugation and stored at -20 ℃ for later sugar analysis.

4.2.5 100 mL scale lab fermentations

The details of this method are reported in Chapter 3 section 2.3.

4.2.6 Determination of fermentation performance of CRS mutants

Sugar consumption by CRS mutants during fermentation was determined by measuring residual sugars in media using an enzymatic assay (Chapter 3). Refractive index (RI) was also measured periodically during the course of the experiment to monitor fermentation progress. CRS mutants that were able to ferment all sugars

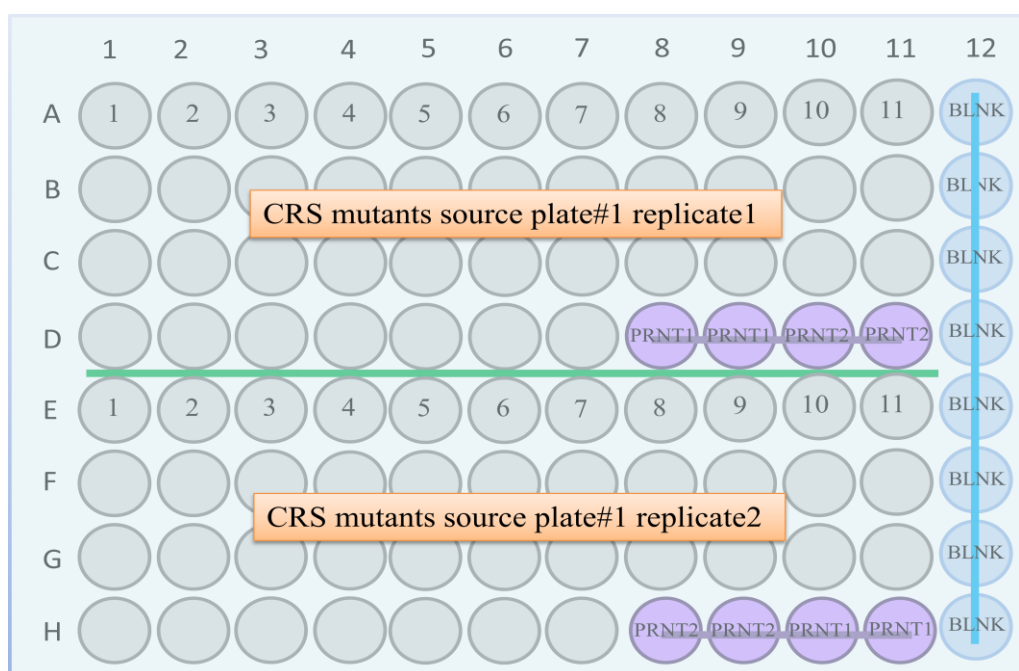within a shorter period of time than the parent were selected for further analysis at a 100 mL scale.

Figure 4.2 An example of a plate layout (source plate # 1). CRS mutants were arranged in this format from row A to H, with each mutant having 2 replicates, arranged on the top- and bottom- halves of the plate, respectively. Each parent strain was grown in duplicate: DST (PRNT1: parent 1) and EC-1118 (PRNT2: parent 2) for each replicate. Column 12 was left empty (BLNK) to be used for sugar assay standards. Ten 96-well plates (from each source plate) were inoculated and monitored for sugar utilisation by the sacrificial sampling.

4.3 Results

4.3.1 Isolation of CRS mutants

As $\Delta ecm33$ grew poorly in the presence of 1 mg mL^{-1} CR (data not shown), this concentration was then used to select CRS strains from EMS generated mutants. Approximately 3,200 mutants from Distinction and 7,300 mutants from EC-1118 were screened for their susceptibility to CR. After 2 rounds of screening 9 EMS mutants of Distinction (0.28%) and 18 EMS mutants from EC-1118 (0.25%) were identified as CRS mutants (Figure 4.3). This represents a mutation frequency. The

mutants were plated for single colonies before two or three colony isolates from each CRS mutant; a total of 77 CRS clones, from DST and EC-1118 were arranged in two source plates and evaluated for fermentation performance using sacrificial micro-fermentation (Liccioli et al. 2011). DST CRS mutants were labelled from #1 to 100, and EC-1118 CRS mutants were labelled from 101 to 200.
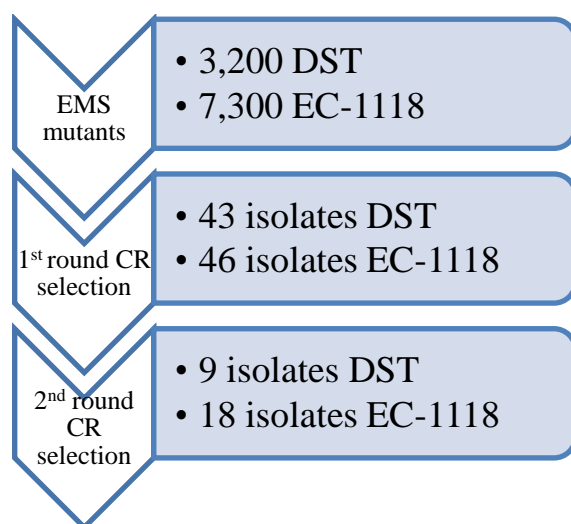

EMS mutants	• 3,200 DST • 7,300 EC-1118
1st round CR selection	• 43 isolates DST • 46 isolates EC-1118
2nd round CR selection	• 9 isolates DST • 18 isolates EC-1118

Figure 4.3 CRS mutant isolation flow chart. The number of EMS mutants of Distinction (DST) or EC-1118 screened for Congo Red sensitivity within each round are indicated.

4.3.2 Characterisation of CRS mutants in micro-fermentations

Only 14 of the 77 CRS mutants (7 from each strain) had significantly shortened fermentation duration (between 18 and 24 h shorter) in comparison to their parent (Table 4.1 and Figures 4.4 A and B). Both parent strains DST and EC-1118 utilised all sugars in 187 h. Of three quicker strains of DST, 37-1 to 37-3, 41-2 and 42-1 to 42-3, represent single colony isolates from mutants 37, 41 and 42, respectively. Of the quicker strains of EC-1118, 110-1 to 110-3 are single colony isolates from mutant 110; whilst 115-2 and 115-3, and 138-1 and 138-2 were clonal isolates from mutants 115 and 138, respectively. The fastest mutant, 37-1 catabolised all sugars in 163.5 h; 13% faster than its parent. A total of 6 out of 27 EMS mutants (9 Distinction

and 18 EC-1118 after the 2nd round of CR selection) were identified as potential HNE candidates having at least 10% shorter fermentation as compared to the parent (Table 4.1). Four candidates with the shortest fermentation duration were chosen for further analysis at a larger scale (37-1, 37-3, 42-2, 115-3, 138-1, 138-2). Two slower performing colonies (107-3 and 113-1) were also included for validation of fermentation performance observed in micro-fermentations.

Table 4.1 CRS strains that have shorter fermentation duration than their parent. Data represents the mean of duplicates, n= 2.

Glycerol stock #	Colony #	Fermentation duration (h)	% of wild type	Background
11	37-1	163.5	87	DST
12	37-2	168.5	90	DST
13	37-3	168.5	90	DST
18	41-2	168.5	90	DST
20	42-1	168.5	90	DST
21	42-2	168.5	90	DST
22	42-3	168.5	90	DST
37	110-1	168.5	90	EC-1118
38	110-2	168.5	90	EC-1118
39	110-3	168.5	90	EC-1118
50	115-2	168.5	90	EC-1118
51	115-3	168.5	90	EC-1118
70	138-1	168.5	90	EC-1118
71	138-2	168.5	90	EC-1118
36	107-3	210	112	EC-1118
46	113-1	210	112	EC-1118
DST	PRNT1	187	100	DST
EC-1118	PRNT2	187	100	EC-1118
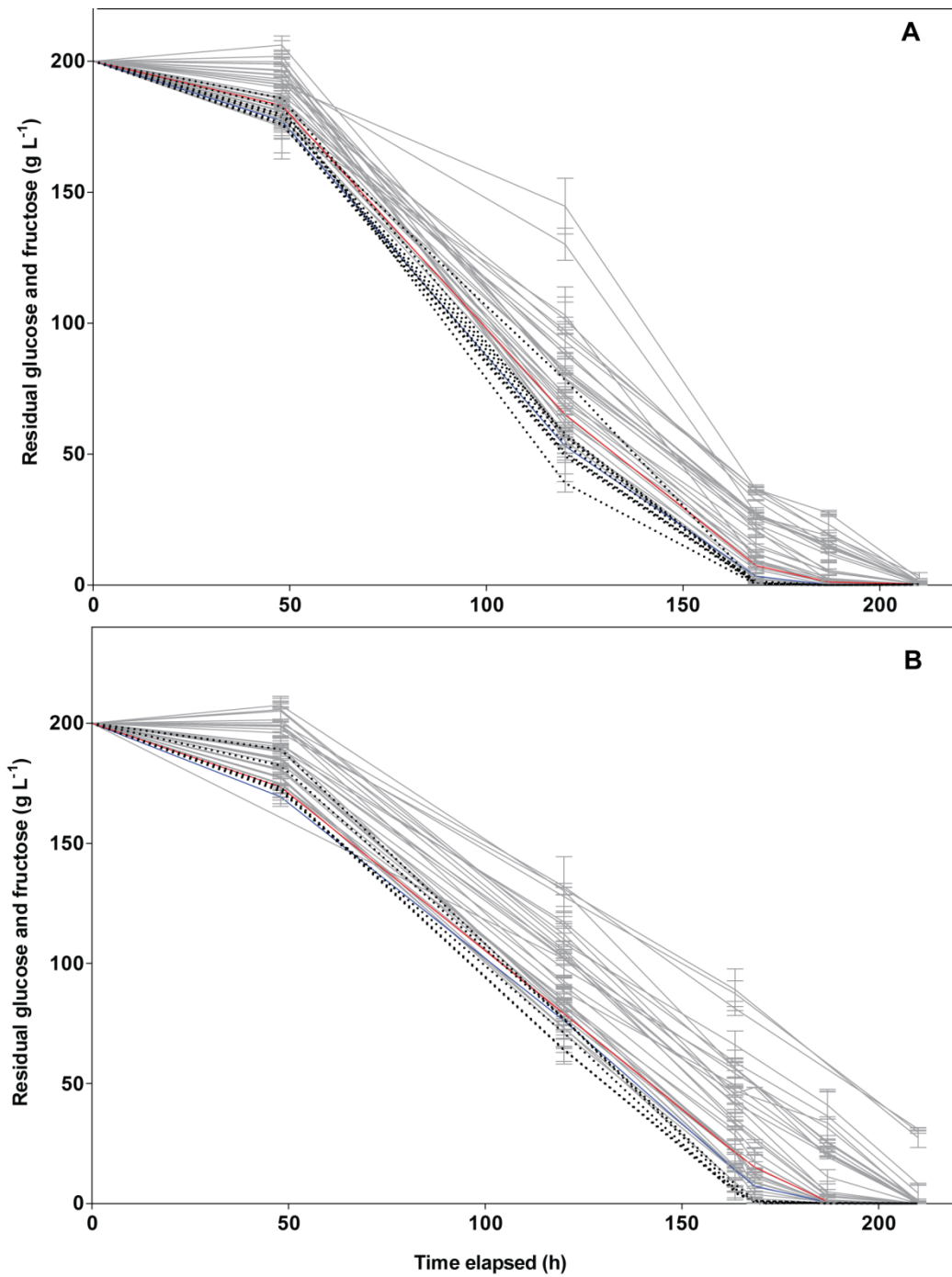
Figure 4.4 Sugar catabolism by CRS mutants from 2nd round CR selection of source plate 1 (A) and source plate 2 (B) during micro-fermentation. DST and EC-1118 are indicated by blue and red lines respectively and CRS mutants with significantly shorter fermentation duration are indicated by the dashed lines. Each data point represents the mean of 2 cultures (± SD).

