Stress, fermentation performance and aroma production by yeast

by

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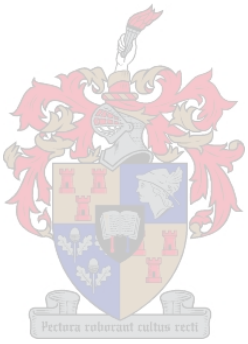

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Declaration

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Summary

Yeast strains contend with numerous stresses during winemaking. An inability to perceive and initiate the physiological changes needed to adapt to stress, has been linked to slow or incomplete (residual sugar > 4 g/L) fermentations. Wine yeast strains differ in genotype; this is manifested as differences in their stress tolerance, and fermentation performance.

The first goal of this study was to evaluate how the initial sugar (200 or 240 g/L) and nitrogen (50, 100, 250, or 400 mg/L) content, and the fermentation temperature (15°C or 20°C) affected the fermentation performance of 17 commercial wine yeast strains. Fermentation performance was evaluated based on the fermentation kinetics (lag phase, maximum fermentation rate and total weight loss by CO_2 evolution), residual sugar content and yeast dry weight. The results demonstrate that the fermentation performances of commercial yeast cultures are significantly and differently affected by initial nitrogen and sugar levels, as well as the fermentation temperature. Additionally, excess nitrogen had a negative impact on the fermentation kinetics and sugar consumption. Nitrogen deficiency is a common cause of slow and incomplete fermentations, as it affects yeast growth and thus fermentation rates. Nitrogen supplements are routinely added at the onset of fermentation, reducing the risk of problematic fermentations. Therefore characterising the fermentative ability of a strain over a range of oenologically relevant conditions, could aid winemakers in selecting a yeast strain capable of fermenting a grape must (of known sugar and nitrogen levels) to completion at the desired fermentation temperature.

Investigations on fermentation related stress generally focus on its influence on fermentation rate and sugar consumption. However, from a winemaking perspective, the strain's ability to produce the desired volatile aroma compounds is equally important. Yet, literature provides little insight into the influence stress has on the volatile aroma profile; this is surprising as wine aroma is closely linked to wine quality and consumer liking.

The final goal of this study was to evaluate changes to the volatile aroma profiles produced by five commercial yeast strains, in response to hyperosmotic and temperature stress. The concentrations of the aroma compounds were quantified using a gas chromatograph coupled to a flame ionization detector. The results show that hyperosmotic and temperature stress caused significant changes in the levels of a number of aroma compounds. Furthermore, the changes observed differed among the evaluated strains, as well as for the fermentation stress treatments studied.

Future aims should be directed towards the potential application of yeast strain selection as a means to avoid problematic fermentations in grape must; in addition to the further characterisation of the relationship between stress and the resultant volatile aroma profile in wine.

Opsomming

Gisrasse moet verskeie stresfaktore afweer tydens die wynmaak proses. Die onvermoë van ‘n wyngis om stres waar te neem en die nodige fisiologiese veranderinge te inisieer om aan te pas by die strestoestande word met slepende of onvolledige fermentasies (met ‘n residuele suiker van meer as 4 g/L) geassosieer. Wyngisrasse verkil in genotipe; wat as groot verskille in die graad van strestoleransie, en dus ook fermentasie sukses geopenbaar word.

Die eerste doelwit van hierdie studie was om te evalueer hoe die suiker (200 of 240 g/L) en stikstof (50, 100, 250, of 400 mg/L), asook die fermentasie temperatuur (15°C of 20°C) die fermentasie prestasie van 17 kommersiële wyngiskulture beïnvloed. Die sukses van fermentasie is geëvalueer op grond van fermentasie kinetika (sloerfase, maksimum fermentasiespoed en totale gewigsverlies as CO_2 verlies), die residuele suiker inhoud en die gis droë massa.

Die resultate demonstreer dat die fermentasie sukses van kommersiële giskulture beduidend en verskillend beïnvloed word deur die aanvangsstikstof en – suikerkonsentrasies, asook die fermentasie temperatuur. Daarbenewens, wanneer stikstof in oormaat teenwoordig is kan dit ‘n negatiewe impak op fermentasietempo en suiker metabolisme hê. Beperkende vlakke van stikstof ‘n algemene oorsaak van slepende of onvolledige fermentasies, aangesien stikstof die groei en gevolglik ook die fermentasiespoed van gis beïnvloed. Stikstofaanvullings word dikwels tot druiwemos toegevoeg aan die begin van gisting, wat die risiko van probleemfermentasies verlaag. Dus kan die karakterisering van die fermentasievermoë van ‘n gisras vir ‘n reeks wynkundig relevante kondisies die wynmaker help om ‘n gisras te selekteer wat in staat is om ‘n druiwemos (waarvan die suiker en stikstofvlakke bekend is) droog te gis by die gewenste temperatuur.

Meeste studies wat fermentasieverwante stress ondersoek, fokus op die die invloed daarvan op fermentasietempo en suikerverbruik. Van ‘n wynmaakperspektief is die gis se vermoë om die gewensde vlugtige aroma komponente te produseer egter ewe belangrik as die vermoë om fermentasie te voltooi. Tog verskaf die literatuur min insig tot die invloed van stres op die vlugtige aromaprofiel; wat verbasend is aangesien die aromaprofiel ‘n belangrike faktor is van die waargenome wynkwaliteit en daarom ook verbruikersvoorkeur.

Die finale doelwit van hierdie projek was om die veranderinge tot die vlugtige aromaprofiel geproduseer deur vyf kommersiële gisrasse in reaksie op hiperosmotiese stres en temperatuur stres te evalueer. Die konsentrasies van die aromakomponente is gekwantifiseer deur gas chromatografie gekoppel aan vlam-ioniserende deteksie. Die resultate wys dat hiperosmotiese- en temperatuur stres beduidende veranderinge meebring in die vlakke van ‘n aantal aromakomponente. Verder is die waargenome veranderinge ook verskillend vir die geëvalueerde gisrasse, asook vir die verskille stresbehandelings wat ondersoek is.

Toekomstige studies behoort gerig te wees op die toepassing van gis seleksie om potensiële probleemfermentasies in druiwemos te voorkom; asook die verdere karakterisering van die verhouding tussen omgewingstresfaktore en die gevolglike vlugtige aromaprofiel in wyn.

This thesis is dedicated to

My family

Biographical sketch

Samantha Fairbairn was born (29 June 1979) in Cape Town and matriculated from Hottentots Holland High School in 1997. She enrolled at the University of Stellenbosch, and obtained a BSc. Honours degree in Microbiology in 2002. Samantha has since worked as a teacher and a technical assistant before enrolling at Stellenbosch University for a MSc in Wine Biotechnology.

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Preface

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately. Chapter 3 is written according to the style of the journal *South African Journal of Enology and Viticulture,* and chapter 4 to *Applied Microbiology and Biotechnology*.

Chapter 1 **General introduction and project aims**

Chapter 2 **Literature review**
Wine fermentation stress

Chapter 3 **Research results**
The impact and interaction between initial nitrogen, initial sugar, and temperature on the fermentation performance of commercial wine yeast

Chapter 4 **Research results**
Impact of environmental stress on aroma production during wine fermentation

Chapter 5 **General discussion and conclusions**

Contents

Chapter 1
General introduction and project aims 1

1.1 Introduction 2

1.2 Project Aims 3

1.3 Literature cited 4

Chapter 2
Wine fermentation stress 6

2.1 Introduction 7

2.2 General stress response 8

2.3 Hyperosmotic stress 10

2.3.1 *Physiological impact of hyperosmotic stress* 10
2.3.2 *Acquisition of hyperosmotic stress tolerance* 10
2.3.3 *Osmotic stress and redox balance: Impact on wine aroma* 11

2.4 Ethanol toxicity 14

2.4.1 *Physiological impact of ethanol toxicity* 14
2.4.2 *Acquisition of ethanol tolerance* 14
2.4.3 *Potential role of ethanol in wine aroma* 16

2.5 Low temperature 16

2.5.1 *Physiological impact of low temperature* 16
2.5.2 *Acquisition of low temperature tolerance* 17

2.6 Conclusion 18

2.7 Literature cited 18

Chapter 3
The impact and interaction between initial nitrogen, initial sugar, and temperature on the fermentation performance of commercial wine yeast 23

3.1 Introduction 24

3.2 Materials and methods 27

3.2.1 *Synthetic grape medium and fermentation treatments* 27
3.2.2 *Dry weight* 27
3.2.3 *Statistical analysis* 28

3.3 Results and discussion 31

3.3.1 *Influence of treatments on fermentation kinetics* 31
3.3.1.1 Impact of treatments on fermentation onset 31
3.3.1.2 Impact of treatments on fermentation onset of individual yeast strains 32
3.3.1.3 Impact of treatments on fermentation rate 36
3.3.1.4 Impact of treatments on fermentation rate of individual yeast strains 38
3.3.1.5 Impact of treatments on the total weight loss 41

3.3.1.6 Impact of treatments on the total weight loss of individual yeast strains 41
3.3.2 *Influence of treatments on sugar consumption* 45
3.3.2.1 Impact of treatments on sugar consumption 45
3.3.2.2 Impact of treatments on sugar consumption of individual yeast strains 46
3.3.3 *Influence of treatments on yeast dry weight* 49
3.3.3.1 Impact of treatments on yeast dry weight production 49
3.3.3.2 Impact of treatments on the dry weight production of individual yeast strains 49
3.4 Conclusion 53
3.5 Acknowledgements 54
3.6 Literature cited 54

Chapter 4
Impact of environmental stress on aroma production during wine fermentation 58
4.1 Introduction 60
4.2 Materials and methods 61
4.2.1 *Yeast strains and growth conditions* 61
4.2.2 *Fermentation conditions* 62
4.2.3 *Chemical analysis* 62
4.2.3.1 Residual glucose and fructose, and glycerol 62
4.2.3.2 Aroma compounds 63
4.2.4 *Statistical analysis* 63
4.3 Results 65
4.3.1 *Fermentation performance* 65
4.3.1.1 Fermentation rate 65
4.3.1.2 Residual glucose and fructose, and glycerol 65
4.3.2 *Aroma compounds* 69
4.4 Discussion 74
4.5 Acknowledgements 76
4.6 References 77

Chapter 5
General discussion and conclusions **80**
5.1 Introduction 81
5.2 Literature cited 83

Chapter 1

Introduction and project aims

Chapter 1

General introduction and project aims

1.1 Introduction

Commercial wine yeast strains are primarily selected for their ability to ferment grape must to dryness, whereby residual sugars are reduced to less than 4 g/L (Pretorius, 2000). For a grape must to be fermented to dryness, the yeast strain is required to adapt and respond to a multitude of environmental stresses, either simultaneously or successively. The environmental stresses commonly encountered during commercial wine fermentations include a high initial sugar concentration, low nitrogen levels, and possible changes in the fermentation temperature, among others. Stress is described as any factor that reduces cell growth. Stress is therefore often linked to problematic fermentations, which are defined by either a slow rate of sugar consumption (sluggish fermentations), or as an incomplete fermentation with a high residual sugar content (stuck fermentations) (Alexandre & Charpentier, 1998; Bisson, 1999; Gibson *et al.*, 2007; Malherbe *et al.*, 2007). The causes of problematic fermentations have been the subject of extensive study due to their economic and logistic consequences (Malherbe *et al.*, 2007; Pizarro *et al.*, 2007).

Yeast strains differ in their innate abilities to sense and effectively adapt to stressful environmental conditions. This ability contributes to the cell's survival, and therefore also its ability to ferment grape must (Ivorra *et al.*, 1999; Carrasco *et al.*, 2001; Zuzuarregui & del Olmo, 2004). Additionally, yeast strains vary in their nitrogen requirements, and their capacity to catabolise sugars (Manginot *et al.*, 1998). This variation in yeast strain metabolic capabilities highlights the importance of determining the grape must composition (at least sugar and nitrogen content), and using that information along with the intended fermentation conditions to select an appropriate yeast starter culture. The selection of the yeast strain "best" adapted to fermenting a characterised grape must to dryness may reduce the risk of problematic fermentations. However, the ability of a strain to ferment grape must to completion is only a reflection of yeast fermentation performance and does not provide insights into the quality of the final product.

From the consumer's perspective wine quality is essential in making a purchasing decision (Swiegers *et al.,* 2005). Wine flavour, consisting of aroma, taste, and mouth-feel, is a vital component of wine quality (Francis & Newton, 2005; Swiegers *et al.*, 2005). Wine flavours are derived from the grapes, produced during alcoholic fermentation, and depend on the

maturation strategy that is used (Rapp & Mandery, 1986; Rapp & Versini, 1996). The volatile aromas produced by wine yeast during fermentation include higher alcohols, esters and volatile fatty acids, among others (Rapp & Versini, 1996). The availability of aroma precursors, the fermentation conditions (Henschke & Jiranek, 1993; Rapp & Versini, 1996; Lambrechts & Pretorius, 2000; Swiegers *et al.*, 2005; Vilanova *et al.*, 2007; Saerens *et al.*, 2008; Bisson & Karpel, 2010) and the genotype of the yeast strain (Soles *et al.*, 1982; Rossouw *et al.*, 2008) all contribute to which particular volatile aroma compounds will be produced by yeast during alcoholic fermentation. However, currently little data is available regarding the impact of environmental stress on the production of volatile aroma metabolites.