4.3.3 Performance of CRS isolates in laboratory scale (100 mL) fermentation

Fermentation of the most promising 4 mutants was analysed in a larger volume (100 mL) as this allowed for better control of experimental conditions, i.e. inoculum rate, maintenance of anaerobisis, etc. Under these conditions, the slower colonies 107-3 and 113-1 had the same fermentation duration as their parent (101.5 h), whereas all CRS mutants previously identified as 'fast' completed fermentation before their parents. For example, 138-1 finished fermentation in 83 h, ~20% faster than its parent EC-1118 (101.5 h). Other 'fast' CRS mutants maintained their phenotype in the larger volume, catabolising all sugars in 90 h, ~ 11.3% faster than their parent (Figure 4.5).
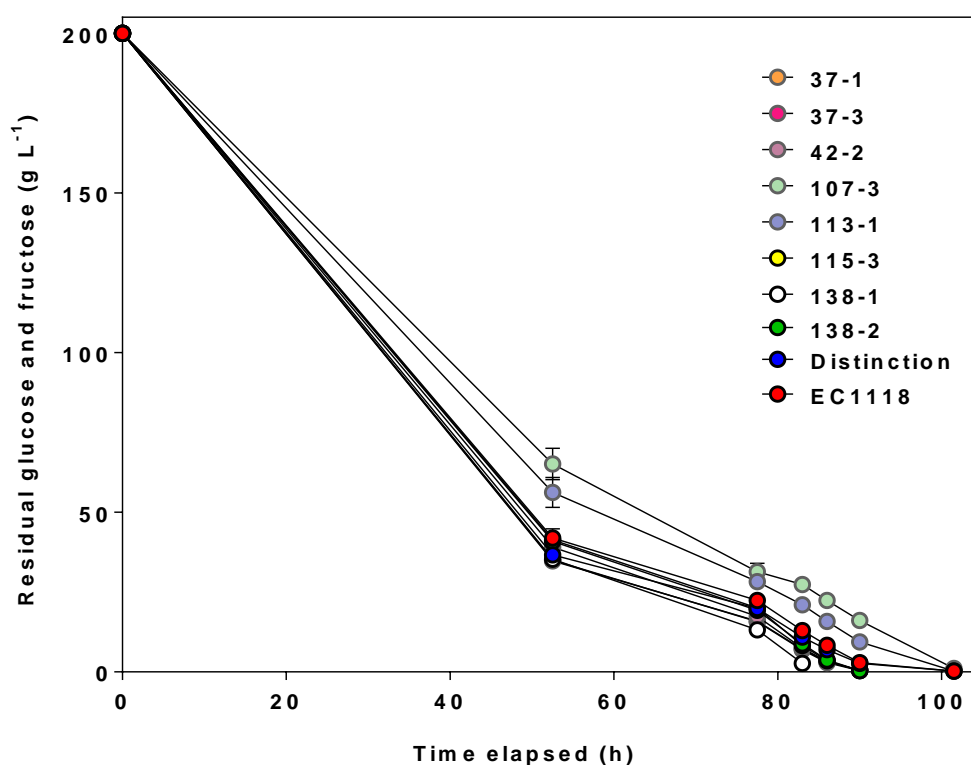
Figure 4.5 Sugar catabolism by 8 CRS mutants in 100 mL scale fermentations. Data points are the mean of triplicate cultures ± SD (n = 3).

4.4 Discussion

In this chapter, the link between HNE phenotype and cell wall chitin content has been investigated. As Δ*ecm33* is identified as an HNE strain with an enhanced fermentation performance, and the disruption of *ECM33* is linked with an increase in cell wall chitin (Chapter 3), speculation arises as to whether chitin production is the major factor associated with the mutant's HNE phenotype. The aim of the study described in this chapter is to generate cell wall mutants as 'proof of concept' on the role of chitin in stress (and fermentation) adaptation. Congo Red (a diazo dye) is commonly used to detect mutants with increased cell wall chitin (Chapter 3 and references therein), whilst ethyl methanesulfonate (EMS) mutagenesis is a common approach to generate random mutants in microorganism or plants (Çakar et al. 2012; Kim et al. 2006; Krieg 1963). Ethyl methanesulfonate results in GC-AT and AT-GC inversions, base pair deletion or insertion into the genome (Kim et al. 2006). Putative mutants are selected from the surviving cells by replica plating onto solid medium in the presence and absence of a specific stress or treatment, which generates either growth sensitivity or resistance of the candidate mutants. The corresponding genome mutation(s) that may be functionally correlated with the phenotype of interest is identified through whole genome or single gene sequencing (Kim et al. 2006).

Putative cell wall mutants were selected following EMS treatment of the commercial wine yeasts Distinction and EC-1118 in this study. It was anticipated that some of these mutants would possess a HNE phenotype, perhaps similar with Δ*ecm33*. A total of >10,000 putative mutants were screened for growth sensitivity (cf. Δ*ecm33* phenotype) towards Congo Red (CR) using a tiered approach with 27 being found CR-sensitive. A further investigation of these 27 isolates via micro-fermentations was performed in comparison with their wild type, Distinction and EC-1118. Amongst the

27 screened, 6 mutants (representing 22% of the total number of mutants) showed decreased fermentation duration. The four fastest mutants (37, 42, 115 and 138) and two slow performers (107-3 and 113-1) were chosen to be further evaluated in 100 mL scale fermentations. Consistently, the faster fermenters again performed better (12~20% quicker than the wild type), while the slower ones performed similarly to the wild types (Figure 4.5). Together, these results demonstrate that micro-fermentation (200 μL) is a rapid and reliable method to select mutants of interest. Furthermore, increased chitin content in the cell wall (which causes growth sensitivity towards CR) is not likely to be the sole reason behind Δecm33's HNE phenotype, because in this study not all of the CRS mutants (only 22% of total, 6 out of 27) showed a fast fermentative behaviour (10%~20% quicker than the wild type). Based on the results shown above, it is proposed that there are other mechanisms driving the HNE phenotype in Δecm33 besides chitin compensation in the cell wall. Therefore, investigation of how other cellular mechanisms may contribute to the HNE phenotype is discussed in the next chapter.

Chapter 5 The effect of overexpression of *PTP2* and *SLT2* on fermentation by a wine yeast

5.1 Introduction

Wine yeasts are often faced with a dynamic environment particularly that found during fermentation. The proper function of cell organelles and related bio-mechanisms are required for maintenance of regular physiological activities. Yeast has its own regulatory system to sense and receive stress signal, which are further amplified and transmitted to the nucleus through a set of conserved modular MAP kinase cascades (Igual and Estruch 2000). Five MAPK signalling pathways in yeast have been identified including the hyper-osmolarity glycerol (HOG) pathway, cell wall integrity pathway (CWI), filamentation/invasion pathway, mating pheromone pathway and cell sporulation assembly pathway (Cullen and Sprague 2012; Haber 2012; Hohmann 2002; Neiman 2011). Some MAPK cascades also share the same proteins so that crosstalk between pathways, and a few studies have focused on signal leakage, decision making and/or correlation between these MAPK pathways (McClean et al. 2007; Saito 2010).

In previous chapters, the *ECM33* gene has been investigated for its involvement with a HNE phenotype during fermentation, with further characterisation of Δ*ecm33* revealing that deletion of *ECM33* leads to changes in the expression of *ECM33*-related genes during fermentation. Proteins encoded by these genes might act either upstream or downstream of Ecm33p-involved HNE pathways, and an investigation of these putative Ecm33p regulators would add further insight into nitrogen use efficiency in wine yeast as well as contribute to understanding the molecular mechanism of Ecm33p by reverse genetics.

Two MAP kinases, Slt2p and Hog1p participating in CWI and HOG signalling pathways, respectively, and genes of each have been demonstrated to be transcriptionally up-regulated in Δ*ecm33* (Chapter 3). Similarly an increase in the gene expression of *PTP2* was also observed (Chapter 3). Ptp2p has been determined to be a negative regulator of both Slt2p and Hog1p (Maeda et al. 1994; Mattison et al. 1999), their involved pathways might both be activated as a result from disruption of *ECM33*. Therefore, it is presumed that the up-regulation of *SLT2* and *HOG1* may be as a result of induced *PTP2* transcription in Δ*ecm33*. For this reason *PTP2* is worthy of further characterisation during fermentation. Moreover, the activation of the Slt2p-mediated CWI pathway has also been reported in cell rescue as discussed in earlier chapters. CWI activation may induce yeast cell wall synthesis during growth or under stresses (Martín et al. 2000). It is also hypothesized that the activation of *SLT2* might be beneficial in Δ*ecm33* by enabling the cell to better tolerate increasing osmolarity and also potentially nutrient starvation during fermentation. In summary, Slt2p is considered an important regulator of or regulatory target of Ecm33p during fermentation. In this chapter, the role of *PTP2* and *SLT2* is characterised in wine yeast during fermentation via gene manipulation in both the presence and absence of *ECM33*.

5.2 Materials and Methods

5.2.1 Media and strains

The haploid wine yeast strain C911D Δ*ura3* was constructed by deletion of *URA3* via homologous recombination, using the methodology described in Chapter 3. This strain was used to harbor the described plasmids. The pDR1123 plasmid (Replogle et al. 1999) containing a *KANMX4* ORF flanked by *URA3* sequences was digested by restriction enzymes *Kpn*I and *Sac*II. The digested fragments were purified

by gel purification using Promega PCR purification (Cat # A9282). The purified $\Delta ura3$::*KANMX4* fragment was then transformed into C911D, and transformants were selected on YPD agar containing 0.2 g L^{-1} geneticin. Uracil auxotrophy was then tested on minimal drop-out minus uracil plates (Adams et al. 1997, Appendix 2). PCR confirmation was also performed with URA3F1 and URAR primers (as listed in Table 5.3). Minimal Drop-out medium (Difco Laboratories, 1998) lacking uracil was used for selection of auxotrophic yeast strains harbouring derivatives of plasmid pJC1 (Crous et al. 1995). Yeast strains, *E. coli* strains and plasmids used in this chapter are summarised in Table 5.1 and 5.2.

Table 5.1 Yeast strains used in this chapter

Yeast Strains	Genotypes	Source
C911D	L-2056 *Δho MATα*	Walker et al. (2003)
C911D *Δura3*	C911D *Δura3::KANMX4 MATα*	This study
C911A *Δura3*	C911D *Δura3::KANMX4 MAT*a	This study
C911D *Δecm33*	C911D *Δecm33::KANMX4 MATα*	Chapter 2, this study
1C	C911D *Δecm33 Δura3::KANMX4 MATα*	This study

Table 5.2 *E. coli* strains and plasmids used in this chapter

E. coli strains or plasmids	Source
TOP10	Life Technology
DH5α	Life Technology
DB3.1	Life Technology
pMDC30	Curtis and Grossniklaus (2003)
pENTR^{TM}/D-TOPO$^{®}$	Life Technology
pJC1	Crous et al. (1995)
pJC1/GW	This study
*PTP2*_pJC1/GW	This study
*SLT2*_pJC1/GW	This study

Table 5.3 Primers for PCR and sequencing

Primers	Sequence (5'-3')
*PTP2*_100bp_up_F	CACAGCCCTTTTCGCTTAGT
*PTP2*_CDS_F	<u>CACC</u>ATGGATCGCATAGCACAGCA
*PTP2*_seq_F1	CTTTTTGCCTCATTGAGAAG
*PTP2*_seq_F2	TCACAAAAGCAGAGGGGTC
*PTP2*_seq_F3	TCCAACCACACTATCAAACTT
*PTP2*_CDS_R	TTAACAAGGTAACGCGTTCTTTATC
*PTP2*_100bp_down_R	GAGAGGTTATGTAATTTATCAAAACG
*SLT2*_100bp_up_F	CAAAATAGTAGAAATAATTGAAG
*SLT2*_CDS_F	<u>CACC</u>ATGGCTGATAAGATAGAGAG
*SLT2*_seq_R	TTAGCTCACGTAGGGATCTTTTA
*SLT2*_seq_F1	GATGTCTTGCATCGTGATTTGA
*SLT2*_seq_F2	GAGAATTACCGTGGATGAGGC
*SLT2*_CDS_R	CTAAAAATATTTTCTATCTAATCCAAACTC
*SLT2*_100bp_down_R	GCTTACATCTATGGTGATTCTATA
URA3F1	AGGGAAGACAAGCAACGAAA
URAR	TTGGTTCTGGCGAGGTATTG
*PTP2*_qPCR_F1	GGTGTTTTCGTTACCTTGGA
*PTP2*_qPCR_R1	GCGTTCTTTATCTGCTTTTGC
pPGK1	ATACATATTTGGTCTTTTCTAATTC
GW_*Xho*I_F1	TACTATCTCGAGATCAAACAAGTTTGTACAAAAAAGC
GW_seq_F1	TCCGTATGGCAATGAAAGAC
GW_seq_F2	AGCGACAGCTATCAGTTGCT
GW_*Xho*I_R1	ATCTGACTCGAGACCACTTTGTACAAGAAAGCTGAA
*ECM33*_Adelweb	AGCCGGTATAAATATTCAATGTCAA
KanB	CTGCAGCGAGGAGCCGTAAT
MAT	AGTCACATCAAGATCGTTTATGG
MATa	ACTCCACTTCAAGTAAGAGTTTG
MATα	GCACGGAATATGGGACTACTTCG

Note: CACC is designed in the forward primer (5'-end) for directional cloning (Invitrogen).

5.2.2 PTP2 *and* SLT2 *gene sequencing in the C911D background*

As C911D has not been fully sequenced, the coding sequences (CDS) of *SLT2* and *PTP2* were initially amplified from genomic DNA plus about 100 base pairs flanking region at both ends of the CDSs using VELOCITY DNA polymerase (Bioline Cat # 21098). Primers used for PCR reactions and sequencing are listed in Table 5.3. Purified PCR products were sequenced commercially (AGRF, Adelaide). Sequences from C911D and S288C (fully sequenced reference strain) backgrounds were aligned using the Geneious Pro program v5.6.5.

5.2.3 PTP2 *and* SLT2 *gene cloning*

PTP2 and *SLT2* were amplified from C911D genomic DNA with VELOCITY DNA polymerase and pre-designed primer sets listed in Table 5.3. Gene products were cloned into the pENTR^{TM}/D-TOPO$^®$ entry vector (pENTR^{TM} Directional TOPO$^®$ Cloning manual Invitrogen^{TM} Life Technologies^{TM}, Cat # K240020), according to the manufacturer's instructions. Following this, *PTP2*_pENTR^{TM} TOPO$^®$ and *SLT2*_pENTR^{TM} TOPO$^®$ constructs were transformed into competent *E. coli* (One Shot$^®$ TOP10).

5.2.4 Modification of the expression vector pJC1

The multicopy plasmid pJC1 (Crous et al. 1995) containing a strong and constitutive promoter *PGK1* and *URA3* and Ap^R (as selectable markers) was used, since the *PGK1* promoter is functional in the presence of glucose and thus suitable for characterisation of gene products during alcoholic fermentation. In order for pJC1 to be a gateway-enabled destination vector (pJC1/GW), a gateway site was added. The gateway site was amplified from the pMDC30 plasmid (Curtis and Grossniklaus 2003), using the GW_*Xho*I_F1 and GW_*Xho*I_R1 primers (Table 5.3). The purified PCR product and pJC1 were both digested with *Xho*I restriction endonuclease, and

pJC1 was dephosphorlated by Antarctic phosphatase (NEB, Cat # M0289S). Ligation of fragments was performed with T4 ligase at 15 ℃ overnight and then transformed into competent *E. coli* DB3.1[TM] (Invitrogen). Transformants were selected on LB agar containing 100 mg L^{-1} ampicillin and clones with the correct orientation were confirmed by agarose gel separation of *Eco*RI-digested plasmid DNA and sequencing with appropriate primers (Table 5.3).

5.2.5 Construction of PTP2 *and* SLT2 *overexpression vectors*

PTP2 was cloned from *PTP2*_pENTR[TM]/D-TOPO[®] into the gateway enabled destination vector pJC1/GW using Gateway[®] LR Clonase II Enzyme Mix (Life Technologies Cat # 11791020), based on the manufacturer's instructions. The constructed *PTP2*_pJC1/GW was then transformed into *E. coli* TOP10[TM] supplied with the vectors (Invitrogen). The *PTP2*_pJC1/GW plasmid was then verified by digestion and sequencing. Purified *PTP2*_pJC1/GW was isolated using Wizard[®] Plus SV Miniprep DNA Purification System (Promega, Cat # A1465), and transformed into the C911D Δ*ura3* wine yeast strain (Gietz and Woods 2005). The presence of the *PTP2* over-expression plasmid in yeast was confirmed by colony PCR reactions using pPGK1 (plasmid backbone) and *PTP2*_CDS_R primers (Table 5.3). A *SLT2* overexpression vector was constructed alike to that for *PTP2*.

Cell viability and plasmid retention were determined after 72 h of fermentations. 72 h cell samples were collected and washed with sterile 1×PBS buffer. The washed cells were counted and then plated onto YPD and minimal DO-uracil plates for cell viability and plasmid retention. Cell viability (%) was calculated by cell colony forming unit (cfu) on YPD plate as described in Chapter 3. Following this, plasmid retention (%) was calculated by the following formula:

Plasmid retention (%) = $\text{cfu}_{\text{minimal DO-uraci}}/(\text{cfu}_{\text{expected}} \times \text{cell viability \%})$

5.2.6 Gene expression analysis

Gene expression analysis was performed using Qualitative Real Time-PCR, details of which are reported in Chapter 3, with variations described in the associated figure legends.

5.2.7 Construction of yeast strain 1C (Δecm33Δura3)

Yeast mating. Yeast strain 1C (*Δecm33Δura3*) was constructed by mating C911A *Δura3* MATa and C911D *Δecm33* MATα. These two haploid strains were streaked for single colonies on YPD agar plates and incubated at 28 ℃ for 2 days. A loop of fresh culture from each strain was then mixed on a fresh YPD plate, with a drop of sterile MilliQ water and incubated at 28 ℃ for 3 h. Successfully mated cells were identified by their characteristic dumbbell shape and moved to a culture free section of the YPD plate with a micromanipulator (Olympus, Model CHT # 4J0148). The diploid cells (C911A *Δura3* MATa × C911D *Δecm33* MATα) were then incubated at 28 ℃ for 2 days.

Yeast sporulation. Putative diploid colonies were inoculated into YPD liquid and incubated at 28 ℃ overnight with shaking (120 rpm). 500 µL of culture was then added into 5 mL pre-sporulation media (0.8% Yeast extract, 0.3% Bactopeptone and 10% glucose; 0.22 µm filter sterilized). Culture tubes were kept horizontal in a rolling drum rotating at room temperature for 4 h. Cells were then centrifuged at 5000 rpm for 5 min, and washed twice in 5 mL of sterile MilliQ water. Finally, the washed cells were resuspended in 10 mL of sporulation medium 2 (0.5% potassium acetate, 10 mL 10× uracil stock (1 g L^{-1}), 0.22 µm filter sterilized), and incubated at room temperature with gentle shaking for 6 days. The formation of asci was checked microscopically.

Yeast ascus digestion and dissection. Once spores were visible, 2 mL of sporulated culture was harvested by centrifugation (5000 rpm, 2 min). Cell pellets were resuspended in 1 mL of sterile MilliQ water. 500 µL of cell resuspension was diluted with 500 µL of sterile MilliQ water and 10 µL β-glucuronidase (Sigma-Aldrich Cat # G7770-2ML), mixed well and incubated at 28 ºC for 15 min. The cells were then placed on ice for later dissection. The digested spore suspension was carefully spread onto thin YPD plates with an inoculation loop and 4 spore asci were isolated and separated with a micromanipulator. The isolated spores were incubated at 28 ºC for 3 days. Genomic DNA was isolated from 10 mL YPD cultures of each of the spore progeny (Adams et al. 1997), which was used for mating type identification by mating type PCR using the MAT, MATa and MATα primers (Table 5.3; Walker et al. 2003). Additionally, the genotype of each spore from one tetrad was identified by two PCR reactions (primers URA3F1 and URAR, *ECM33*_Adelweb and KanB; Table 5.3) to verify both *ECM33* and *URA3* had been deleted. The genotype C911D Δ*ecm33* Δ*ura3::KANMX4 MATα* was then used as the background strain for *PTP2* and *SLT2* OEX vector transformation.

5.2.8 Small scale of fermentations (100 mL)

Please refer to Chapter 3 section 2.3 for methodology.

5.2.9 HPLC analysis

Please refer to Chapter 3 for HPLC methodology. Statistical analysis was performed by Prism 6 using Two-way ANOVA.

5.2.10 Statistical methods

Excel 2007 (Microsoft® Office) and GraphPad Prism 6 (GraphPad Software Inc., La Jolla, C.A., USA) were applied for statistic analysis. Significant differences of the specific data set were compared using One-way ANOVA, Two-way ANOVA

or Student t-test, three of which are integrated in Prism 6 software. Indication is shown for the specific statistic analysis applied.

5.3 Results

5.3.1 DNA sequence of wine yeast derived PTP2

The expression of some of the key genes putatively identified in this study to be affected by deletion of *ECM33* (e.g. *PTP2* and *SLT2*). It is worth to analyse the effects of modification of their expression to improve yeast fermentation performance. The sequences of these two genes were initially analysed in the wine yeast *S. cerevisiae* C911D. *PTP2* CDS ± 100 bp was amplified by PCR from genomic DNA of C911D (a derivative of the wine yeast strain L2056) and sequenced by AGRF. The sequence of *PTP2* (C911D) was aligned with *PTP2* from S288C (Figure 5.1, sequence alignment refer to Appendix 5).
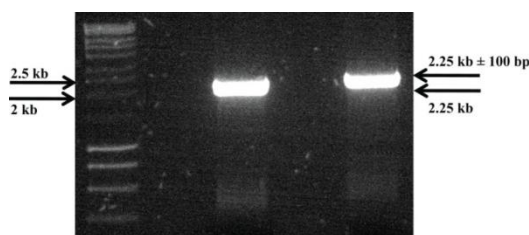
Figure 5.1 Confirmation by gel electrophoresis of PCR amplification products of *PTP2* (2.25 kb) and *PTP2* ± 100 bp from C911D genomic DNA using primers *PTP2*_100bp_up_F and *PTP2*_100bp_down_R

In both S288C and C911D, the CDS of *PTP2* was 2,253 bp nucleotides in length but with 7 single nucleotide polymorphisms (SNPs) (Appendix 5). This difference results in 6 amino acid changes in Ptp2p from C911D, including arginine to glycine (R9G), asparagine to serine (N10S), serine to threonine (S395T), isoleucine to threonine (I585T), serine to asparagine (S650N), and glutamic acid to aspartic acid (E651D) (Appendix 6).

5.3.2 DNA sequence of wine yeast derived SLT2

Similar with *PTP2*, *SLT2* CDS was analysed in the wine yeast strain C911D strain. The sequence of *SLT2* CDS ± 100 bp was determined following the method as described above for sequencing *PTP2* in C911D (data not shown). The CDS of C911D *SLT2* was aligned to S288C as above. C911D derived *SLT2* was 1449 bp in length, compared to 1455 bp for S288C. A small deletion of six nucleotides (CAGCAG) was present in C911D *SLT2* (position 1,141 to 1,146 bp, Appendix 7), corresponding to the omission of 2 glutamines (Q) at positions 376 and 377, which also corresponded to a glutamine-rich region within the protein sequence. This glutamine-rich region in Slt2p has been suggested to correlate with the yeasts capability for adaption to changing environments (de Llanos et al. 2010). Additionally, 4 synonymous SNPs did not change their corresponding amino acids between S288C Slt2p and C911D Slt2p at 477 (G to A), 576 (C to T), 585 (T to C) and 759 (A to T). Paired-alignment of the Slt2p translated protein sequence from C911D (483 aa) to S288C (485 aa) was performed using Geneious Alignment (Geneious Pro program v5.6.6). Besides the absence of G^{376} and G^{377} in C911D, no further difference in protein sequence was found in comparison to S288C (Appendix 8).

5.3.3 PTP2 *overexpression (OEX) analysis*

As shown in Chapter 3, the deletion of *ECM33* induced up-regulation of some genes such as *SLT2*, *HOG1* and *PTP2*. The former, *SLT2* and *HOG1* are known to encode MAP kinases involved in CWI and HOG pathways, respectively. Ptp2p has been reported to negatively regulate Slt2p and Hog1p via dephosphorlating both and/or either a phospho- threonine or phospho-tyrosine residue: Thr^{190} and Tyr^{192} of Slt2p; Thr^{174} and Tyr^{176} of Hog1p (Brewster et al. 1993; Hahn and Thiele 2002; Martín et al. 2000). Deletion of *PTP2* is also known to exacerbate the growth defect

of both *SLT2* and *HOG1* overexpression strains (Jacoby et al. 1997; Mattison et al. 1999). To examine the role of *PTP2* in yeast fermentation, *PTP2* was overexpressed using the pJC1/GW vector. The C911D derived *PTP2* CDS was cloned into the intermediate vector, pENTR/D/TOPO and afterwards sub-cloned into pJC1/GW, whereby *PTP2* was constitutively expressed using the *PGK1* promoter, which is widely used for gene overexpression in yeast (Kingsman et al. 1990; Nissen et al. 2000; Romanos et al. 1992). The overexpression of *PTP2* was confirmed by QRT-PCR (Figure 5.2 a). Using this system *PTP2* was overexpressed by ~ 10^3-fold compared with C911D Δ*ura3* containing empty vector pJC1/GW (control).