This study is part of a broader program at the Institute of Wine Biotechnology, Stellenbosch University, investigating yeast nutritional requirements in the wine matrix, and its influence on fermentation performance and aroma production under winemaking conditions.

1.2 Project aims

This study focused on the influence of some of the factors commonly associated with problematic grape must fermentations on fermentation performance of commercial yeast starter cultures, in addition to the impact of environmental stress on the volatile aroma profile.

The main aims of this project were therefore:

i. To investigate the impact of the initial nitrogen content, osmotic pressure (initial sugar content), and temperature on the fermentation performance of 17 commercial active dry yeast cultures. To our knowledge this is the first study to evaluate stress by varying stress levels and combinations using a multifactorial experimental design. This will potentially identify strains that are suited to fermenting a grape must of specific characteristics, providing winemakers with a tool to select the yeast strain best adapted to ferment that specific grape must and ensuring a complete fermentation.

ii. To elucidate the effect of hyperosmotic and temperature stresses on fermentation performance and the production of fermentation derived volatile aroma compounds, providing information whether stress exposure impacts wine aroma and whether the observed changes are conserved among different commercial *Saccharomyces* yeast strains. To our knowledge this is the first study to assess the influence of stress on the fermentation aroma under winemaking conditions in a synthetic wine matrix.

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Chapter 2

Literature review

Wine fermentation stress

Chapter 2

Wine fermentation stress

2.1 Introduction

Stress is any environmental condition compromising a cell's capacity to survive and grow (Ruis & Schüller, 1995; Hohmann & Mager, 2003). From the onset of alcoholic fermentation, yeast cells are bombarded with many such stresses experienced either simultaneously or in quick succession of each other. Furthermore, the inhibitory influence of an individual stress may be magnified when present in combination with other stresses (Bisson, 1999). Fermentation associated stresses include hyperosmotic stress (high initial sugar), ethanol toxicity, temperature extremes, low pH and nutrient limitations (nitrogen, oxygen, vitamins, and minerals) (Attfield, 1997; Bisson, 1999; Gibson *et al*., 2007; Malherbe *et al*., 2007).

Commercial wine yeast strains are selected based on their ability to conduct reliable and complete fermentations in addition to the production of desirable aroma compounds (Pretorius, 2000; Fleet, 2008). An inability to overcome fermentation related stresses is often manifested as a stuck or a sluggish fermentation. A stuck fermentation ends prematurely with a high residual sugar content, whereas a sluggish fermentation proceeds at a very slow rate (Bisson, 1999).

Yeast cells have developed mechanisms to sense environmental cues, and initiate physiological responses to counteract the harmful effects of stress. The inherent ability of yeast strains to conduct fermentation has been inversely correlated with their intrinsic stress tolerance (Ivorra *et al.*, 1999; Carrasco *et al.*, 2001; Zuzuarregui & del Olmo, 2004). Since yeast strains differ in their ability to detect and respond to the different stresses experienced during fermentation, stress tolerance could be a meaningful means of screening potential wine yeast starter cultures (Zuzuarregui & del Olmo, 2004).

While the ability to complete fermentation of a high sugar, low pH grape juice is the most important attribute of wine yeast strains, yeast strains also contribute significantly to wine flavour, and therefore quality, of the final wine (Francis & Newton, 2005; Swiegers *et al.*, 2005). This impact of yeast is mainly linked to the *de novo* production of volatile and non-volatile aroma and flavour compounds such as esters, aldehydes, higher alcohols and organic acids (Rapp & Versini, 1996). While many studies have focused on the impact of stress on the kinetics and completion of fermentation, there is very little published information on the impact of the major fermentation stresses on aroma production by individual yeast strains.

This chapter will primarily focus on reviewing the general stress response and the physiological changes that yeast cells undergo when exposed to the major stresses experienced during wine fermentation, hyperosmotic stress, ethanol toxicity, and changes of, or growth at low temperatures. Where possible, the review will also propose how such stresses may impact on the production of aromatic compounds, based on our existing knowledge of the regulation of the relevant genetic and metabolic networks.

2.2 General stress response

Stress has far reaching consequences on the yeast cell. It may compromise membrane integrity, cause a loss of protein and enzyme function, and lead to growth inhibition (Attfield, 1997; Gasch, 2003).

Exposure to stress induces a general and a specific gene expression response, which may result in an improved tolerance to subsequent stress exposures. The expression of genes responsible for repairing the ravages of stress and the acquisition of stress tolerance are coordinated via a number of signal transduction pathways and specific transcription factors that activate several gene expression response elements, the stress response element (STRE), the heat shock element (HSE) and the AP-1 response element (oxidative stress) (Estruch, 2000).

STRE mediates the expression of a number of genes falling within the general stress response (Martinez-Pastor *et al*., 1996). The STRE is found in the promoter region of numerous stress-responsive genes and consists of either a single or multiple copies of the CCCCT/ AGGGG nucleotide sequence. It serves as a binding site for the transcription factors Msn2p or Msn4p, which are activated by heat shock, osmotic stress, oxidative stress and nutrient starvation (Ruis & Schüller, 1995). However, the initiation of gene expression by Msn2p or Msn4p via the STRE does not always fall within the general stress response. In response to hyperosmotic stress, the high osmolarity glycerol pathway (HOG) modulates the expression of genes containing STRE. This does not form part of the general stress response but is an example of a specific stress response (Figure 1).

The heat shock element (HSE) is a second transcription element activated by stress. The HSE contains at least three nucleotide sequence repeats of nGAAn, and serves as the binding site for the heat shock transcription factor (Hsf1) (Morimoto, 1998; Bauer & Pretorius, 2000; Estruch, 2000; Rangel, 2010). The Hsf1 induces the expression of heat shock proteins (HSP) which generally serve as molecular chaperones that aid protein folding. Many of these molecular chaperones are present in the absence of stress. However, their expression is increased upon exposures to stress. The expression of HSP's are induced by other stresses in

addition to temperature stress; therefore they also contribute to "general" stress tolerance (Bauer & Pretorius, 2000; Estruch, 2000; Rangel, 2010).

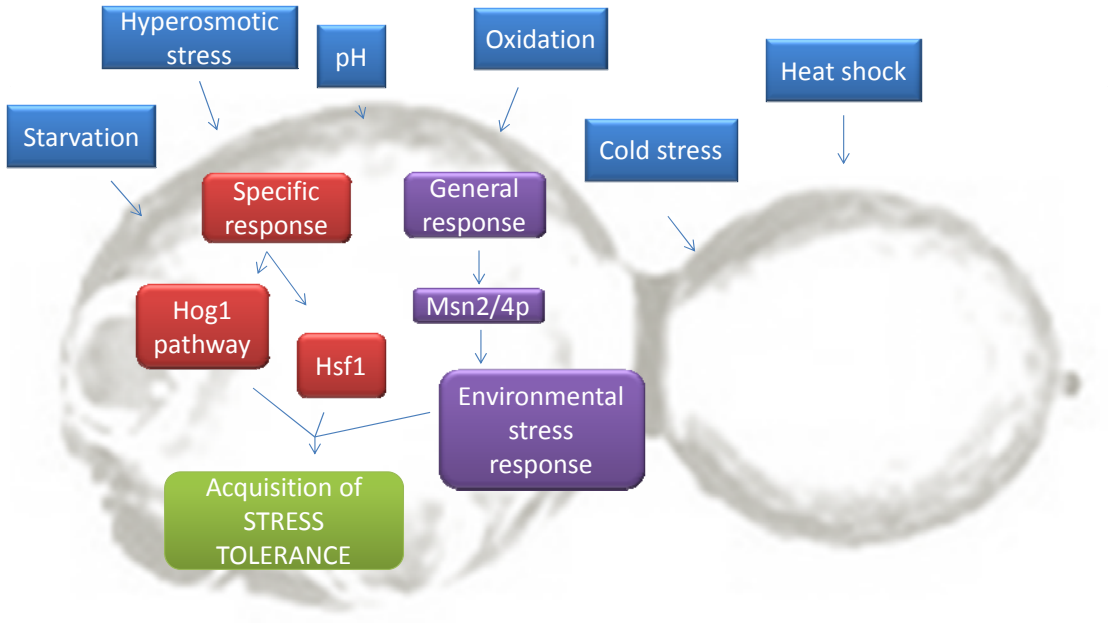

Figure 1 Environmental stresses initiate by both a general and a specific stress response; may result in an overall improved stress tolerance. Adapted from Siderius and Mager (1997) and Teixeira *et al.*, (2010)

Genomic expression studies characterising yeast response to numerous stresses, (oxidative and osmotic stress, heat shock, nitrogen starvation, and stationary phase) have identified a shared environmental stress response (ESR) (Gasch *et al.*, 2000; Causton *et al.*, 2001; Gasch, 2003). This ESR is characterised by the repression of genes responsible for cellular growth and protein synthesis (Gasch *et al.*, 2000; Causton *et al.*, 2001; Gasch & Werner-Washburne, 2002). It is also characterised by an increased expression of genes involved in carbohydrate metabolism (for energy, glycogen and trehalose generation), fatty acid metabolism, protein folding and degradation (heat shock proteins), nucleic acid repair, the maintenance of the internal osmolarity, cytoskeleton reorganisation, signalling, defence to reactive oxygen species and maintenance of redox potential (Gasch *et al.*, 2000; Causton *et al.*, 2001). The expression studies conducted by Gasch *et al.*, (2000) and Causton *et al.*, (2001) evaluated each stress individually, and observed a transient response to stress. However, under fermentative conditions, where the cell is continually adapting to the ever changing environment, this long term response is termed the fermentation stress response (Marks *et al.*, 2008). In this case the global expression changes were observed throughout the fermentation, which is indicative of the dynamic nature of must fermentation (Marks *et al.*, 2008).

This general stress response, or rather ESR, has been proposed as the mechanism behind cross-protection (Gasch *et al.*, 2000; Causton *et al.*, 2001; Gasch & Werner-Washburne, 2002;

Gasch, 2003). The exposure to mild levels of stress initiates physiological changes to the cell conferring tolerance to subsequent exposures of lethal levels of the same stress and possibly also to other stresses. This cross protection also suggests the existence of a general stress response, which can be initiated by an array of environmental stresses (pH, heat, osmotic, nitrogen starvation and oxidation) (Ruis & Schüller, 1995; Martinez-Pastor *et al.*, 1996). A recent study reported that Msn2p and Msn4p initiate gene expression individually in a stress specific manner, and such specific induction of gene expression may require a reassessment of the nature of the "generic" general stress response (Berry & Gasch, 2008). Furthermore, a study on the genome expression profiles of deletion mutants in response to various types of stress revealed that only a small number of the gene products that were expressed are required to adapt to the current stress (Giaever *et al.*, 2002). Berry and Gasch (2008) assert that a single stress exposure induces gene expression primarily directed towards the pre-emptive protection of the cell from future stress, which would account for the existence of cross-protection.

2.3 Hyperosmotic Stress

2.3.1 Physiological impact of hyperosmotic stress

In winemaking, the high initial sugar concentration causes a hyperosmotic stress response immediately upon yeast inoculation into grape must. The sudden loss of cell volume (or turgor pressure) damages the plasma membrane in terms of its structure and permeability (Wood, 1999), as well as the actin cytoskeleton. The actin cytoskeleton is vital for budding, and damage to this network would also contribute to the cessation of growth observed following hyperosmotic stress (Hohmann, 1997; Tamás & Hohmann, 2003).

2.3.2 Acquisition of hyperosmotic stress tolerance

In an attempt to stem water efflux from the cell, water is released from the vacuole, providing some time for the cell to adapt to its environment (Hohmann, 1997; Attfield, 1998). To limit the loss of water, the glycerol export channel Fps1 closes, and glycerol production is activated via the high osmolarity glycerol pathway (HOG). Glycerol serves as an osmoprotectant by increasing the internal solute concentration, and in so doing it limits the efflux of water. The accumulation of glycerol continues until the influx of water restores the cell size to a critical level and yeast growth can be resumed (Hohmann, 1997; Rep *et al.*, 2000; Mager & Siderius, 2002; Tamás & Hohmann, 2003; Hohmann *et al.*, 2007). Consequently, the higher the initial sugar concentration the longer the lag phase before fermentation commences.

The capacity to sense and counteract the ill effects of osmotic stress is determined by strain genotype. If the cell is unable to respond rapidly to hyperosmotic stress, it may result in stuck or sluggish fermentations (Lafon-Lafourcade, 1983; Llauradó *et al.*, 2002; Gibson *et al.*, 2007).

A genome expression study on the adaptation of *Saccharomyces cerevisiae* to high sugar stress found that in addition to glycerol uptake and synthesis, the genes for trehalose, and glycogen synthesis were also upregulated (Rep *et al.*, 2000; Erasmus *et al.*, 2003). Trehalose is a disaccharide accumulated in response to osmotic stress, oxidative stress, heat stress, cold shock, dehydration, carbon starvation, as well as during the stationary phase (Hounsa *et al.*, 1998; Causton *et al.*, 2001; Erasmus *et al.*, 2003; Rangel, 2010). It binds to proteins, preventing protein denaturation and aggregation, as well as decreasing membrane permeability. Trehalose levels do not necessarily correlate with its synthesis, as it is usually rapidly degraded, releasing the proteins and allowing HSP's to facilitate the folding of native or denatured proteins (Singer & Lindquist, 1998). Glycogen is a storage carbohydrate playing a crucial role in cell survival during periods of nutrient limitation. Additionally, glycogen catabolism has been linked with the formation of sterols (Pretorius, 2000), which are in turn associated with improved cell vitality as well as an overall improvement in ethanol tolerance (Pretorius, 2000; Gibson *et al.*, 2007).