In Chapter 3, preliminary QRT-PCR data highlighted the interplay between *ECM33* and other genes such as *PTP2*, *SLT2* and *HOG1*. To examine how these interactions present in the overexpression system and whether overexpressing *PTP2* would influence expression of these genes, the transcripts of *ECM33*, *SLT2* and *HOG1* were determined in the *PTP2* OEX strain. The relative transcription levels of these four genes were measured in cells sampled from a 16 hour culture (i.e. cells from stationary phase) in minimal drop-out minus uracil media. The transcriptional abundance of *SLT2* was found to be slightly decreased in the *PTP2* OEX strain compared with the control strain (Figure 5.2 c). However, overexpression of *PTP2* did not affect either *ECM33* or *HOG1* expression (Figure 5.2 b and d).
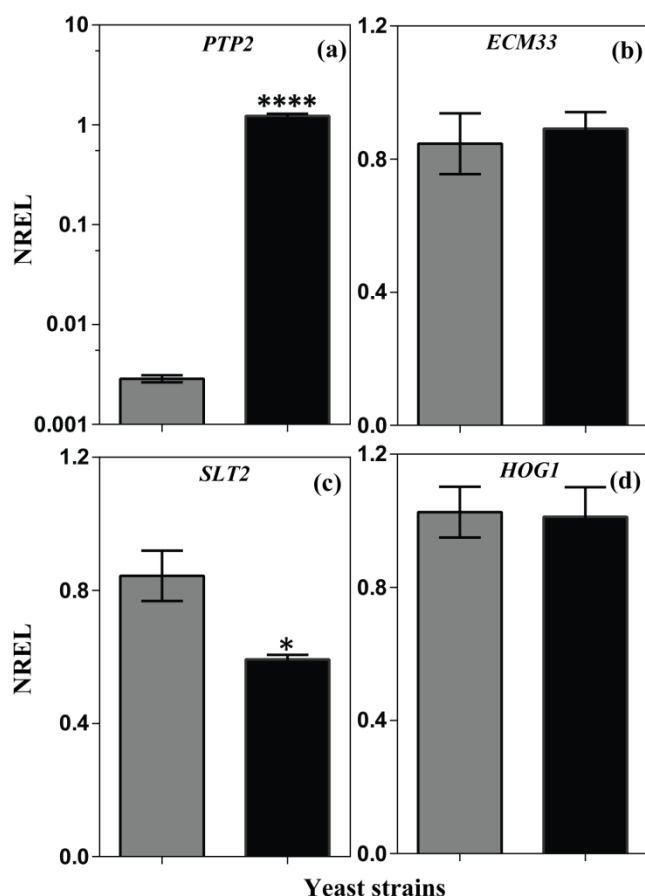
Figure 5.2 Normalized relative expression level (NREL) of key genes involved in the CWI and HOG pathways in C911D Δ*ura3* harbouring either empty vector control pJC1/GW (■) or *PTP2*-pJC1/GW (■): (a) *PTP2*, (b) *ECM33,* (c) *SLT2* and (d) *HOG1*. Data points represent the mean ± SD, n = 4. Statistical analysis was performed using Student t-test: *, $p < 0.05$; ****, $p < 0.0001$.

5.3.4 The influence of overexpression of PTP2 on fermentation performance (Fermentation 1)

The alcoholic fermentation performance of wine yeast C911D Δ*ura3* with either the *PTP2* overexpression vector *PTP2*_pJC1 or an empty control vector was evaluated. When *PTP2* was overexpressed, there was minimal impact on sugar utilization. During the early part of fermentation (after 48 h), a small but statistically significant difference in sugar consumption was observed with *PTP2* overexpressed. For example at the 55.5 h time point the control had consumed an extra 14 g L^{-1} of sugar as compared to the *PTP2* OEX strain (Student t-test, *** $p < 0.001$). Over the

remainder of fermentation sugar utilisation by the latter strain increased, which resulted in the strain finishing fermentation at a similar time (156 h) to the control. A reduction in growth was also noted when *PTP2* was overexpressed in these cells (Figure 5.3). At the midpoint of fermentation (72.5 h), cell viability and plasmid retention were examined as well as cell dry weight at the end of fermentation. A slight decrease (11%) in plasmid retention was found in the *PTP2* OEX strain. Decrease of cell biomass was found in *PTP2* overexpression strain compared with control strain (Student t-test, ** $p < 0.01$). There was insignificant difference in cell viability or plasmid retention between *PTP2* OEX and the control in C911D Δ*ura3* background (Fermentation 1, Table 5.4).
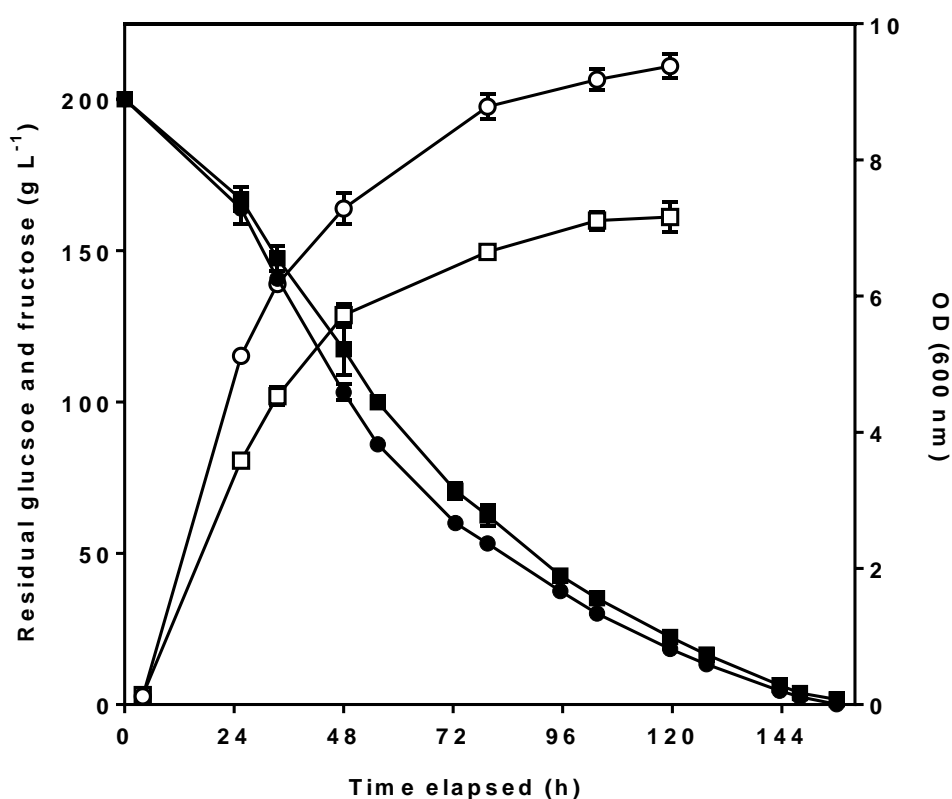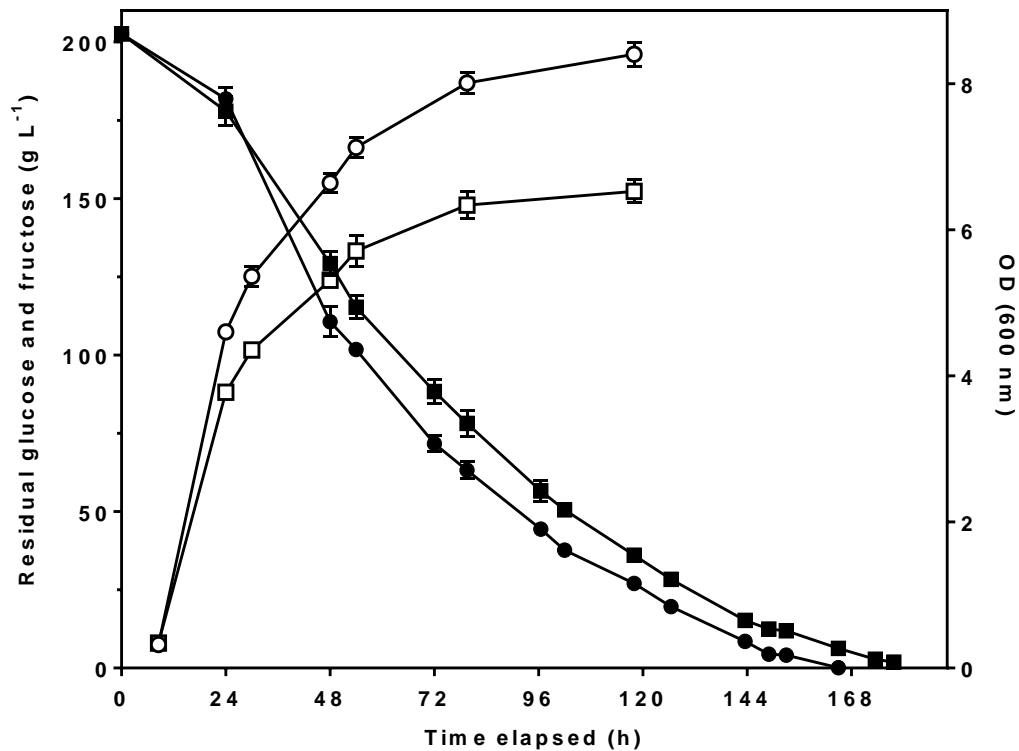

Figure 5.3 Sugar catabolism by C911D Δ*ura3* with control vector (●) or *PTP2* OEX (■) and optical density (OD_{600} nm) of vector control (○) and *PTP2* OEX (□) are shown. Fermentations were conducted in CDGJM with 450 mg L^{-1} nitrogen lacking uracil. Data points represent the mean of triplicates ± SD.

Table 5.4 Fermentation duration, cell viability, plasmid retention and cell dry weight (biomass) measured in three separate fermentation experiments. Data is the mean ± SD, n = 3-5. Statistical analysis was performed using Student t-test (Fermentation 1 and 2) or One-way ANOVA (Fermentation 3).

	Fermentation 1		Fermentation 2		Fermentation 3			
	C911D Δura3	C911D Δura3 + *PTP2* OEX	C911D Δura3	C911D Δura3 + *SLT2* OEX	C911D Δura3 (WVC)	C911D Δecm33 Δura3 (MVC)	MVC + *PTP2* OEX	MVC + *SLT2* OEX
Fermentation duration (h)	156	156	165	178	153	102	126	126
(% of control strain)	100	100	100	108	100	67	82	82
Biomass (grams of cell/litre)	4.4 ± 0.1	4.1 ± 0[b]	4.2 ± 0	3.4 ± 0.1[d]	4.4 ± 0.1	4.5 ± 0.1	4.2 ± 0.1[b]	3.8 ± 0.1[d]
Cell viability% (72 h)	47 ± 15	42 ± 26	63 ± 9	57 ± 15	64 ± 12	49 ± 8	54 ± 18	57 ± 10
Plasmid retention% (72 h)	24 ± 12	13 ± 8	39 ± 16	33 ± 10	91 ± 4	80 ± 15	30 ± 10[c]	75 ± 13

[a] indicates significant difference (sig. dF) p<0.05; [b] indicates sig. dF p <0.01;

[c] indicates sig. dF p <0.001; [d] indicates sig. dF p < 0.0001

5.3.5 The influence of overexpression of SLT2 *on fermentation performance (Fermentation 2)*

In a similar experiment to that for *PTP2* overexpression, the *SLT2* gene was initially sub-cloned into the entry vector (*SLT2*-pENTR/TOPO) and afterwards transferred into the overexpression vector, *SLT2*-pJC1/GW. The cloning of the *SLT2* gene into the plasmids was confirmed by colony PCR, restriction enzyme digestion and sequencing (AGRF). DNA sequence analysis verified the correct amplification of the *SLT2* CDS as that found in C911D (data not shown). The overexpression of *SLT2* also resulted in reduced cell growth during alcoholic fermentation (Figure 5.4). This lower biomass may explain why *SLT2* OEX had a slower rate of sugar catabolism and consequently a longer fermentation, 178 h *vs*. 165 h (Figure 5.4 and Table 5.4). No significant difference in cell viability and plasmid retention at 72 h, but a decreased dry cell weight was observed in the *SLT2* OEX strain at the end of fermentation (Student t-test, **** $p < 0.0001$, Fermentation 2, Table 5.4).

Figure 5.4 Sugar catabolism by C911D Δ*ura3* with control vector (●) or *SLT2* OEX (■) and optical density (OD$_{600}$ nm) of vector control (○) and *SLT2* OEX (□) are shown. Fermentations were conducted in CDGJM with 450 mg L^{-1} nitrogen lacking uracil. Data points are the mean of 5 replicates ± SD.

5.3.6 Construction of C911D Δecm33 Δura3

Due to reduced cell growth when *PTP2* or *SLT2* was overexpressed in C911D Δ*ura3*, it was hypothesised that deletion of *ECM33* might restore growth fitness in these strains. To examine this idea, both genes were overexpressed separately in C911D Δ*ecm33* Δ*ura3*. Initially, the C911D Δ*ecm33* Δ*ura3* strain was constructed. Deletion of *URA3* from C911D Δ*ecm33* was achieved through classical genetic methods, mating and sporulation (as described in section 5.2.7). In brief, haploid strains C911D Δ*ecm33* (MATα) and C911A Δ*ura3* (MATa) were mated to form a diploid strain. Following this, haploids were isolated via sporulation and dissection. Then genotypes and mating types were analysed by two PCR reactions and growth on

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minimal DO-uracil plates. PCR products from ECM33A and KanB or mating type primers sets were separated by gel electrophoresis, to identify which strains retained Δ*ecm33*::*KANMX4* cassettes and their mating types. Strains 1C was identified as C911D Δ*ecm33* Δ*ura3*::*KANMX4* MATα, via the PCR analyses (data shown in Appendix 3)together with its inability to grow on minimal drop-out minus uracil plates.

5.3.7 Fermentation performance of C911D Δecm33 Δura3 with either PTP2 *or* SLT2 *overexpressed (Fermentation 3)*

PTP2 and SLT2 overexpression vectors were transformed into C911D Δ*ecm33* Δ*ura3* and their presence was checked by colony PCR with the primers pPGK1 (forward) and *PTP2*_CDS_R or *SLT2*_CDS_R as reverse (see Table 5.3; data not shown). The verified strains were then examined for their fermentation performance in comparison to both C911D Δ*ecm33* Δ*ura3* with an m̲utant empty v̲ector c̲ontrol (MVC) and C911D Δ*ura3* (w̲ild type empty v̲ector c̲ontrol, WVC). Similar to previous experiments, deletion of *ECM33* in C911D Δ*ecm33* Δ*ura3* resulted in a significantly shortened fermentation duration (33%) quicker, 102 h *vs*. 153 h (Figure 5.5 (a); Table 5.4). The overexpression of *PTP2* or *SLT2* in C911D Δ*ecm33* Δ*ura3* decreased sugar utilisation efficiency compared with MVC. Both of the overexpression strains finished fermentation sooner than the wildtype (i.e. 126 h *vs* 153 h), but they were actually slower than the C911D Δ*ecm33* Δ*ura3* (102 h). As such, neither OEX construct reduced fermentation duration, but rather appeared to reduce the benefits of the *ecm3* deletion (Table 5.4 and Figure 5.5 a). The overexpression of *PTP2* and *SLT2* decreased cell density similar to that seen in C911D Δ*ura3* background (Table 5.4 and Figure 5.5 b).

Both cell viability and plasmid retention at the 72.5 h time point were examined. There was no significant differences between strain cell viability, however, the overexpression of *PTP2* in C911D Δ*ecm33* Δ*ura3* lowered plasmid retention rate compared with C911D Δ*ecm33* Δ*ura3* with the empty vector (MVC) (One-way ANOVA, ***, p = 0.0006 < 0.001). After fermentation finished, cell biomass was also examined. Either overexpression of *PTP2* or SLT2 decreased cell dry weight (grams cell per litre), i.e. C911D Δ*ecm33* Δ*ura3* cells were 0.3 g and 0.7 g heavier than *PTP2* OEX and *SLT2* OEX strains, respectively (One-way ANOVA, ** $p_{PTP2\ OEX}$ = 0.0021 < 0.01; **** $p_{SLT2\ OEX}$ < 0.0001) (Table 5.4).
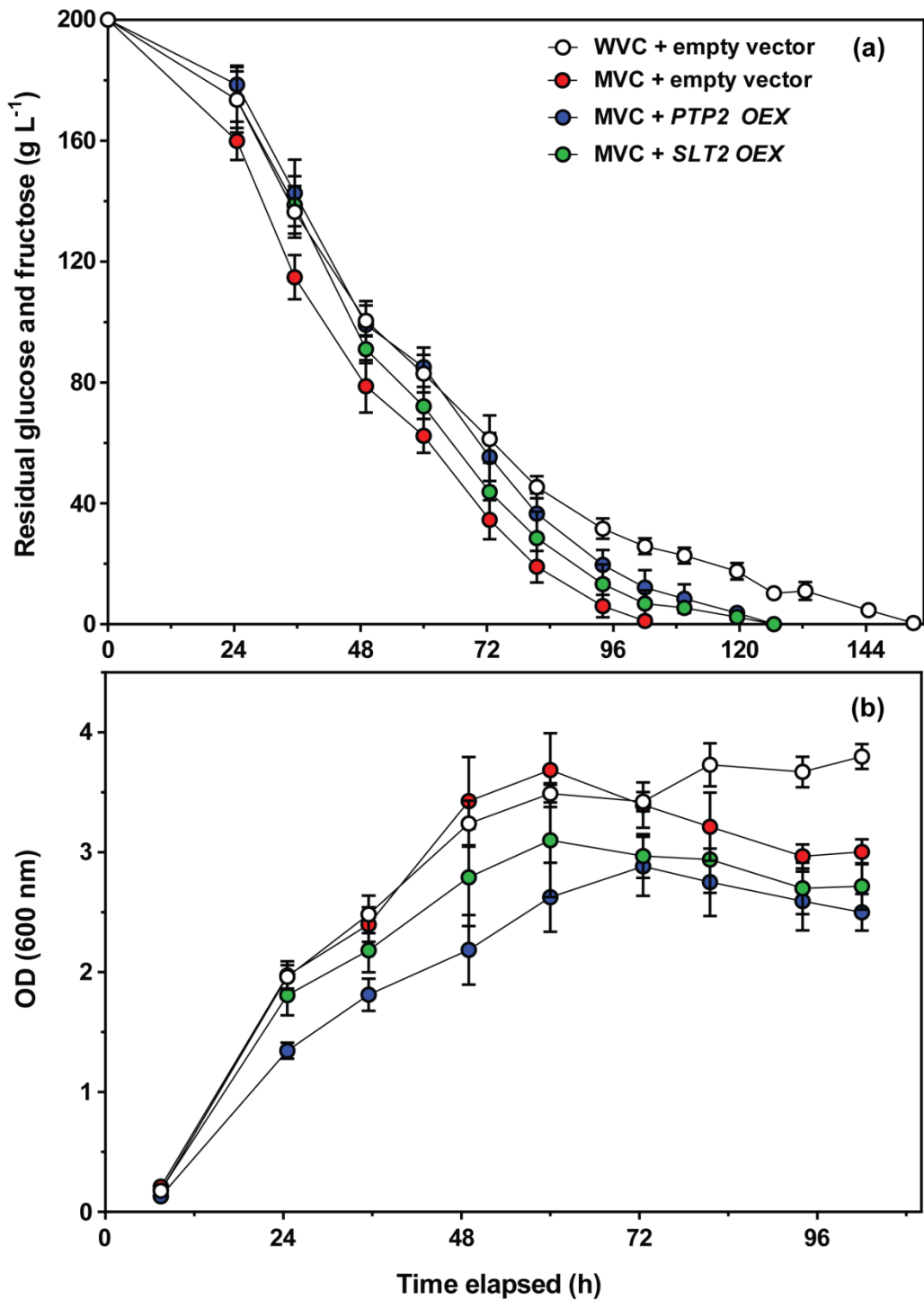
Figure 5.5 Comparison of sugar catabolism and growth (as optical density OD 600 nm) by overexpressing *SLT2* and *PTP2* to WVC (C911D Δ*ura3*, white circle) and MVC (C911D Δ*ura3* Δ*ecm33*, red circle), both of which were contained the empty control vector. MVC containing *PTP2* OEX vector (blue circle), or *SLT2* OEX vector (green circle) are shown. Fermentations were conducted in CDGJM supplied with 200 g L^{-1} sugars and nitrogen as a mixture of ammonia and amino acids lacking uracil. Data points are the mean ± SD, n = 4.

Metabolites from terminal fermentation samples (residue total sugars < 2 g L^{-1}, dry) were analysed using HPLC to determine whether glycerol and ethanol productions were affected through deletion of *ECM33* together with the overexpression of *PTP2* and *SLT2*. Final ethanol concentrations increased in both *PTP2* and *SLT2* OEX strains e.g. 125.5 g L^{-1} (*PTP2* OEX) and 124.9 g L^{-1} (*SLT2* OEX) *vs.* 120.2 g L^{-1} (MVC) (**** $p < 0.0001$, Two-way ANOVA). Deletion of *ECM33* did not alter glycerol or ethanol levels. Thus, MVC produced 5.2 g L^{-1} of glycerol *vs.* WVC for 5.6 g L^{-1}, and 120.2 g L^{-1} *vs.* 119.9 g L^{-1} of ethanol, respectively. Slightly lower glycerol yields were found when *PTP2* was overexpressed compared with WVC strain e.g. 4.5 g L^{-1} *vs.* 5.6 g L^{-1} (* $p = 0.0133 < 0.05$, Two-way ANOVA), however, no statistical difference of glycerol production was observed among other strains (Figure 5.6). Whilst no differences were noted for acetic acid production, acetaldehyde levels in samples from OEX strains in the MVC background were generally less than in WVC. When organic acid production was examined, the citric acid content of fermentation samples from the *PTP2* OEX strain was calculated to be 2.18-fold more than MCV, 3.9 g L^{-1} *vs.* 1.8 g L^{-1} (**** $p < 0.0001$, Two-way ANOVA) (Figure 5.6).
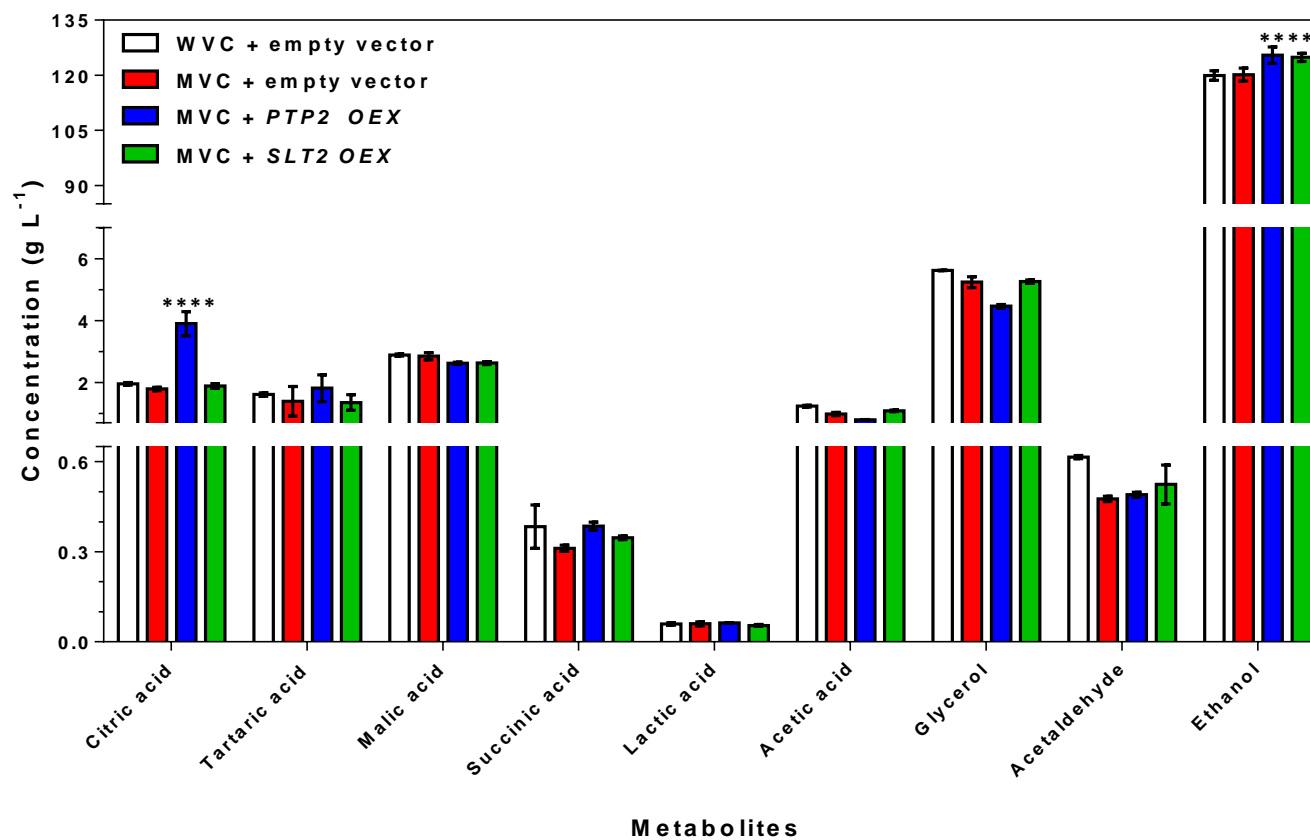
Figure 5.6 Major fermentation metabolites produced by WVC (C911D Δ*ura3*), MVC (C911D Δ*ura3* Δ*ecm33*) and MVC harbouring a *PTP2* or *SLT2* overexpression plasmid.