2.3.3 Osmotic stress and redox balance: Impact on wine aroma

Glycerol is produced in response to osmotic stress and as a precursor to phospholipid (a plasma membrane component) formation. However, during alcoholic fermentation it is primarily a by-product of the maintenance of the redox balance, specifically the relative levels of NAD^+ and NADH (Figure 2) (Hohmann, 1997). The catabolism of sugar to ethanol causes no net change in the levels of NAD^+/NADH since the NAD^+ reduced during glycolysis is re-oxidised when ethanol is produced from acetaldehyde. However, several intermediates of this pathway, in particular pyruvic acid, are metabolised by alternative pathways resulting in an imbalance in the redox potential. Elevated levels of either NAD^+ or NADH must be reversed in order for the cell to continue to grow and ferment. In the case of a NADH surplus, this generally achieved via the production of glycerol, whereby NADH is reduced to NAD^+ (Hohmann, 1997).

When exposed to osmotic stress, cells respond with the accumulation of glycerol, which results in a surplus of NAD^+. This redox imbalance is corrected by the oxidation of acetaldehyde to acetic acid to avoid the additional production of NAD^+ via the metabolism of acetaldehyde to ethanol. Acetic acid production has been linked to the initial sugar content, and constitutes approximately 90% of the volatile acids in wine. This increase in the production of acetic acid

has a negative impact on wine flavour, as it may impart a vinegar character to the wine when present above the sensory threshold (Swiegers & Pretorius, 2005).

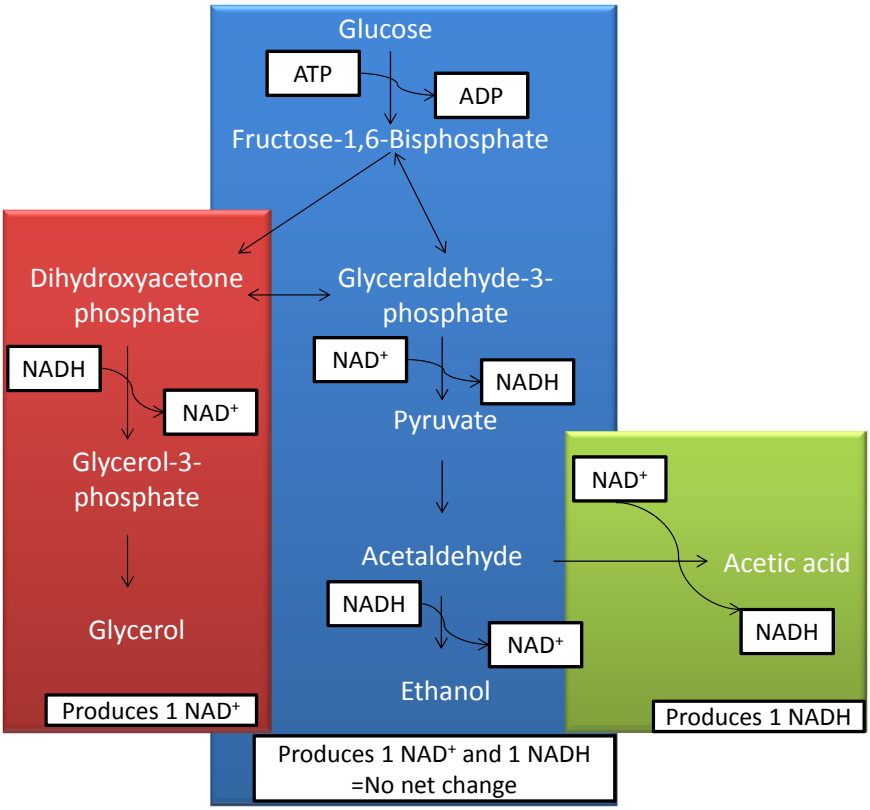

Figure 2 A simplified representation of glycolysis, illustrating the driving force of redox balance in metabolism. Adapted from Hohmann, (1997)

The metabolic networks leading to the formation of other volatile aroma compounds are reasonably understood and described (Lambrechts & Pretorius, 2000). However, the regulation of these networks is not well characterised. It has been suggested that redox homeostasis is involved in the regulation of these aroma producing networks (Lambrechts & Pretorius, 2000).Jain *et al*., (2011) evaluated the influence of the substitution of the glycerol biosynthetic pathway with alternative NAD^+ regenerating pathways, on the production of primary and secondary metabolites. Compared to the wild type strain, the growth of the mutant strains were significantly affected by the redox imbalance. The alternative NAD^+ producing pathways provided a slight improvement in yeast growth; however they could not match the growth of the wild type. The imbalance in NAD^+/NADH levels, generally drove the production of higher alcohols (isobutanol) in an attempt to reduce NADH to NAD^+ (Figure 3), this is in agreement with the results reported by Styger et al., (2011). The production of a fusel alcohol versus a fusel acids is therefore also dependent on the redox requirements of the yeast (Bisson & Karpel, 2010). The production of esters requires NAD^+, hence a shortage thereof resulted in lower levels

of esters produced by the mutant strains than the wild type strain (Jain *et al.*, 2011). In a wine context, the accumulation of NAD^+ as a consequence of glycerol production may therefore influence the production of esters and fusel acids (Figure 3) in an attempt to restore the redox balance.

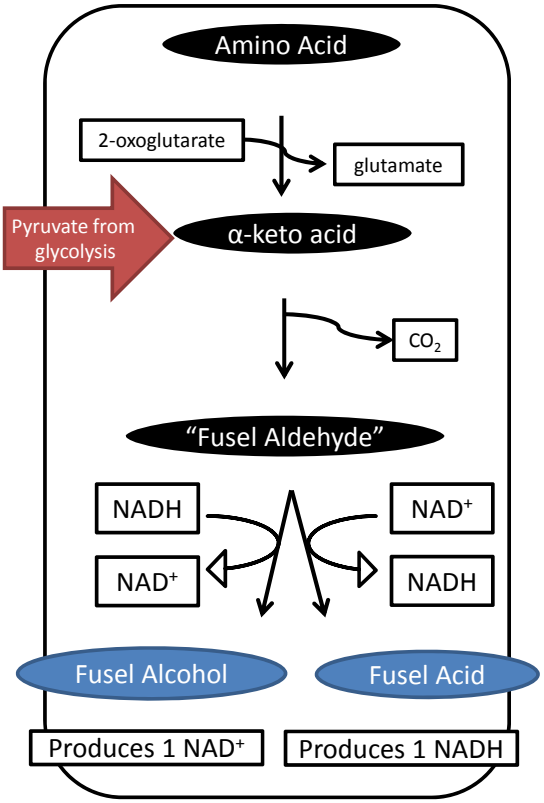

Figure 3 The Ehrlich pathway: The importance of redox balancing in the catabolism of amino acids to fusel alcohols and acids. Adapted from Hazelwood *et al.*, (2008)

2.4 Ethanol Toxicity

2.4.1 Physiological impact of ethanol toxicity

As fermentation progresses, the sugar concentration decreases and the ethanol concentration increases (Bisson, 1999). Ethanol primarily targets the plasma membrane, by increasing its permeability. This causes a decline in the transport of nitrogen and sugar into the cell (Bisson, 1991; Hallsworth, 1998; Bisson, 1999). Changes in the plasma membrane permeability also cause an increased influx of protons in to the cell which dissipates the proton motive force used to transport amino acids into the cell. In order to regulate cytoplasmic pH the cell pumps out protons via the ATPase, and ceases the simultaneous import of amino acids and protons (Bisson, 1991) (Figure 4). Similarly, the V-ATPase pumps protons into the vacuole (Ma & Liu, 2010). Ethanol also denatures proteins, including those involved in transport (Hallsworth, 1998). This loss in the functionality of transport systems ultimately contributes to a loss of cell viability and a reduction in yeast growth (Bisson, 1999; Gibson *et al*., 2007; Stanley *et al*., 2010).

2.4.2 Acquisition of ethanol tolerance

The genome expression profile in response to short term ethanol stress (7% v/v) displayed a reduction in the expression of those genes involved in protein synthesis, cellular growth and RNA metabolism according to a study by Alexandre *et al.,* (2001). Conversely, an increase in the expression of genes associated with energy metabolism, protein transport, ionic homeostasis, and stress response was observed. The genes within the functional stress response group included those related to the expression of numerous HSP's and trehalose synthase. This alludes to the negative impact ethanol has on protein structure (Alexandre *et al*., 2001). Trehalose also reduces membrane permeability, improving the cell's ethanol tolerance by reducing the efflux of nutrients from the cell (Mansure *et al*., 1994; Sharma, 1997). A second transcriptomic study, evaluating the expression patterns after an one hour long exposure to 5% (v/v) ethanol reported an increase in the expression of genes associated with transport, cell surface interactions and lipid metabolism in addition to genes involved in energy metabolism, ionic homeostasis, and stress response as stated above (Chandler *et al*., 2004).

Deletion studies have identified numerous genes involved in cell wall and membrane synthesis conferring ethanol tolerance. Interestingly, these genes are generally down regulated upon exposure to ethanol (Chandler *et al.*, 2004; Ma & Liu, 2010). Ethanol tolerance has also been correlated with the fatty acid and sterol composition of the membrane

(Alexandre *et al.*, 1994), and strains adapt to ethanol by increasing the sterol and unsaturated fatty acid content which provide structural stability to the plasma membrane. However, this process is hindered by the absence of oxygen since synthesis of sterols and unsaturated fatty acids requires the presence of molecular oxygen (Alexandre *et al.*, 1994).

The ethanol induced loss of functional transport systems induces the expression of high affinity hexose transporters, which are usually only expressed under conditions of glucose limitation. This suggests that cells experiencing ethanol stress enter a pseudo-starved state, as the cell is unable to access nutrients from the surrounding medium (Chandler *et al.*, 2004). Furthermore, Marks *et al.,* (2008) proposes that ethanol serves as a signal for the cell to enter stationary growth phase once it reaches the 2% (v/v) level. This may be a pre-emptive mechanism to ensure long term survival, as cells in the stationary phase are generally more stress tolerant.

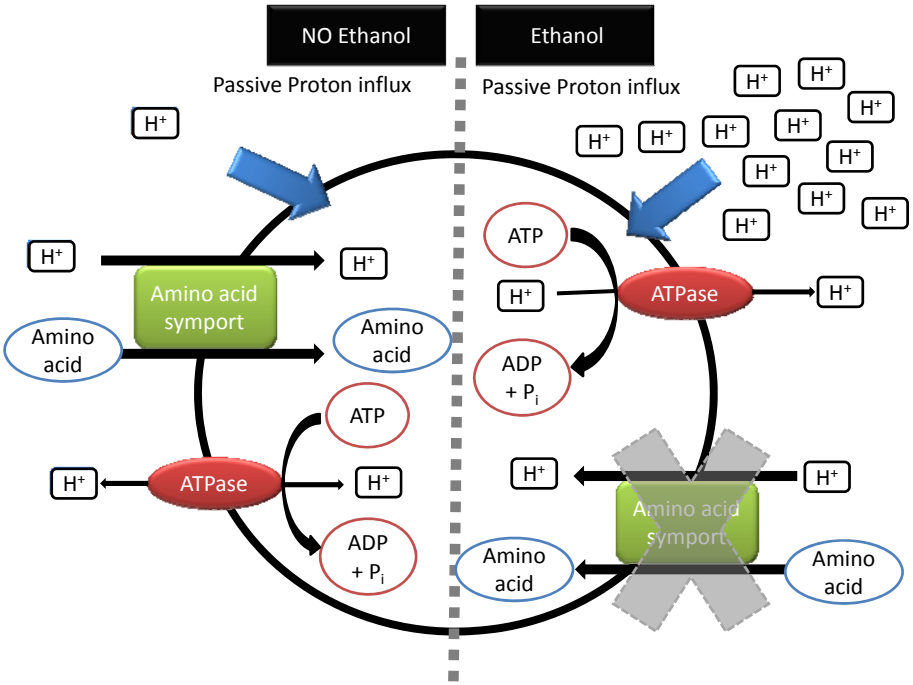

Figure 4 The impact of ethanol on the transport of protons and amino acids into the cell. Adapted from Bisson (1991)

2.4.3 Potential role of ethanol in wine aroma

The impact of ethanol on wine aroma perception has been studied, and at low levels, ethanol enhances the sensory perception of aroma compounds. However, when in excess it has a masking effect, and in high amounts can directly lead to a burning sensation (Swiegers *et al*., 2005).

On the other hand, only very limited data are available regarding the impact of ethanol on the *de novo* production of aroma compounds by yeast. Ethanol toxicity causes a loss of the transport of sugar and nitrogen into the cell, both of which are crucial to the formation on aroma compounds (Bisson & Karpel, 2010). Thus it is not surprising that ethanol is listed among the factors impacting higher alcohol formation (Fleet & Heard, 1993), additionally, it is likely that ethanol toxicity also impacts the formation of esters, acetaldehyde, organic acids and diacetyl; as their production is also dependent on sugar and nitrogen metabolism (Bisson & Karpel, 2010).