Fermentations were conducted in CDGJM supplied with 200 g L^{-1} sugars and 450 mg L^{-1} nitrogen minus uracil. Metabolites were detected by HPLC analysis. Data are the

mean of quadruplicates ± SD. Statistical analysis was performed using Two-way ANOVA. Significant differences are indicated, ****, p < 0.0001.

5.4 Discussion

As described previously, the transcriptional abundances of both *SLT2* and *HOG1* responsible for encoding MAPKs in the CWI pathway and HOG pathway, respectively, have been shown to be up-regulated in Δ*ecm33* during fermentation (Chapter 3, Figure 6). The *SLT2*-mediated MAPK signalling pathway can be induced in a number of ways, such as heat shock or hypo-osmotic shock, cell wall damage by chemicals (like Calcofluor White and Congo Red), or cell wall weakening by deletion of genes such as Δ*gas1* (β-glucan synthesis) and Δ*gpi7* (GPI-anchored protein synthesis) (Benachour et al. 1999; Davenport et al. 1995; Martín et al. 2000; Ram and Klis 2006; Ram et al. 1994). The MAP kinase Hog1p is involved in the high osmolarity glycerol (HOG) pathway, and is activated by hyper-osmotic stress (Hohmann 2002). Interestingly, Ptp2p has been reported to be a negative regulator of both Slt2p and Hog1p (Jacoby et al. 1997; Mattison et al. 1999). Furthermore, deletion of *ECM33* and *PTP2* in combination results in a more severe fitness defect, and no interaction or correlation has been observed between *ECM33* and other two MAPK suppressors *PTP3* and *MSG5* (http://drygin.ccbr.utoronto.ca/) (Baryshnikova et al. 2010; Costanzo et al. 2010; Koh et al. 2010).

In this study the transcriptional abundance of *PTP2* was increased at the later stages of the fermentation when *ECM33* was deleted (Chapter 3, Figure 6). So, it was proposed that *ECM33* might normally function to regulate expression of *PTP2* and in turn *SLT2* and *HOG1* and thus, when deleted, stimulates cell wall maintenance. To test this, *PTP2* was cloned and overexpressed in C911D Δ*ura3* background to examine the effect on fermentation. Overexpression of *PTP2* resulted in reduced cell density after 24 h and perhaps was the cause for a slower sugar catabolism observed early in fermentation (Figure 5.3). Nevertheless, it appears that overexpression of

PTP2 enhanced yeast sugar utilisation at later stages of fermentation (after 50 h) so that eventually the strain harbouring a *PTP2* overexpression plasmid and the control yeast had a similar fermentation duration of about 156 h (Figure 5.3; Table 5.4). These results suggest that *PTP2* may influence yeast sugar catabolism in the later stages of fermentations, and further indicate that the fermentation duration is not always determined by the number of cells. This presumption is worthy of further investigation. Supposedly overexpression of *PTP2* leads to down regulation of the CWI pathway, and subsequently yeast may become more fragile and easy to lyse thus explaining the observed lower cell density. This result however is not consistent with the study of Mattison et al. (1999), who observed no growth difference between a *PTP2* overexpression strain and the control. Such differences could arise for a number of reasons, including: 1) in the present study *PTP2* was driven by the *PGK1* promoter, with glucose and fructose supplied as carbon sources, in comparison to a galactose induced GAL^+ promoter; 2) a wine yeast derivative C911D was the background strain used here, whilst Mattison used the lab yeast derivative BBY48; 3) this study focused on fermentation progress over ~7 days, versus examination of an overnight culture.

Mattison and co-workers (1999) have proposed that Ptp2p acts as a negative regulator on Slt2p (alias Mpk1p). Ptp2p inactivates Mpk1p by dephosphorlating the phosphotyrosine residue of Slt2p both *in vivo* and *in vitro*, and the transcriptional abundance of *PTP2* is greatly increased in a Slt2p-dependent manner after heat shock (Mattison et al. 1999). Similarly, in this study, gene expression analysis reveals that overexpression of *PTP2* decreases *SLT2* expression (Figure 5.2 c), suggesting that *PTP2* transcript levels may directly inhibit transcription of *SLT2*. However, no effect on *ECM33* was observed in the *PTP2* overexpression strain (Figure 5.2 b). This may also imply that *ECM33* acts upstream of *PTP2* in the CWI pathway, or that *PTP2* and

ECM33 perhaps instead interact at post-transcriptional level. This notion is supported by another study showing that Ptp2p dephosphorylates the phosphotyrosine of Hog1p after osmotic shock, and *PTP2* transcripts are upregulated in a wild type strain under osmotic stress (Jacoby et al. 1997). Overexpressing *PTP2* has also been found to suppress the growth defect of Δ*sln1* (a constitutively activated HOG pathway) by using a low copy number plasmid (Jacoby et al. 1997). In addition, Jacoby et al. (1997) have examined the phosphorylation level of Hog1p regulated by Ptp2. For instance, the dephosphorylation durations were examined in Δ*ptp2*, Δ*ptp3*, and Δ*ptp2* Δ*ptp3* double deletant after osmotic stress treatments, revealing that Ptp2p was an effective regulator compared with Ptp3p, and Hog1p was dephosphorylated as quickly as wild type when *PTP2* present (Jacoby et al. 1997). Wurgler-Murphy and colleagues (1997) reported similar findings. As suggested previously and together with the data reported here that the transcript of *HOG1* was not affected by overexpression of *PTP2* (Figure 5.2 d), this implicates that regulation of Hog1p by Ptp2p may occur at the posttranslational level.

As no obvious improvement on yeast fermentation performance was found with the overexpression of *PTP2* in a wild type background, *SLT2*, whose protein is reported to be the target of Ptp2p in the CWI pathway (Mattison et al. 1999), was overexpressed to determine if yeast sugar utilisation efficiency would improve. However, growth was observed to be decreased in yeast with *SLT2* overexpressed, caused prolonged fermentation duration (178 h *vs*. 165 h). This finding is in agreement with Watanabe et al. (1995) who have showed that constitutive activation of the Slt2p-pathway causes poor cell growth.

The overexpression of either *PTP2* or *SLT2* in C911D Δ*ura3* led to a low cell density in fermentations. It was speculated that deletion of *ECM33* might rescue the

growth inhibition. However, the growth defect remained as well as a decreased fermentation rate and extended fermentation duration (Figure 5.5 a and b). This suggests that the overexpression of either *PTP2* or *SLT2* from a strong constitutive promoter in a high copy number plasmid is likely to require a large amount of energy and present a metabolic burden to the yeast (host) cell, which consequently reduces growth and influences metabolic progress (Görgens et al. 2001). This analysis also indicates that the HNE phenotype of Δ*ecm33* is most likely attributed to a combined function of gene networks and probably not to be attained by the simple overexpression of one related gene. Meanwhile, cells with *PTP2* or *SLT2* overexpressed were found to be lighter than control, consistent with the lower cell density (Table 5.4, biomass grams of cells per litre; Figure 5.3, 5.4 and 5.5 b).

The *PTP2* OEX strain was found to have a lower plasmid retention rate than *SLT2* OEX, suggesting that overexpression of *PTP2* might be selected against due to either a phenotypic burden of the presence of the gene or simply due to its larger size than *SLT2* (2.25 *vs.* 1.5kb). In addition, more citric acid was produced by *PTP2* OEX than the control (2 g L^{-1}) (Figure 5.6). This indicates that the upregulation of *PTP2* may contribute to citric acid metabolism. Lawrence and co-workers (2004) have shown that deletion of *PTP2* leads to enhanced growth sensitivity to 300 mM citric acid compared with the parent, suggesting the activity of Ptp2p is important for yeast fitness, whose deletion could aggravate the growth defectiveness by hyperactivity of Hog1p induced by citric acid stress (Lawrence et al. 2004).

Further metabolic analysis found that ethanol production was increased together with decreased glycerol yield in both *PTP2* and *SLT2* overexpression strains (Fermentation 3; Figure 5.6). This is consistent with results from Nissen et al. (2000) who have found a negative correlation between ethanol and glycerol production in

yeast during anaerobic fermentation. This implies that Ptp2p or Slt2p may cooperate with Ecm33p in certain common metabolic pathways such as biomass synthesis, NADH/NAD$^+$ formation and glycerol production, which influence final ethanol.

Chapter 6 General discussion, final conclusions and future research directions

6.1 General discussion and final conclusions

This project investigated the genetic basis of efficient nitrogen utilisation by a HNE strain C911D Δ*ecm33*, during anaerobic sugar (alcoholic) fermentation, and further explored which signalling pathways were involved in such a well-adapted behaviour.

A number of HNE genes have been identified in our lab and by other researchers, reviewed in Chapter 1, one of which, *ECM33* was the subject of further investigation in this study. Δ*ecm33* mutants were generated in both laboratory and wine yeast strains and subsequently characterised as described in Chapters 2 and 3, respectively. In Chapter 4, chitin mutants (or Congo Red Sensitive: CRS) were isolated and screened for fermentation performance, in order to determine the correlation between increased chitin and improved fermentation performance. Whilst a number of CRS isolates performed statistically better than the wild type (by 10-20%), they did not perform as well as the mutant Δ*ecm33*, whereby fermentation was completed up to ~30% faster. Thus, it is proposed that increased chitin in Δ*ecm33* dose not the only contributor to the HNE phenotype of this mutant, and there would be other mechanisms participating in fermentation stress adaptation and nitrogen utilisation.

Gene expression during fermentation under limited nitrogen was examined in Δ*ecm33* and wild type by Q-RTPCR. Based on the Q-RTPCR data described in Chapter 3, it was speculated that the modification of some key genes involved in the CWI and HOG pathways could alter yeast fermentative behaviour. Thus to test this,

the reverse approach via gene manipulation was used, whereby *PTP2* and *SLT2* were overexpressed using the constitutive promoter *PGK1*, in both wild type and Δ*ecm33* deletant backgrounds, and fermentation outcome examined. In either wild type or Δ*ecm33* deletant background, overexpression of *PTP2* or *SLT2* reduced cell fitness during fermentation. The overexpression of *PTP2* was observed to be associated with a positive effect on yeast fermentation performance during the later stage of fermentation. *SLT2* overexpression did not improve the yeast's ability to catabolise sugar. This may be because of the relative position of *SLT2* in the CWI signalling pathway (KEGG website: http://www.genome.jp/kegg/pathway.html), such that overexpression may induce alterations of several up- and/or down-stream activities. Therefore, the HNE phenotype of C911D Δ*ecm33* again might not be caused by the alteration of a single genetic pathway.

This project aimed to isolate wine yeast strains with HNE phenotype under limited nitrogen conditions, and further indentify and characterise these genes and how they are responsible for the HNE phenotype. These objectives have been achieved by the following:

6.1.1 Isolation and characterisation of HNE candidates

The fermentation performance of 9 HNE candidates was investigated. The quickest mutant, C911D Δ*ecm33*, was defined as an HNE wine yeast strain for its highlighted HNE phenotype, with a 15~31% quicker fermentation compared to the wild type. The morphology and transcriptional profile of Δ*ecm33* were characterised and determined.

6.1.2 Investigation of HNE mechanism in Δecm33 *mutant*

One reason for the HNE mechanism of *Δecm33* could be the increased chitin in the cell wall. This hypothesis has been tested by isolation and characterisation of <u>C</u>ongo <u>R</u>ed <u>S</u>ensitive (CRS) mutants for HNE phenotype. Chitin content may contribute in part to the HNE phenotype, as 6 out of a total of 22 CRS mutants (22%) isolated from EMS-treated commercial wine yeast strains demonstrated a 10-20% increased efficiency in sugar catabolism during fermentation.

Another reason for the HNE phenotype could be the activation of other cellular mechanisms due to alteration of key genes involved in the CWI, HOG and CNM pathways. Two genes *PTP2* and *SLT2* were overexpressed. *PTP2* resulted in a slightly enhanced fermentation performance in the later stages of fermentation in both strains, whilst overexpression of *SLT2* did not improve yeast fermentative behaviour, but decreased yeast cell density during alcoholic fermentation.

In brief, the HNE phenotype of *Δecm33* mutant during fermentation is unlikely to be a result of alteration of a single metabolic pathway in the *Δecm33*, but more likely is a consequence of co-efficiency of multiple metabolic pathways.

6.2 Future research directions

High sugar content together with limited nitrogen are regard as the major risk factors for sluggish and/or stuck fermentation. In relation to climate change and changing trends of harvest decisions, high sugar content grapes have been noted as a penitential issue for wine making. Alston and co-workers (2011) have reported that the sugar content of harvested grapes has increased by 9% on average in the past 28 years in California. Similar trend has also been observed in Australian grapes (Webb et al. 2011).

Wine yeasts are able to sense stresses, sophisticatedly transmit signals into their nucleus and make relevant decisions for adaptation to harsh conditions where necessary. One method of response yeast has developed during evolution is conveyed via signalling pathways. The ability to sense the external milieu and adapt accordingly ensures the survival of the wine yeast during an extended wine fermentation, whereby the juice's sugar content is progressively decreased with its uptake and metabolism.

Based on the discoveries from this study, the following approaches may be applied for further investigation of *ECM33* deletion effects on other genetic pathways in order to thoroughly clarify the role of Ecm33p in the yeast HNE phenotype. A putative schematic regulation network of Ecm33p involved is described in Appendix 4. Based on this information, future work could be applied:

(1) Overexpression of candidate genes following integration into the yeast genome

In this study, both *PTP2* and *SLT2* genes were overexpressed using the constitutive *PGK1* promoter in the 2 micron episomal plasmid, pJC1/GW. However, the use of this plasmid expression system has some drawbacks, namely, potential genetic instability and the increased metabolic burden to the host cell (Friehs 2004; Rozkov et al. 2004). Another approach to analyse gene function is to integrate the expression cassette into the host cell genome by homologous replacement of the endogenous gene, so as to overcome such issues (Romanos et al. 1992). Whilst beyond the scope of this study due to time constraints, different scenarios could be investigated, including stable integration of *PTP2* and *SLT2* into the C911D genome. For example, increasing the copy number

of the endogenous gene, introduction of mutations within the promoter and coding sequence to increase both expression and protein stability (Poole et al. 2009), as well as stronger promoters with similar temporal expression to that of the candidate genes. As such the overexpression of the candidate gene might be regulated systematically by the yeast itself.

Additionally, *ECM33* gene function is also worthwhile to study, specifically by its overexpression in C911D Δ*ecm33* or wild type C911D to examine whether the robust fermentation phenotype exhibited in the deletant can be reversed or even prolonged when *ECM33* is overexpressed. This work would help to re-confirm the role of *ECM33* in the wine yeast HNE phenotype. Another approach to examine the role of *ECM33* in HNE is by introduction of point mutations in the gene's promoter region in order to manipulate *ECM33* expression, and perhaps yeast fermentation performance.

(2) Profiling of gene expression in response to low nitrogen conditions in C911D Δ*ecm33* during fermentation

C911D Δecm33 *gene expression analysis via microarray technology*
Yeast DNA microarray analysis provides an accessible tool to study gene response to low YAN stress (DeRisi et al. 1997; Rossignol et al. 2003). In the Rossignol et al. (2003) study, thousands of genes were expressed and regulated in an orderly manner at different stages of alcoholic fermentation. Genes involved in the target of rapamycin (TOR) signalling pathway relating to nitrogen metabolism were observed to have altered transcriptional abundance during stationary phase upon nitrogen depletion.

Other genes in key metabolic pathways were also found to be changed along with cell growth phase, nitrogen and carbon source depletion (Rossignol et al. 2003). A genome-wide gene expression analysis of the nitrogen efficient wine yeast C911D Δ*ecm33* during fermentation would prove useful in understanding the important gene clusters contributing to the HNE phenotype in this strain, as well as the interaction of a myriad of biological processes in response to fermentation stress, including osmotic and nutrient stress. This information, together with 'data mining' of datasets relevant to fermentation ('omics datasets including genetic mapping of quantitative trait locus (QTLs) (Roncoroni et al. 2013) would provide the basis for further characterisation of select candidate genes in order to help to deeply understand the HNE mechanism of this yeast strain, and more importantly provide guidance for wine yeast strain development.

C911D Δecm33 transcriptomic analysis via RNA-Seq RNA-Seq or deep sequencing is a new approach to transcriptional analysis, which can recognize gene isoforms, novel extrons, and single base differences of transcriptional boundaries on large transcriptomic maps, in comparison to these limitations of microarray technologies (Ozsolak and Milos 2010; Wang et al. 2009). For this project, work could be under taken to quantify the C911D Δ*ecm33* transcriptome during fermentation with limited YAN. As well as characterising and quantifying the transcriptional response of C911D Δ*ecm33* to low YAN stresses, correlating HNE genes and genes associated with other stress responses, novel HNE genes may be disclosed.

(3) Posttranslational investigation of the HNE mechanism of C911D Δ*ecm33* during fermentation

In this study, no obvious fermentative phenotype changes were observed by overexpressing *PTP2* or *SLT2* in either wild type or the Δ*ecm33* deletant. Previously, Pardo and co-workers (2004) showed that deletion of *ECM33* increased the activation level of Slt2p by three-fold through quantification of Slt2p phosphorylation. Ecm33p has been reported as a GPI-anchored protein located on the cell wall (Terashima et al. 2003); loss of which, through gene disruption may directly or indirectly alter communication between the cell wall and the nucleus through the CWI and/or HOG signalling pathways (Chapter 3 QRT-PCR data). Speculation arises as to whether this communication occurs at the posttranslational level. It is plausible that the HNE phenotype is a consequence of reprogramming cell signalling patterns or channels, which may be only regulated at the protein level when Ecm33p is either lacking (as in Δ*ecm33*) or non-functional (not tested). In order to continue this research, it would be worthwhile to identify potential activators which interact/bind with Ecm33p via investigation of protein-protein interactions using yeast two-hybrid methodology (Fields and Sternglanz 1994). Also potential binding proteins associate with Ecm33p-GST could be purified and precipitated using anti-GST antibody, and further identified via mass spectrometry. This information would provide clues as to how loss of Ecm33p triggers alteration of specific key pathways to enable wine yeast to possess such robust fermentative vigour at low YAN conditions.

(4) Application of putative HNE hypothesis based on understanding the genetic features of the Δ*ecm33* mutant

This study on Δ*ecm33* determined that Ecm33p is unlikely to directly affect nitrogen utilization, but that the deletion of this gene indeed contributes to the HNE phenotype of wine yeast. The HNE phenotype exhibited by this mutant is proposed to be a consequence of several metabolic pathways altered by disruption of *ECM33* gene. As such a further investigation of the metabolic pathway altered in Δ*ecm33* may help to provide insight into how many biological processes involve Ecm33p. As the metabolic function of this GPI anchor protein remains unclear, this study provides new knowledge on this gene (and protein) with regards to fermentation. Furthermore, this information will allow a proper integrated manipulation/alteration of these metabolic processes and networks of Ecm33p-involved via EMS mutagenesis, which can assist in the development of a commercial HNE strain, with a similar HNE phenotype as observed in Δ*ecm33*.

(5) Wine yeast strain improvement based on the Δ*ecm33* HNE phenotype

The ability of yeast to utilise nitrogen efficiently is a desirable wine yeast attribute sought by winemakers. Whilst improved nitrogen utilisation does not appear to be the sole reason behind the HNE phenotype exhibited by Δ*ecm33,* deletion of this gene using an approach such as 'self-cloning' would produce a strain acceptable for industry use (Akada 2002; Walker et al. 2005). The Δ*ecm33::KANMX* deletion cassette used to construct C911D Δ*ecm33* does not allow for the removal of the antibiotic selectable marker (Wach et al. 1994). However, construction of Δ*ecm33* deletant using a deletion cassette similar to that designed by Walker et al. (2005) would allow for the 'looping out' of the dominant

selectable marker by homologous recombination to leave a 'seamless' deletion of *ECM33*. Further improvements could be attained through classical breeding strategies to backcross the mutated allele to other yeast backgrounds to sequentially combine other good oenological attributes such as cold tolerance, low H_2S production, etc.

Overall, the work undertaken in this project combined with other possible related approaches would finally help us to draw a comprehensive framework on the basis of the HNE mechanism and associated interactions. This knowledge also provides guidance for geneticists to breed more reliable, improved wine yeast strains to meet current and future winemaker needs.

Appendix

1. Amino acid and ammonium mix for CDGJM

Amino acids	Amount per litre (mg)	Nitrogen content per litre (mg)	×25 (500 mL) stock
Alanine	100.0	15.7	1.3
Arginine	750.0	241.5	9.4
Asparagine	150.0	21.8	1.9
Aspartic acid	350.0	36.8	4.4
Glutamic acid	500.0	47.5	6.3
Glutamine	200.0	38.4	2.5
Glycine	50.0	9.3	0.6
Histidine	150.0	40.7	1.9
Isoleucine	200.0	21.4	2.5
Leucine	300.0	32.1	3.8
Lysine	250.0	48.0	3.1
Methionine	150.0	14.1	1.9
Phenylalanine	150.0	12.8	1.9
Proline	500.0	61.0	6.3
Serine	400.0	53.2	5.0
Threonine	350.0	41.3	4.4
Tryptophan	100.0	13.7	1.3
Tyrosine	20.0	1.5	0.3
Valine	200.0	24.0	2.5
Ammonium sulphate	153.3	32.5	3.8
Total N content	850 g N L^{-1}		

2.   Minimal drop-out Medium

Minimal Drop-out Medium

Components	Weight or Vol. for 1 L
Ammonium sulphate (NH_2SO_4)	5 g
Glucose	20 g
Salts	1.7 g
Trace elements	1 mL
Vitamin stock	10 mL
10× Drop-out stock solution	100 mL
Inositol (10 mM)	6.5 mL

pH was adjusted to 6 for solid media.

Trace elements stock

Components	$\mu g\ L^{-1}$
Boric acid	500
Copper sulphate	40
Potassium iodide	100
Ferric chloride	200
Manganese sulphate	400
Sodium molybdate	200
Zinc sulphate	400

The components listed above were weighed and dissolved in 1 L milliQ water, filter sterilised and

stored at 4 ºC.

Vitamin powder stock

Components	Weight
Biotin	5 mg
Calcium pantothenate	1 g
Folic acid	5 mg
Nicotinic acid	1 g
ρ-aminobenzoic acid	0.5 g
Pyridoxine HCl	1 g
Riboflavin	0.5 g
Thiamine HCl	1 g

The dry powder was kept in dark at 4 ºC. 100 mg of the powder was dissolved in 500 mL of MilliQ

water for 100× Vitamin stock.

10× Drop out stock solution (in 500 mL milliQ water)

Amino acids	Weight (mg for 500 mL)
adenine	100 mg
arginine	100 mg
histidine	100 mg
leucine	300 mg
lysine	1150 mg
methionine	100 mg
threonine	1500 mg
tryptophane	100 mg
uracil	100 mg

The amino acids listed above were weighed, **except** the one to be dropped out (e.g. uracil or leucine in this study) and dissolved in 500 mL milliQ water, filter sterilised and stored at 4 ºC.