2.5 Low temperature

2.5.1 Physiological impact of low temperature

White wine fermentations are often conducted between 10°C and 15°C to ensure the retention of volatile aroma compounds which would be released in larger amounts at higher temperatures. When cooled to temperatures below 20°C, the cell experiences a temperature and duration specific cold stress response (Aguilera *et al*., 2007; Gibson *et al*., 2007). This cold stress response is not as conserved as the heat shock response (Piper, 1995; Kregel, 2002; Rangel, 2010), and is also poorly characterised for yeast (Aguilera *et al*., 2007). Temperature, like ethanol, primarily acts upon the plasma membrane. Exposure to high temperatures or ethanol results in an increase in membrane permeability, whereas lower temperatures result in a decrease in membrane permeability (Shinitzky, 1984; Gibson *et al.*, 2007). Consequently, cells fermenting at low temperatures are less susceptible to ethanol exposures than those fermenting at high temperatures. However, the reduced membrane permeability also hinders the transport of essential nutrients into the cell by trans-membrane proteins (Hazel, 1995). Several studies have shown that low temperature increases fermentation duration due to a decline in metabolic activity and, consequently, a lowering of yeast biomass production (Llauradó *et al.*, 2002; Beltran *et al.*, 2006; Pizarro *et al.*, 2008).

2.5.2 Acquisition of low temperature tolerance

Yeast cells adapt to lower temperatures by attempting to maintain a constant level of membrane fluidity. During the early stages of low temperature adaptation, the cell stabilises the membrane by increasing the degree of fatty acid unsaturation, and increases permeability by decreasing fatty acid chain length (Sahara *et al.*, 2002; Torija *et al.*, 2003; Al-Fageeh & Smales, 2006; Tai *et al.*, 2007; Beltran *et al.*, 2008; Redón *et al.*, 2011). Additionally, low temperature causes the formation of secondary structures within RNA molecules, reducing translation efficiency. The cell counters this by increasing the expression of genes involved in ribosomal proteins, RNA processing and translation (Sahara *et al.*, 2002; Schade *et al.*, 2004; Aguilera *et al.*, 2007; Gibson *et al.*, 2007).

A transcriptomic study comparing the expression patterns during the course of fermentations conducted at 13°C and 25°C, reported the increased expression of genes associated with membrane permeability at 13°C relative to 25°C during the initial stages of fermentation. Conversely, during the later stages of fermentation the level of expression of the genes associated with membrane permeability was greater at 25°C than 13°C (Beltran *et al.*, 2008). Despite a lack of cell division, strains fermenting at 13°C were better able to survive compared to those at 25°C, where a decline in viable cells was observed compared to a maintained maximal population size at 13°C throughout the fermentation. This transcriptomic data suggests that the early onset of the stress response, based on the *MSN2* expression levels, at lower temperature compared to the induction upon entering the stationary phase for fermentations at higher temperatures, better prepares the cells to survive (Beltran *et al.*, 2008).

Low temperature tolerance is further characterised by trehalose and phospholipid synthesis, the expression of heat shock proteins, induction of oxidative stress response and cell wall mannoproteins synthesis during the later stages of cold shock (Schade *et al.*, 2004).

2.6 Conclusions

Wine yeast strains experience a dynamic environment during grape must fermentation. The cell is required to counteract the deleterious effects of temperature change, low pH, hyperosmotic stress, ethanol toxicity, limited oxygen and nitrogen availability, and the presence of competing or sometimes antagonistic micro-organisms throughout fermentation (Alexandre & Charpentier, 1998; Bisson, 1999; Gibson *et al.*, 2007). Its ability to respond to these stresses, via so-called "general" and specific means, will determine whether it survives and completes alcoholic fermentation.

Overall, wine yeast strains attain "general" stress tolerance via the accumulation of HSP and trehalose to prevent and repair protein denaturation. In addition to this "general" response, hyperosmotic stress also induces the expression of the enzymes of glycerol production as part of its specific stress response. In the case of ethanol toxicity and low temperature, as both stresses impact the plasma membrane, it is not surprising that the cell regulates plasma membrane permeability to offset the harmful effects of stress.

Future work should be directed towards establishing the impact of major fermentation stresses on the production of volatile aroma compounds.

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Chapter 3

Research results

The impact and interaction between initial nitrogen, initial sugar, and temperature on the fermentation performance of commercial wine yeast

Chapter 3
The impact and interaction between initial nitrogen, initial sugar, and temperature on the fermentation performance of commercial wine yeast

3.1 Introduction

The metabolic process central to winemaking is the conversion of sugar to ethanol and CO_2. This conversion is mediated by yeast, primarily *Saccharomyces cerevisiae*. The use of active dry yeast (ADY) is common practice in the wine industry, as it aids onset of fermentation as well as fermentation to dryness, where the residual sugar content is reduced to less than 4 g/L (Pretorius *et al.*, 1999). During fermentation, yeast cultures are exposed to numerous stresses, both simultaneously and in succession (Bisson, 1999). These stresses include nutrient deficiencies (minerals, vitamins, nitrogen, and oxygen), low pH, ethanol toxicity, temperature extremes, and a high osmotic pressure (Kunkee, 1991; Attfield, 1997; Alexandre & Charpentier, 1998; Bauer & Pretorius, 2000; Malherbe *et al.*, 2007). These are also the factors associated with problematic fermentations, which have been studied extensively, due to the economic losses and logistical problems they cause (Malherbe *et al.*, 2007).

Nitrogen deficiency is reportedly the most common cause of problematic fermentations. As nitrogen plays an integral role in biomass production, cell maintenance, and sugar catabolism, it also influences the fermentation rate (Bisson, 1991; 1999). Yeast assimilable nitrogen (YAN) consists of ammonia, free amino acids (excluding proline and hydroxyproline), and low molecular weight peptides. *S. cerevisiae* is unable to utilize larger peptides due to its poor extracellular proteolytic activity, and proline because of the anaerobic state of fermentation (Bell & Henschke, 2005). It is generally agreed that 140 mg/L YAN is the threshold level below which the risk of stuck or sluggish fermentations increases (Agenbach, 1977; Bely *et al.*, 1990a). However, this level was established for clarified must with moderate sugar levels, and thus should only be considered as a guide (Bell & Henschke, 2005), as a higher sugar concentration requires more nitrogen to ferment to dryness (Bisson & Butzke, 2000).

Low YAN content is the most common cause of problematic fermentations, and is most commonly overcome with diammonium phosphate (DAP) supplementation. It is routinely added to the grape must before the onset of fermentation, frequently without first determining the YAN content of the grape must. When nitrogen is deficient, DAP addition reduces the risk of problematic fermentations (Bisson, 1999). However, when nitrogen is in excess it may result in microbial instability, and, in some cases, a decline in the fermentation performance of a yeast strain (Taillandier *et al.*, 2007).

Commercial starter cultures differ significantly in their inherent nitrogen requirements

(Jiranek *et al.*, 1991; Manginot *et al.*, 1998). This strain specific response to nitrogen emphasizes the importance of evaluating all commercial yeast strains to ascertain their respective nitrogen requirements, and in so doing prevent excessive or insufficient nitrogen supplementation (Jiranek *et al.*, 1991; Manginot *et al.*, 1998; Taillandier *et al.*, 2007).

A high initial sugar concentration initiates a hyperosmotic stress response immediately upon inoculation. To limit the loss of water, glycerol channels close, and glycerol production is activated. Glycerol serves as an osmoprotectant, by increasing the internal solute concentration, and in so doing limits the efflux of water. The accumulation of glycerol continues until the influx of water restores cell size to a critical level and yeast growth is resumed (Hohmann, 1997; Mager & Siderius, 2002). Consequently, the higher the initial sugar concentration the longer the lag phase before fermentation commences, due to the cessation of growth while the cell adapts to the prevailing osmotic conditions. This may in some cases even result in stuck or sluggish fermentations (Lafon-Lafourcade, 1983; Llauradó *et al.*, 2002). As the fermentation progresses, the sugar concentration decreases and the ethanol concentration increases. Ethanol primarily targets the plasma membrane, increasing its permeability. This ultimately results in a reduction in cell viability and growth, caused by a decline in the transport of nitrogen and sugar into the cell (Bisson, 1991; Hallsworth, 1998; Stanley *et al.*, 2010). The cells respond to ethanol toxicity by synthesizing trehalose, unsaturated fatty acids and heat shock proteins to restore membrane permeability (Bisson, 1999; Gibson *et al.*, 2007; Stanley *et al.*, 2010).

Temperature and ethanol both act upon the plasma membrane. Exposure to high temperatures or to ethanol results in an increase in membrane permeability, whereas lower temperatures result in a decrease in membrane permeability (Gibson *et al.*, 2007). Consequently, cells fermenting at high temperatures are more susceptible to ethanol exposure than those fermenting at low temperatures. White wine fermentations are generally conducted between 10°C and 15°C compared to 25°C or higher for red wines. Fermentation temperature impacts the retention of aroma compounds; additionally transcriptomic studies have found temperature dependant differences in the expression of aroma related genes (Torija *et al.*, 2003; Beltran *et al.*, 2006, Molina *et al.,* 2007).

Several studies have shown that low temperature increases fermentation duration due to a decline in metabolic activity and, consequently, a lowering of yeast biomass production (Fleet & Heard, 1993; Llauradó *et al.*, 2005; Beltran *et al.*, 2006; Pizarro *et al.*, 2008).

Yeast strains differ in their ability to sense and effectively respond to all of the abovementioned stresses (Ivorra *et al.*, 1999; Carrasco *et al.*, 2001). This ability to sense and respond to stress has been linked to fermentation performance (Zuzuarregui & del Olmo, 2004), as stress resistance would contribute to whether strains are able to survive and ultimately ferment grape must. Strains also vary in their nitrogen requirements (Agenbach, 1977; Bezenger & Navarro, 1988; Bely *et al.*, 1990b; Bely *et al.*, 1991; Jiranek *et al.*, 1991; Manginot *et al.*, 1998) , and their ability to catabolise sugars (Manginot *et al.*, 1998). This emphasizes the importance of the selection of an appropriate yeast

starter culture capable of fermenting a must, of known parameters (YAN, total sugars etc.), adequate nitrogen supplementation, or a combination of both strategies.

The aim of this study was to investigate the impact of the initial nitrogen content (50, 100, 250 or 400 mg/L), hyperosmotic pressure (200 or 240 g/L sugar), and temperature (15°C or 20°C) on the fermentation performance of 17 commercial active dry yeast cultures using a multifactorial experimental design. Past studies have assessed the response of yeast strains, including some wine yeast strains, to individual stresses. To our knowledge this is the first study to evaluate the impact of different stresses when applied at different levels and in different combinations. Considering the complex and integrated nature of molecular stress response pathways, a combined application of stresses may indeed result in responses that are qualitatively and quantitatively very different from those described for individual stresses. In this study, the fermentation performances of strains were characterised on the basis of fermentation kinetics data (weight loss due to CO_2 evolution), the residual sugar levels and the dry weight produced as determined at the end of fermentation. The fermentation kinetics data was used to generate the EC50, hill-slope, and top values for each of the fermentations. The EC50 value represents the time required for the fermentation to reach the half-way mark, illustrating how rapidly a strain is able to adapt to its environmental conditions. The hill-slope (maximum fermentation rate) equals the gradient of the curve, and reflects the extent to which the grape must and fermentation conditions impact yeast growth and grape must fermentation. The top value equals the total weight loss, which provides a relative indication of fermentation completeness (dependant on the initial sugar content). This approach will identify strains that are capable of fermenting a grape must with specific nitrogen and sugar levels, providing winemakers a tool with which to select a yeast strain best adapted to ferment a specific grape juice and ensuring complete fermentation.

3.2 Materials and methods

3.2.1 Synthetic grape medium and fermentation treatments

The synthetic grape must used in this study is described in Tables 1 and 2. The pH of the medium containing the carbon sources, acids, and salts was set to 3.3 with potassium hydroxide, before autoclaving. The vitamin, mineral, lipid and amino acid stocks were filter sterilized and added to the autoclaved medium.

The small scale fermentations were performed in triplicate at 15°C or 20°C. The must differed in the initial sugar content and contained equimolar amounts of glucose and fructose amounting to either 200 g/L or 240 g/L. Fermentations also differed in the initial nitrogen content, which was proportionally decreased or increased to reach desired level of 50 mg/L, 100 mg/L, 250 mg/L or 400 mg/L nitrogen. The 16 fermentation treatments are summarised in Table 3. All the yeast strains used (Table 4) were rehydrated according to manufacturers' instructions and inoculated at 20 g/hL. All yeast strains were supplied by Anchor yeast, except Lalvin EC1118 (Lallemand) and AWRI796 (Maurivin).

The fermentations were weighed regularly to monitor fermentation progress, as CO_2 evolution. After 21 days, the fermented synthetic wine samples were scanned using the Winescan FT120 instrument (FOSS Analytical A/S software version 2.2.1) equipped with a purpose-built Michelson interferometer (FOSS Analytical A/S, Hillerød, Denmark) to generate a Fourier transform mid infrared (FT-MIR) spectra. Quantified chemical data for residual glucose and fructose levels were predicted from infrared spectra by commercial calibrations or in-house adjustments using the Winescan FT120 2001 version 2.2.1 software.