3.   Identification of genotype and mating type of a single tetrad
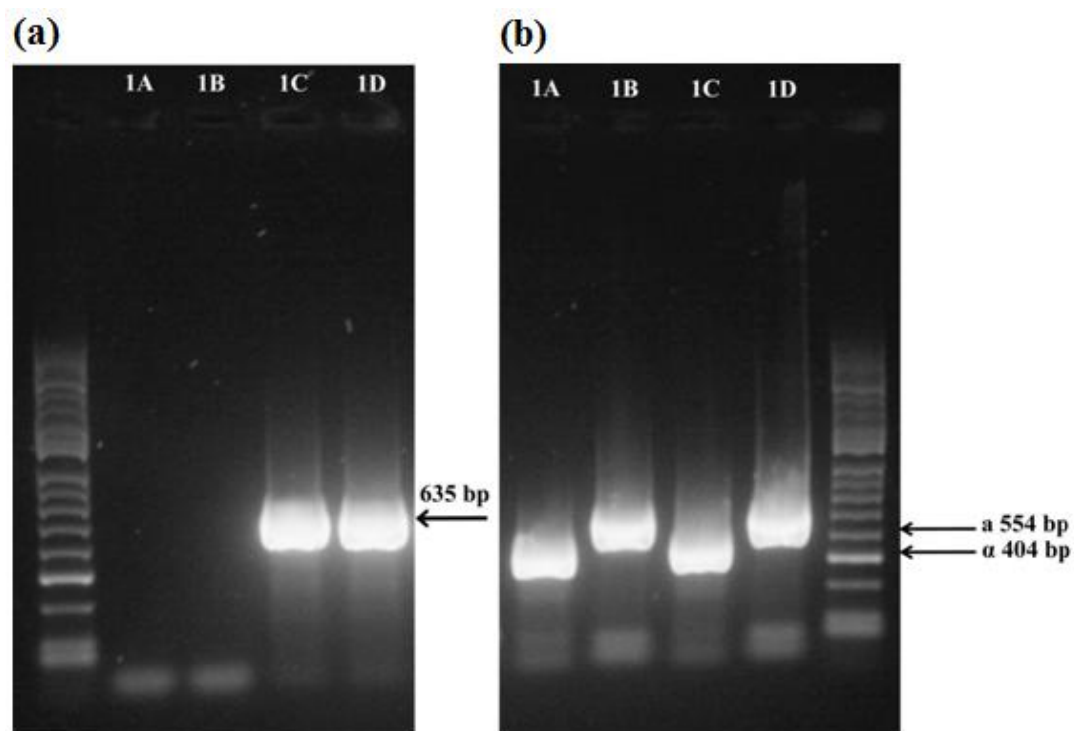
PCR identification of (a) *ECM33* genotype and (b) mating type of 4 spore progeny derived from a single tetrad (1A-1D) dissected from a C911D Δ*ecm33* and C911A Δ*ura3* cross. The 635bp band generated with partial flanking region of *ECM33* and partial *KANMX* cassette. The 554 bp band represents mating type a, 404 bp is mating type α.

4. **A putative model for the role of Ecm33p in regulation of the cell wall integrity pathway and the high osmolarity pathway**
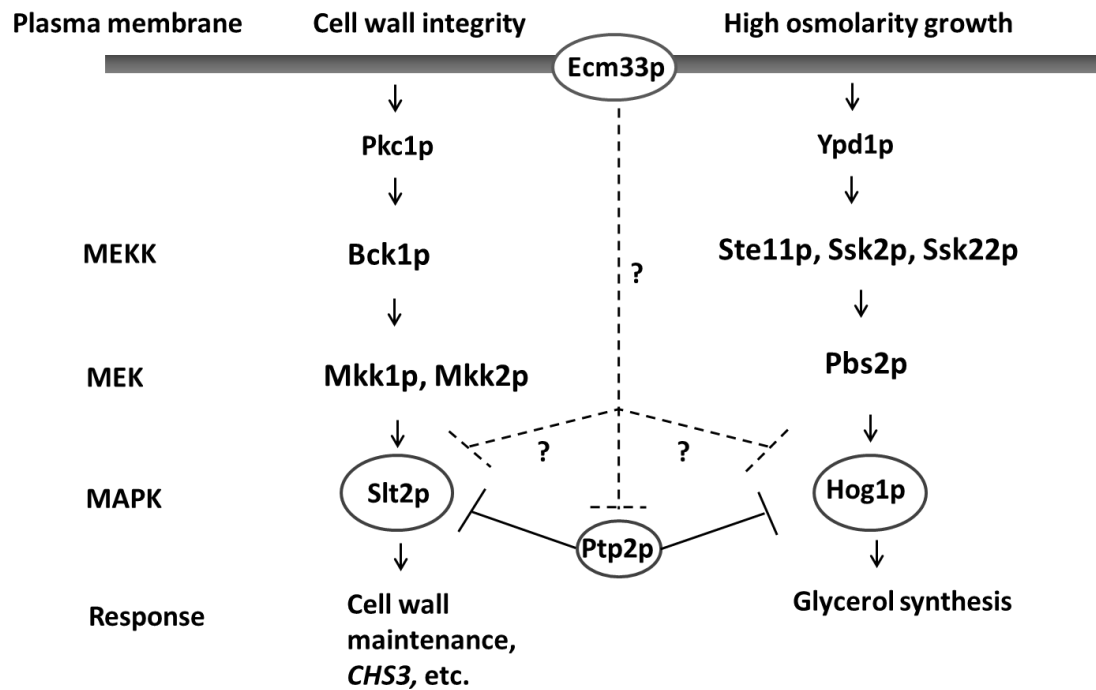
Participating components and related processes are indicated. Dotted lines and question marks represent uncharacterized routes (Adapted from Igual and Estruch 2000, Takada et al. 2010).

5. *PTP2* coding sequence alignment of S288C and C911D yeast strains using Geneious Pro program v5.6.6, the green line represents 100% sequence homology whilst the blank gaps highlight single nucleotide polymorphisms between the two DNA sequences

Continued:

6. **Protein sequence alignment of Ptp2p from S288C and C911D using Geneious Pro program v5.6.6, the green line denotes identical protein sequence and the blank gaps, amino acid substitutions**

Consensus: MDRIAQQYXXGKRDNNGNRMASSAISEKGHIQVNQTRTPGQMPVYRGETINLSNLPQNQIKPCKDLDDVNIRRNNSNRHSKILLLDLCAGPNTNSFLGNI
Identity

1. Ptp2p_S288C: MDRIAQQYRNGKRDNNGNRMASSAISEKGHIQVNQTRTPGQMPVYRGETINLSNLPQNQIKPCKDLDDVNIRRNNSNRHSKILLLDLCAGPNTNSFLGNI
2. Ptp2p_C911D: MDRIAQQYGSGKRDNNGNRMASSAISEKGHIQVNQTRTPGQMPVYRGETINLSNLPQNQIKPCKDLDDVNIRRNNSNRHSKILLLDLCAGPNTNSFLGNI

Consensus: NAKDITVLSLPLPSTLVKRSNYPFENLLKNYLGSDEKYIEFIKIIKDYDIFIFSDSFSRISSCLKTTFCLIEKFKKFICHFFPSPYLKFFLLEGSLNDSK
Identity

1. Ptp2p_S288C: NAKDITVLSLPLPSTLVKRSNYPFENLLKNYLGSDEKYIEFIKIIKDYDIFIFSDSFSRISSCLKTTFCLIEKFKKFICHFFPSPYLKFFLLEGSLNDSK
2. Ptp2p_C911D: NAKDITVLSLPLPSTLVKRSNYPFENLLKNYLGSDEKYIEFIKIIKDYDIFIFSDSFSRISSCLKTTFCLIEKFKKFICHFFPSPYLKFFLLEGSLNDSK

Consensus: APSLGKNKKNCILPKLDLNLNVNLTSRSTLNLRINIPPFNDSNKIFLQSLKKDLIHYSFNSLQKFFQFNMPADLAPNDTILPNWLKFCSVKENEKVILKK
Identity

1. Ptp2p_S288C: APSLGKNKKNCILPKLDLNLNVNLTSRSTLNLRINIPPFNDSNKIFLQSLKKDLIHYSFNSLQKFFQFNMPADLAPNDTILPNWLKFCSVKENEKVILKK
2. Ptp2p_C911D: APSLGKNKKNCILPKLDLNLNVNLTSRSTLNLRINIPPFNDSNKIFLQSLKKDLIHYSFNSLQKFFQFNMPADLAPNDTILPNWLKFCSVKENEKVILKK

Consensus: LFNNFETLENFEMQRLEKCLKFKKKPLHQKQLSQKQRGPQSTDDSKLYSLTSLQRQYKSSLKSNIQKNQKLKLIIPKNNISSSPSPLSSDDTIMXPINDY
Identity

1. Ptp2p_S288C: LFNNFETLENFEMQRLEKCLKFKKKPLHQKQLSQKQRGPQSTDDSKLYSLTSLQRQYKSSLKSNIQKNQKLKLIIPKNNISSSPSPLSSDDTIMSPINDY
2. Ptp2p_C911D: LFNNFETLENFEMQRLEKCLKFKKKPLHQKQLSQKQRGPQSTDDSKLYSLTSLQRQYKSSLKSNIQKNQKLKLIIPKNNISSSPSPLSSDDTIMTPINDY

Consensus: ELTEGIQSFIKNRYSNILPYEHSRVKLPHSPKPPAVSEASTTETKIDKSYPMCPVDAKNHSCKPNDYINANYLKLTQINFDFKYIATQAPLPSTMDDFWK
Identity

1. Ptp2p_S288C: ELTEGIQSFIKNRYSNILPYEHSRVKLPHSPKPPAVSEASTTETKIDKSYPMCPVDAKNHSCKPNDYINANYLKLTQINFDFKYIATQAPLPSTMDDFWK
2. Ptp2p_C911D: ELTEGIQSFIKNRYSNILPYEHSRVKLPHSPKPPAVSEASTTETKIDKSYPMCPVDAKNHSCKPNDYINANYLKLTQINFDFKYIATQAPLPSTMDDFWK

Consensus: VITLNKVKVIISLNSDDELNLRKWDIYWNNLSYSNHTIKLQNIWENICNINGCVLRVFQVKKTAPQNDNISQDCDLPHNGDLTSXIMAVSEPFIVYQLQY
Identity

1. Ptp2p_S288C: VITLNKVKVIISLNSDDELNLRKWDIYWNNLSYSNHTIKLQNIWENICNINGCVLRVFQVKKTAPQNDNISQDCDLPHNGDLTSITMAVSEPFIVYQLQY
2. Ptp2p_C911D: VITLNKVKVIISLNSDDELNLRKWDIYWNNLSYSNHTIKLQNIWENICNINGCVLRVFQVKKTAPQNDNISQDCDLPHNGDLTSTTMAVSEPFIVYQLQY

Consensus: KNWLDSCGVDMNDIIKLHKVKNSLLFNPQSFITSLEKDVCKPDLIDDNNXXLHLDTANSSPLLVHCSAGCGRTGVFVTLDFLLSILSPTINHSNKIDVWN
Identity

1. Ptp2p_S288C: KNWLDSCGVDMNDIIKLHKVKNSLLFNPQSFITSLEKDVCKPDLIDDNNSELHLDTANSSPLLVHCSAGCGRTGVFVTLDFLLSILSPTINHSNKIDVWN
2. Ptp2p_C911D: KNWLDSCGVDMNDIIKLHKVKNSLLFNPQSFITSLEKDVCKPDLIDDNNNDLHLDTANSSPLLVHCSAGCGRTGVFVTLDFLLSILSPTINHSNKIDVWN

Consensus: MTQDLIFIIVNELRKQRISMVQNLTQYIACYEALLNYFALQKQIKNALPCX
Identity

1. Ptp2p_S288C: MTQDLIFIIVNELRKQRISMVQNLTQYIACYEALLNYFALQKQIKNALPC*
2. Ptp2p_C911D: MTQDLIFIIVNELRKQRISMVQNLTQYIACYEALLNYFALQKQIKNALPC*

7. *SLT2* **coding sequence alignment of S288C and C911D yeast strains using Geneious Pro program v5.6.6, the green line indicates identical nucleotide sequence and the blank gaps highlight single nucleotide substitutions or polymorphisms (SNPs) and a small hexa-nucleotide deletion in C911D**

Continued:

8. Protein sequence alignment of Slt2p from S288C and C911D using Geneious Pro program v5.6.6, the green line shows identical protein sequences and the blank gap represents 2 missing glutamine (Q) residues in the translated sequence from C911D

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