3.2.2 Dry weight

The biomass was determined (in triplicate) as cell dry weight. A 4 ml sample was spun down in a pre-weighed microcentrifuge tube; the resulting pellet was dried in an oven at 30˚C for approximately two weeks. In order to ensure that all moisture was removed the microcentrifuge tube was spun in the speedy vac set at high heat for 5 minutes and weighed.

Table 1: The synthetic grape must used as previously described by Henschke and Jiranek (1993) for the carbon, acids, salts, trace elements, vitamins, and lipid sources.

		per litre
Carbon Sources	Glucose	100 or 120 g
	Fructose	100 or 120 g
Acids	KH Tartrate	2.5 g
	L-Malic acid	3 g
	Citric acid	0.2 g
Salts	K_2HPO_4	1.14 g
	$MgSO_4.7H_20$	1.23 g
	CaCl2.2H2O	0.44 g
Minerals	$MnCl_2.4H_2O$	200 µ g
	$ZnCl_2$	135 µ g
	$FeCl_2$	30 µ g
	$CuCl_2$	15 µ g
	H_3BO_3	5 µ g
	$Co(NO_3)_2.6H2O$	30 µ g
	$NaMoO_4.2H_2O$	25 µ g
	KIO_3	10 µ g
Vitamins	Myo-Inositol	100 mg
	Pyridoxine.HCl	2 mg
	Nicotinic acid	2 mg
	Ca Pantothenate	1 mg
	Thiamin.HCl	0.5 mg
	PABA.K	0.2 mg
	Riboflavin	0.2 mg
	Biotin	0.125 mg
	Folic Acid	0.2 mg
Lipids	Ergosterol	10 mg
	Tween 80	0.5 mL

3.2.3 Statistical analysis

The fermentation kinetics (weight loss due to CO_2 evolution) data was used to fit three-parameter logistic dose response curves across all combinations of treatments. The three parameters were EC50, hill-slope, and the top value. EC50 value represents the time required for the fermentation to lose half of the total weight loss (fermentation mid-point). The hill-slope (maximum fermentation rate) equals the gradient of the curve and the top value equals the total weight loss. The effect of the different

treatments on the above mentioned parameters as well as residual sugars (sum of residual glucose and fructose) and dry weight were assessed using factorial analysis of variance (ANOVA). In all cases a significance level of 5% was used.

All results are presented in two ways, first looking at the general impact of the treatments on the parameter in question, and secondly comparing the performance of individual strains. Due to the size and complexity of the data set, only the lower order interactions are reported on, in some cases this is despite a significant higher order interaction. Additionally, when a variable (temperature, nitrogen, sugar or strain) appears to have been "omitted", the data for that variable has been pooled. This may hide other significant patterns.

Table 2: The nitrogen supplementations used as previously described by Bely *et al*., (1990b), was proportionally increased or decreased to obtain a final concentration of 50, 100, 250 and 400 mg/L.

Nitrogen Sources:		**300 mg/L**
Amino Acids	Tyrosine	18.326 mg/L
	Tryptophane	179.333 mg/L
	Isoleucine	32.725 mg/L
	aspartic acid	44.506 mg/L
	glutamic acid	120.428 mg/L
	Arginine	374.374 mg/L
	Leucine	48.433 mg/L
	Threonine	75.922 mg/L
	Glycine	18.326 mg/L
	Glutamine	505.274 mg/L
	Alanine	145.299 mg/L
	Valine	44.506 mg/L
	Methionine	31.416 mg/L
	phenylalanine	37.961 mg/L
	Serine	78.54 mg/L
	Histidine	32.725 mg/L
	Lysine	17.017 mg/L
	Cysteine	13.09 mg/L
	Proline	612.612 mg/L
Ammonium Chloride	NH_4Cl	0.46 g/L

Table 3: Fermentations were conducted in triplicate at 15°C or 20°C. All treatments contained sugars (200 or 240 g/L total), nitrogen (50, 100, 250 or 400 mg/L nitrogen), salts, minerals and fatty acids.

		Treatment description															
Temperature	**˚C**	15	20	15	20	15	20	15	20	15	20	15	20	15	20	15	20
Sugar	**g/L**	200	200	200	200	200	200	200	200	240	240	240	240	240	240	240	240
Nitrogen	**mg/L**	50	50	100	100	250	250	400	400	50	50	100	100	250	250	400	400

Table 4: Strains used in this study (compiled from commercial specification sheets).

Commercial name	Strain	Recommended wine styles	Recommended temperature
IWBT PR7 [1] **(Exotics SPH)**	*Saccharomyces cerevisiae, S. paradoxus hybrid*	White wine	16-20°C
VIN 2000	*S. cerevisiae* hybrid	White wine	13-16°C
VIN 7	*S. kudriavzevii* and *S. cerevisiae* hybrid[2]	White wine	13-16°C
VIN 13	*S. cerevisiae* hybrid	White/ Rose wine	12-16°C
WE 14	*S. cerevisiae*	Natural sweet white wine	16-20°C
WE 372	*S. cerevisiae*	Red wine/ semi-sweet white	18-28°C
AlchemyI	*Saccharomyces spp.* blend	White wines	13-16°C
AlchemyII	*Saccharomyces spp.* blend	White wines	13-16°C
228	*S. cerevisiae*	Brandy base wine production	15-20°C
AWRI 796	*S. cerevisiae*	Red/ white wine	15-18, 20-30°C
EC1118	*S. cerevisiae bayanus*	Sparkling, fruit wine, and ciders	15-25°C
N 96	*S. cerevisiae bayanus*	Sparkling and ice wines	12-28°C
NT 45	*S. cerevisiae*	Red wines	14-28°C
NT 50	*S. cerevisiae* hybrid	Red wines	14-28°C
NT 112	*S. cerevisiae* hybrid	Red wines	24-28°C
NT 116	*S. cerevisiae* hybrid	White wines	12-16°C
NT 202	*S. cerevisiae* hybrid	Red wines	20-28°C

[1] Exotic SPH will be referred to by its IWBT name PR7 from this point forward

[2]Bradbury *et al*. (2006)

3.3 Results and discussion

3.3.1 Influence of treatments on fermentation kinetics

3.3.1.1 Impact of treatments on fermentation onset

The number of days required for the fermentation to reach its halfway mark is represented as the EC50 value. This value was used as an estimate of the duration of the lag phase or the onset of fermentation. Figure 1 illustrates the influence of the initial nitrogen and sugar concentrations, as well as temperature on the onset of fermentation when combining the EC50 values of all the strains (see Table 4) within a specific treatment (see Table 3). Similarly, in subsequent figures, when a variable (initial sugar, nitrogen, temperature or yeast strains) is not described, that is an indication that the data for that variable has been pooled.

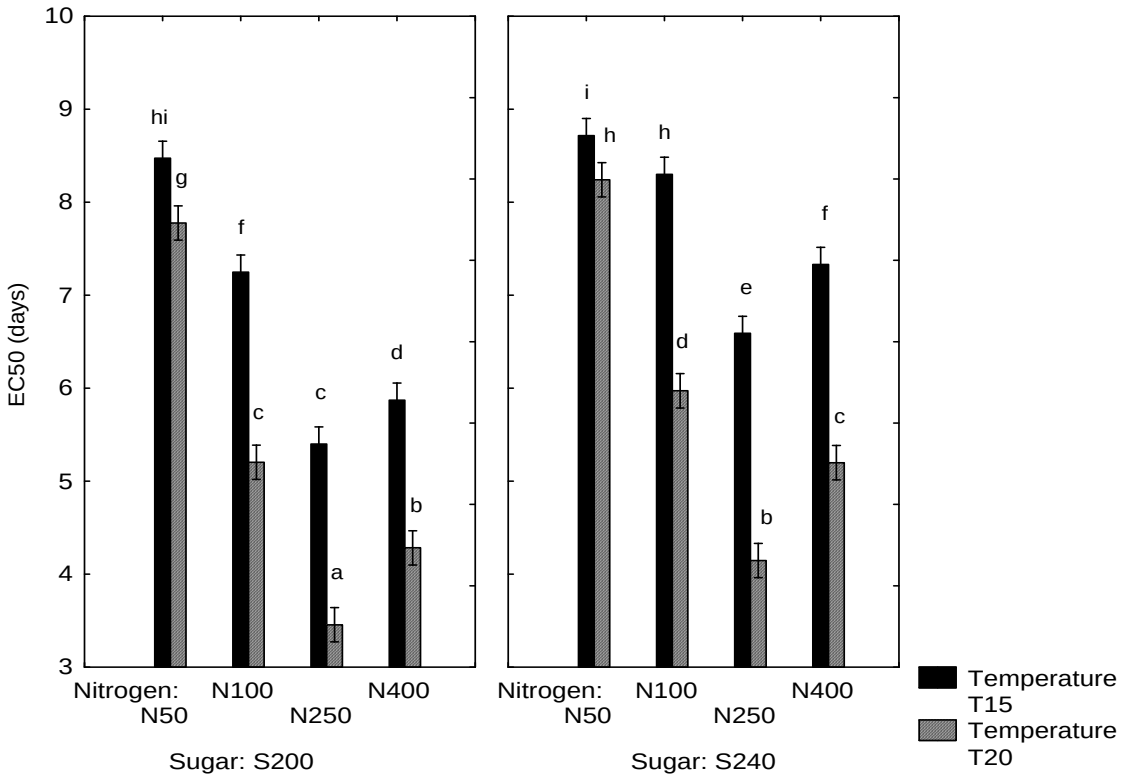

Figure 1: Influence of the nitrogen (50, 100, 250 or 400 mg/L), sugar (200 or 240 g/L), and temperature (15°C or 20°C) treatments on the fermentation onset (EC50). The data for all strains within a specific treatment are pooled. Error bars indicate 95% confidence intervals, and the letters denote a significant difference on a 5% significance ($p<0.05$) level.

Low temperature (15°C), low nitrogen levels (50 and 100 mg/L) and high sugar content (240 g/L) are environmental stresses commonly linked to reduced yeast growth. This reduction in yeast growth explains the extended fermentation lag phases observed in Figure 1 for the fermentations containing a single or combinations of these stresses. At both sugar levels and temperatures, the increase in the nitrogen concentration caused a decrease in the lag phase (Bely *et al.*, 1991; Beltran *et al.*, 2005). When nitrogen was raised to 400 mg/L, a relative increase in the lag time was observed when compared to the 250 mg/L lag phases. This suggests that excess nitrogen has a detrimental impact on the onset of fermentation.

3.3.1.2 Impact of treatments on the fermentation onset of individual yeast strains

The variation in the initial nitrogen levels resulted in different lag phases for the strains evaluated (Figure 2). Nitrogen is essential for biomass production, cell maintenance, and sugar catabolism (Bisson, 1991; 1999), it is not surprising then that the onset of fermentation was hampered by the low initial nitrogen content (50 mg/L). Within the 50 mg/L nitrogen treatment, WE372, NT1116, and AlchemyI were among the strains with the quickest fermentation onset, and N96, VIN7 and NT45 were among the strains with the slowest onset. For the other nitrogen levels, 100, 250 and 400 mg/L, VIN2000, NT116, and WE372 were among the strains with a short lag phase. Conversely; VIN7, NT45, and WE14 were among the strains with the longer lag phase.

As the nitrogen content increased a corresponding decrease in lag phase was observed (Bely *et al.*, 1991; Beltran *et al.*, 2005). However, when nitrogen was raised to 400 mg/L, the lag phase was either statistically similar to that of the 250 mg/L treatments (228, AlchemyII, NT112, NT45, PR7, VIN2000, and VIN13) or increased significantly, as was the case for the majority of the strains (EC1118, AWRI796, N96, NT116, WE372, NT202 NT116, NT50, WE14, VIN7 and AlchemyI). For the strains NT116, NT50, VIN7 and WE14 the lag phase increased to durations comparable to that of the 100 mg/L treatments. VIN7 was consistently among the strains with the longer lag phase. This is not surprising, as its inability to rapidly respond to hyperosmotic stress has been reported in other studies (Erasmus *et al.*, 2004; Erasmus & van Vuuren, 2009).

Fermentation temperature influences the onset on fermentation (Figure 3) by affecting yeast growth (Fleet & Heard, 1993). Fermentations conducted at 20°C had a shorter lag phase compared to those at 15°C. VIN2000, VIN13 and WE372 are among the strains with the shortest lag phase and VIN7, NT50 and WE14 were among those with a long lag phase, irrespective of the fermentation temperature.

Fermentations containing the lower sugar level (200 g/L) were initiated more readily than those containing 240 g/L (Data not shown). This is indicative of exposure to increased osmotic stress, where yeast strains require additional time to adapt to the environmental conditions (Mager & Siderius, 2002). For both sugar concentrations, VIN2000, VIN13 and WE372 were among the strains with the shortest lag phase, and VIN7, NT50 and WE14 were among those with a long lag phase.

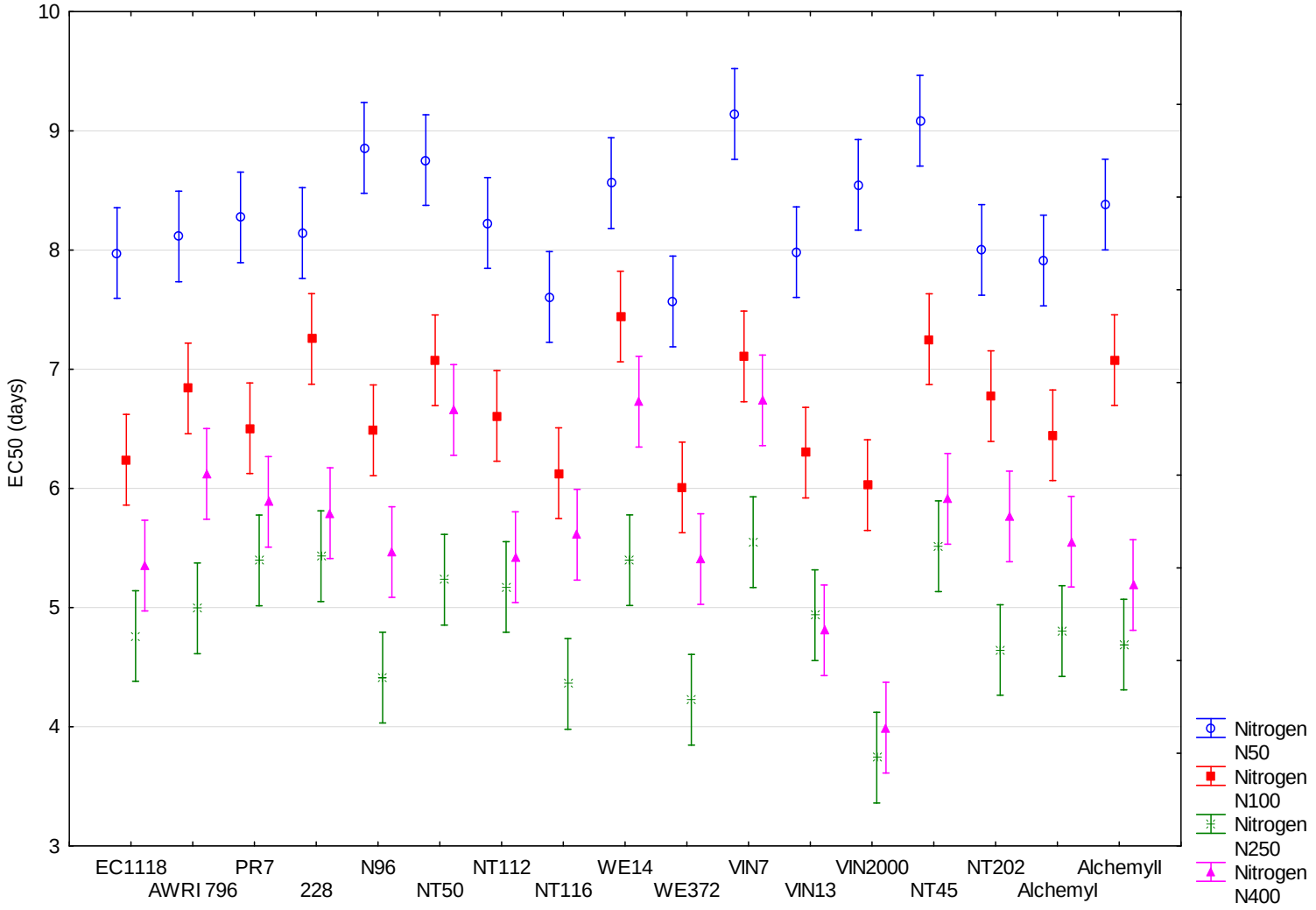

Figure 2: The fermentation onset (EC50 value) of 17 commercial cultures fermenting synthetic grape must containing 50, 100, 250, or 400 mg/ nitrogen. The data for sugar and temperature was combined, providing one value per strain for each nitrogen level. Error bars indicate 95% confidence intervals for the means.

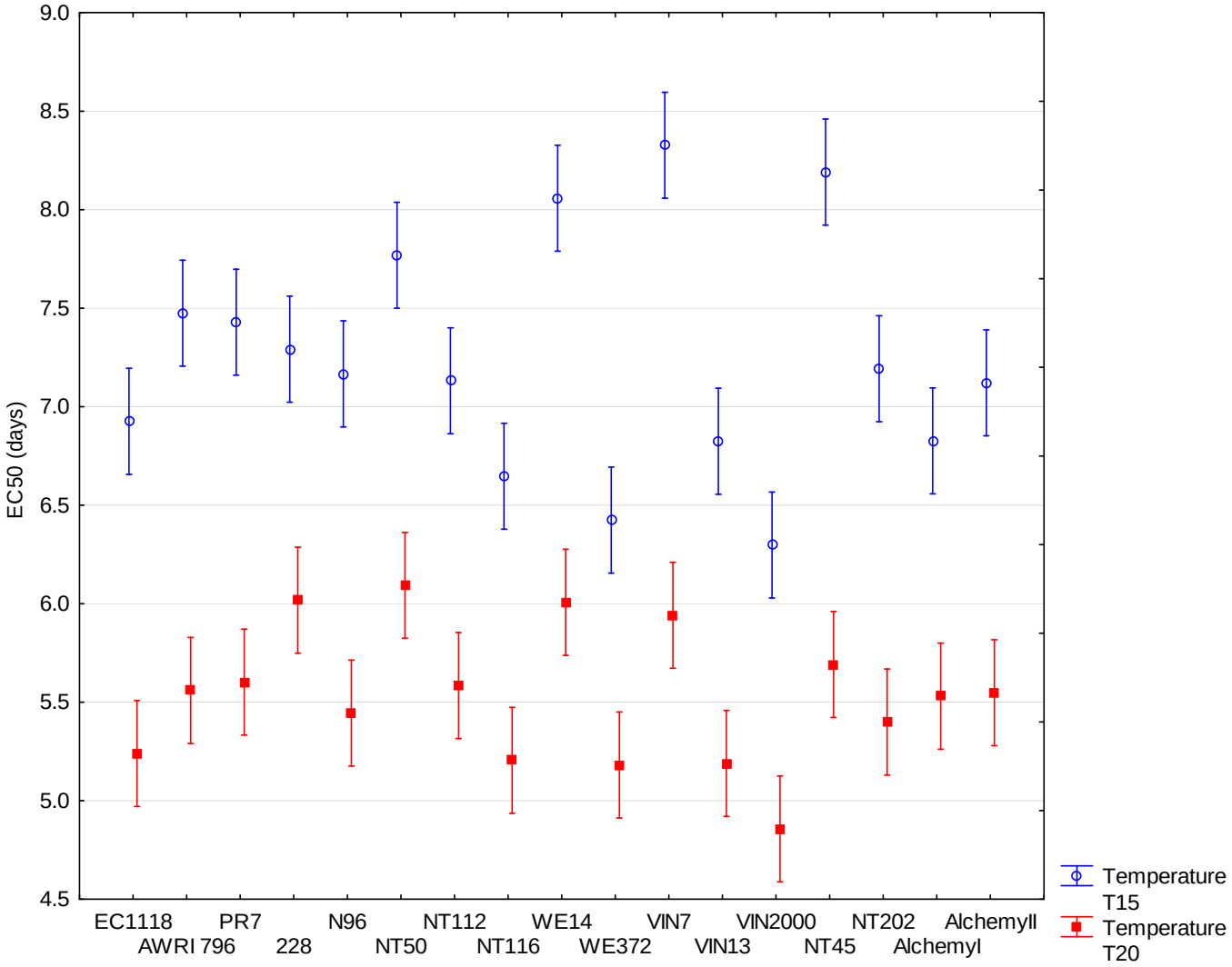

Figure 3: The fermentation onset (EC50) of 17 commercial cultures fermenting synthetic grape must at 15°C or 20°C. The nitrogen and sugar data has been pooled, resulting in a single value for each strain at the evaluated temperatures. Error bars indicate 95% confidence intervals for the means.

3.3.1.3 Impact of treatments on fermentation rate

The influence of nitrogen, sugar and temperature on the maximum fermentation rate is depicted in Figure 4. The maximum fermentation rates (hill-slope) for both sugar levels respond in a similar pattern to the temperature and nitrogen treatments; however the responses do differ in their magnitude. At the lowest nitrogen level (50 mg/L) for both sugar levels, there was no significant difference between fermentations at 15°C and 20°C, suggesting that nitrogen rather than the other stresses had a limiting effect on the maximum fermentation rate. The fermentation rate increased as the nitrogen level increased. However, at 400 mg/L a decline in the fermentation rate occurred.

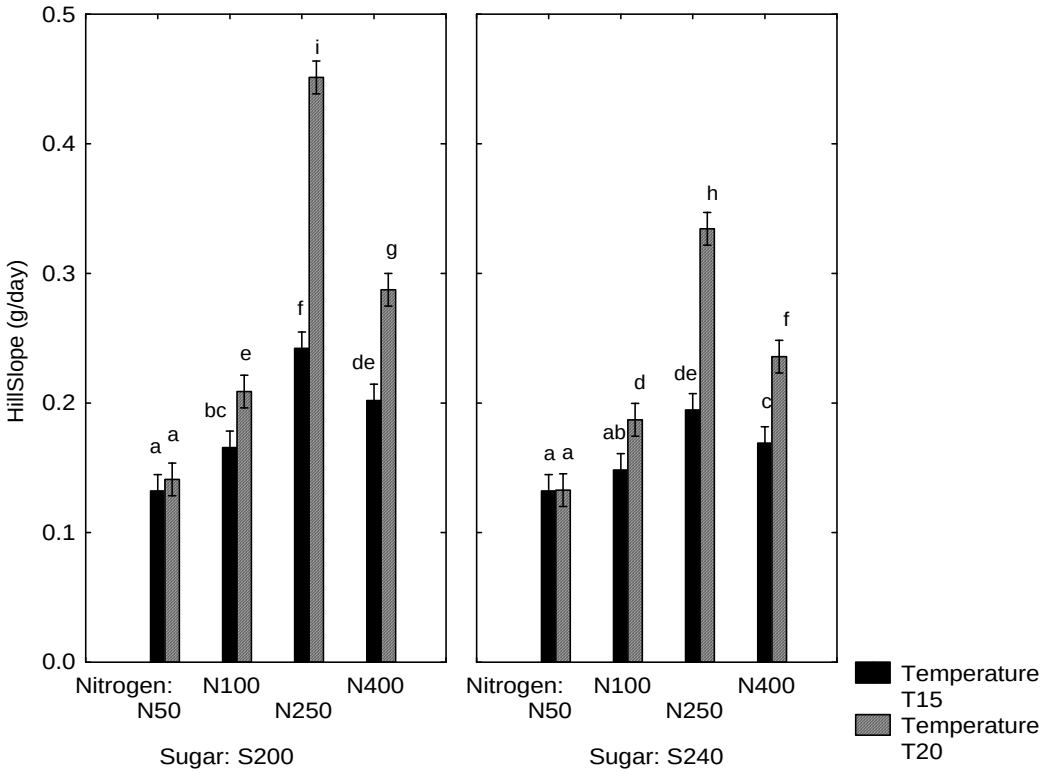

Figure 4: Influence of the nitrogen (50, 100, 250, 400 mg/L), sugar (200 or 240 g/L), and temperature (15°C or 20°C) treatments on the maximum fermentation rates (hill-slope). The data for all strains within a specific treatment were pooled. Error bars indicate 95% confidence intervals for the means. Letters denote significant differences on a 5% ($p<0.05$) significance level.

Figure 5 shows the correlation between the timing of fermentation onset and maximum fermentation rate. The data shows that the EC50 value is inversely correlated with the maximum fermentation rate, i.e. that a rapid onset of fermentation correlates with a rapid maximal fermentation rate. Thus when fermentations have a long lag phase, it is generally followed by a sluggish fermentation rate, possibly related to a fermentation limiting condition (low temperature, hyperosmotic stress, ethanol toxicity or nutrient depletion, among others).

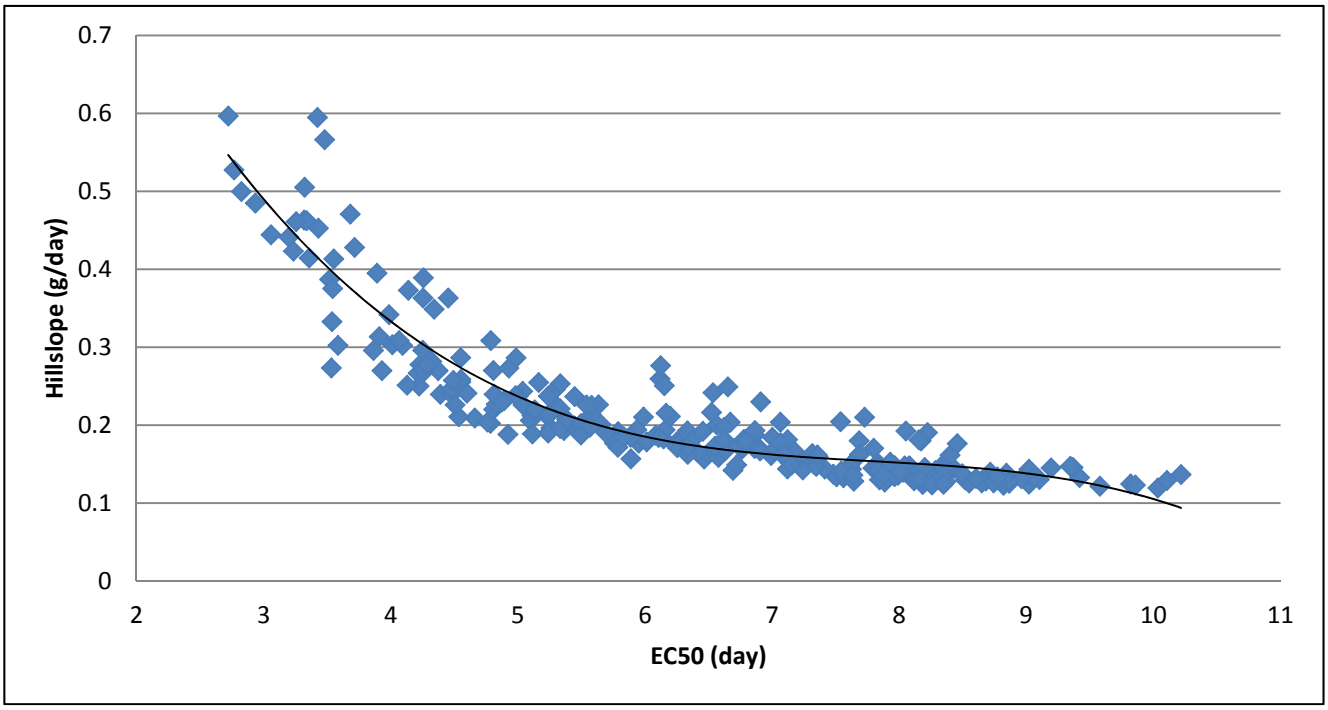

Figure 5: The relationship between the hill-slope (g/day) and the EC50 value (day).

3.3.1.4 Impact of treatments on the fermentation rates of individual yeast strains

The maximum fermentation rates of individual strains did not differ significantly from each other for the 50 and 100 mg/L nitrogen treatments (Figure 6). In most cases the fermentation rate was significantly greater for the strains fermenting must containing 250 mg/L nitrogen compared to the 400 mg/L must. The only exceptions were NT112 and VIN13 that displayed similar fermentation rates at 250 mg/L and 400 mg/L nitrogen. Furthermore, nitrogen excess (400 mg/L) reduced the maximal fermentation rate of NT116, NT50, PR7, VIN7, WE14, AlchemyI and WE372 to a rate that was statistically similar to that of their 100 mg/L nitrogen fermentations.

In response to the temperature, fermentations at 20°C fermented more rapidly than those at 15°C (Figure 7). The only exception was strain 228, for which fermentations proceeded at a similar rate at both temperatures. At 15°C the maximal fermentation rate of 228 is among the strains with rapid fermentation rates and at 20°C it is among the strains with slow rates. The stable fermentation rate may be as a result of superior low temperature tolerance, relative to the other strains.

Generally, the maximal fermentation rates were negatively affected by the higher initial sugar level, except in case of 228, AlchemyI and NT202 (data not shown). Irrespective of the sugar level, VIN2000, NT45 and EC1118 were among the strains with faster fermentation rates. When the initial sugar content was 200 g/L; PR7, NT112, Alchemy1 and NT202 were among the slower fermenters, whereas NT50, WE372, AWRI796, VIN7 were among the slow fermenters when the sugar content was 240 g/L. This difference in fermentation rate in response to the increased sugar level may be indicative of an increased sensitivity to osmotic stress and its resultant ethanol toxicity.

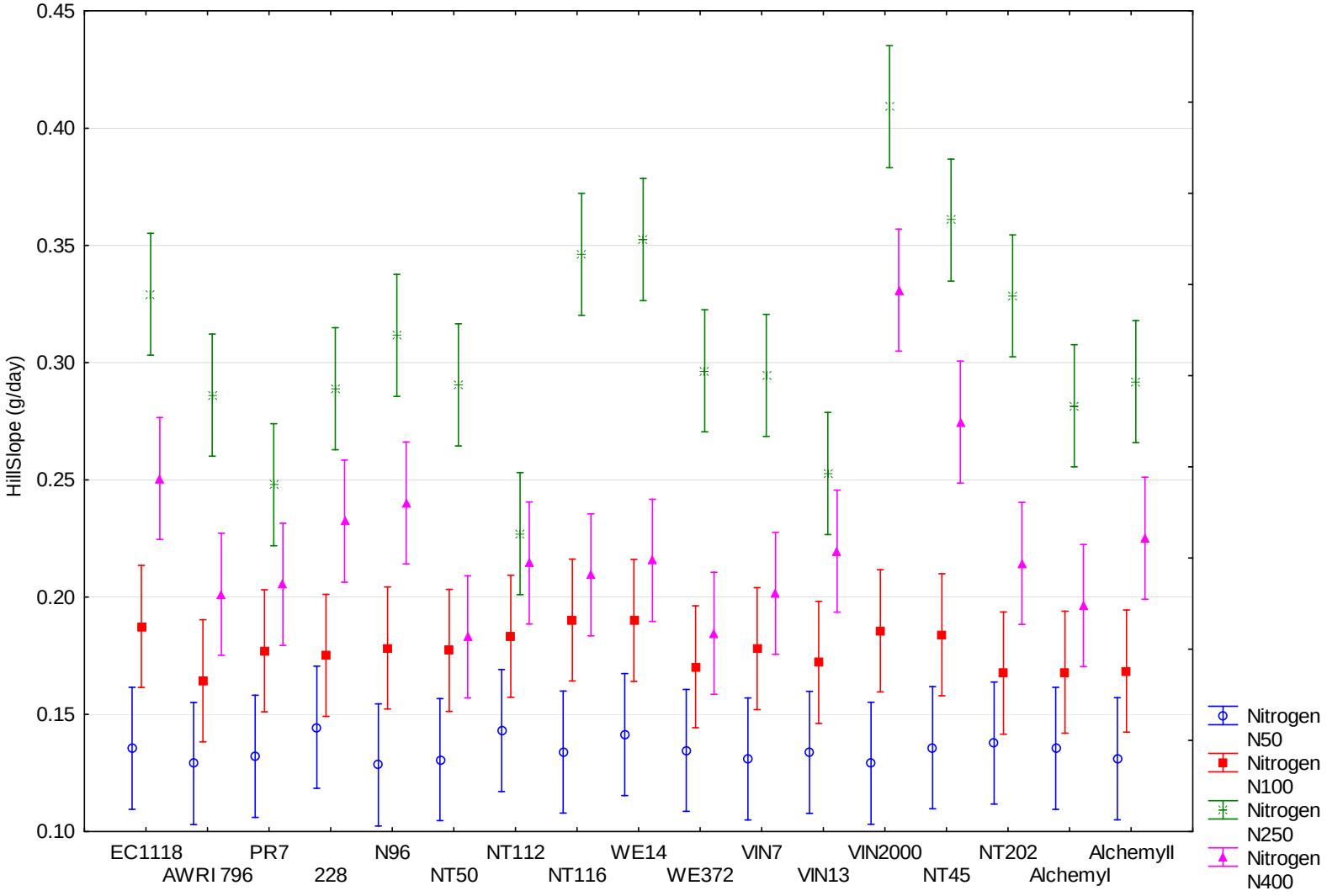

Figure 6: The maximal fermentation rate (hill-slope) of 17 commercial cultures fermenting synthetic grape containing 50, 100, 250, or 400 mg/L nitrogen. The sugar and temperature data has been combined, providing a single value for each strain at the evaluated nitrogen level. Error bars indicate 95% confidence intervals for the means.

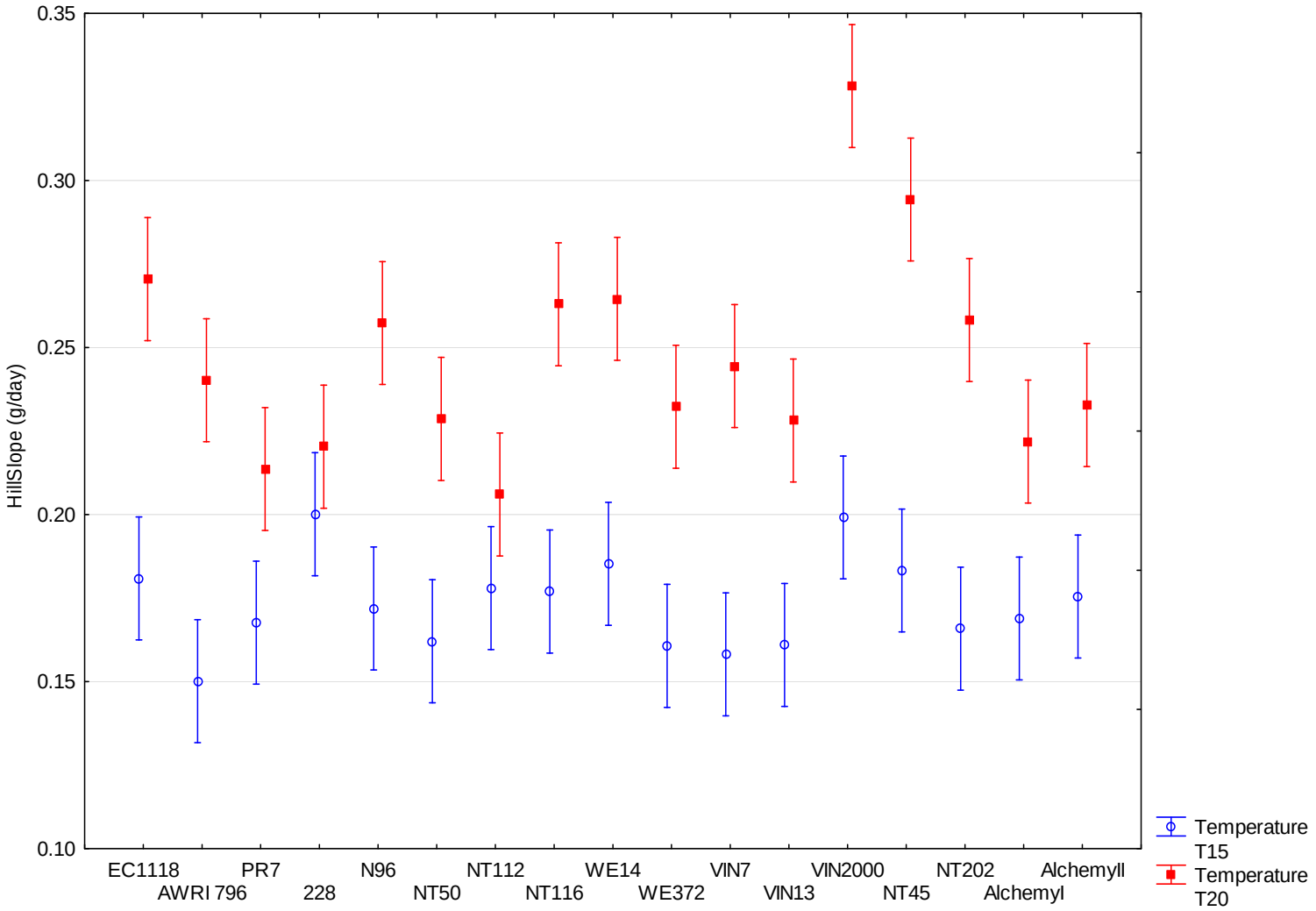

Figure 7: The maximal fermentation rate (hill-slope) of 17 commercial cultures fermenting synthetic grape fermenting at 15°C or 20°C. The nitrogen and sugar data has been pooled, providing a single value for each strain at the evaluated temperatures. Error bars indicate 95% confidence intervals for the means.

3.3.1.5 Impact of treatments on the total weight loss

The total weight loss (top value) was influenced by the initial nitrogen and sugar levels, and temperature (Figure 8 A and B). The total weight loss was consistently greater for the fermentations at 20°C compared to those conducted at 15°C (Figure 8 A). Fermentations at 20°C containing the lowest nitrogen content (50 mg/L) had a similar total weight loss as those conducted at 15 °C containing 100 mg/L nitrogen. This is indicative of the profound influence temperature has on fermentation performance despite the nitrogen deficiency. Fermentations at 20°C reached similar top values for treatments containing 100, 250 and 400 mg/L nitrogen, as did fermentations at 15°C containing 250 and 400 mg/L nitrogen. Despite the differences in lag phase (Figure 1) and maximum fermentation rates (Figure 4), the fermentation duration was generally long enough for the sluggish fermentations to reach completion when nitrogen was above 50 mg/L.

The maximum weight loss was similar for both sugar levels when nitrogen was limiting (Figure 8 B). The increase in nitrogen resulted in an increase in the total weight loss.

3.3.1.6 Impact of treatments on the total weight loss of individual yeast strains

The fermentation performances of the individual strains were hampered by the nitrogen deficient conditions (50 mg/L), as seen by the low total weight loss (Figure 9). Fermentations conducted with the strains WE372, and 228 were most severely hampered by the low nitrogen levels (50 and 100 mg/L), in terms of total weight loss. The strains VIN7, EC1118 and NT50 were among those that lost the most overall weight, suggesting that they used the nitrogen more efficiently. Despite a slow initial response (EC50 and hill-slope), VIN7 is among the strains with the highest total weight loss at very low nitrogen levels (50 mg/L), suggesting that when give time VIN7 is able to overcome adverse fermentation conditions. At the higher nitrogen levels, the similar top values for the weight loss indicates a similar degree of completion of fermentation.

Fermentations at higher temperature resulted in a higher total weight loss (Figure 10). At both temperatures VIN7 and AlchemyII were among the strains that lost the most weight and 228 and WE372 were among the strains that lost the least amount of weight.

The interactions between the initial sugar concentrations and the strains are not significant for the top values (data not shown).

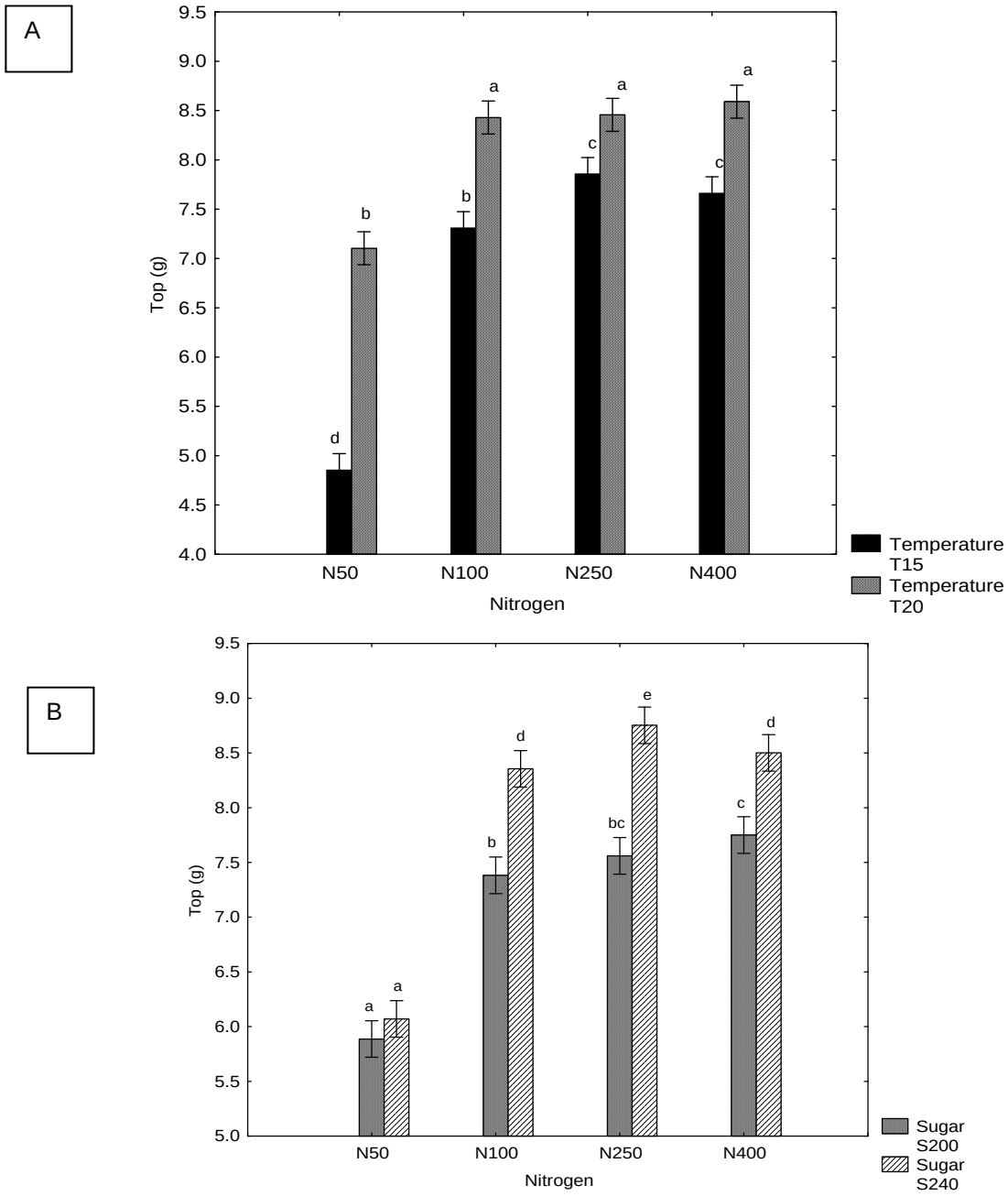

Figure 8: (A) Influence of nitrogen (50, 100, 250, or 400 mg/L), and temperature (15°C or 20 °C) on the total weight loss (top values). The sugar and strain data has been combined (B) Influence of nitrogen (50, 100, 250, or 400 mg/L), and sugar (200 or 240 g/L) on the total weight loss (top values). The temperature and strain data has were combined. Error bars indicate 95% confidence intervals for the means. Letters indicate significant differences on a 5% ($p<0.05$) significance level.

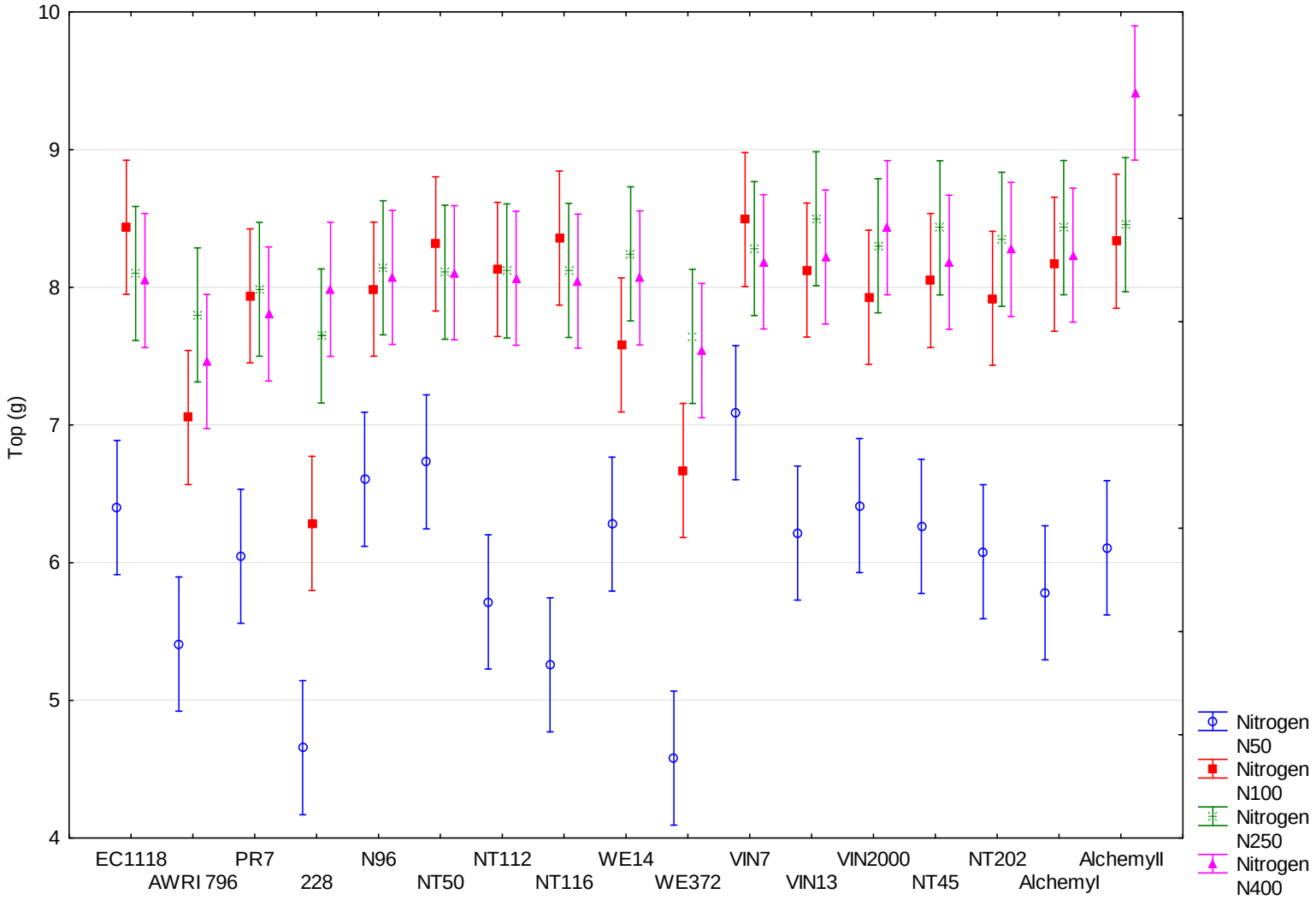

Figure 9: The total weight loss, measured as the top value, of 17 commercial cultures fermenting synthetic grape containing 50, 100, 250, or 400 mg/L nitrogen. The sugar and temperature data were combined, resulting in a single value for each strain at the evaluated nitrogen levels. Error bars indicate 95% confidence intervals for the means.

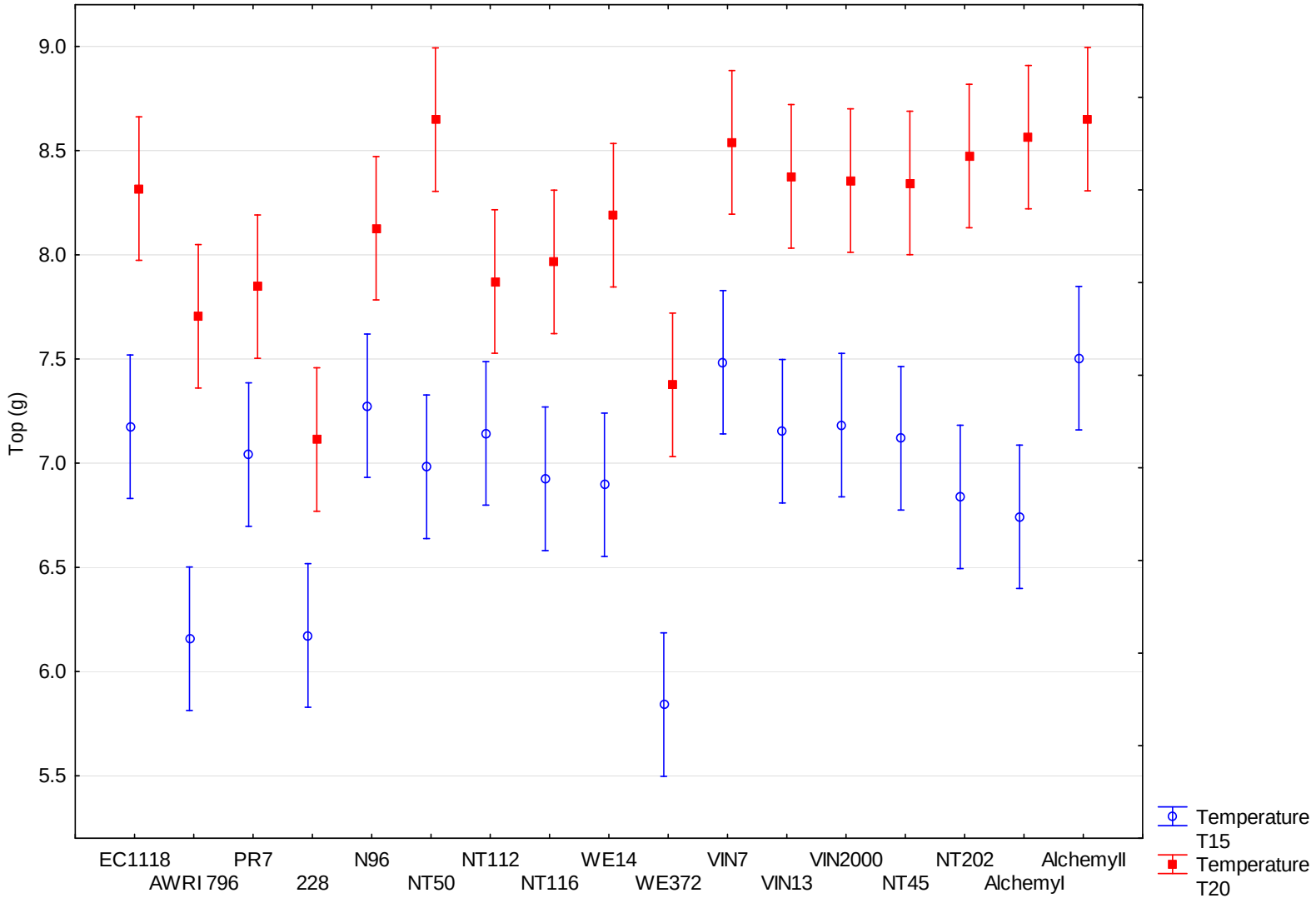

Figure 10: The total weight loss (top value) of 17 commercial cultures fermenting synthetic grape at 15 and 20°C. The sugar and nitrogen data were combined, resulting in a single value for the evaluated temperatures. Error bars indicate 95% confidence intervals for the means.

3.3.2 Influence of treatments on sugar consumption

3.3.2.1 Impact of treatments on sugar consumption

Fermentation temperature plays a pivotal role in sugar metabolism (Figure 11). The higher temperature (20°C) consistently resulted in a lower level of residual sugars at the end of fermentation compared to those at 15°C.

Nitrogen is essential for the production and maintenance of biomass, therefore nitrogen also affects the rate of sugar consumption (Bisson, 1991; 1999). As the nitrogen content increased, a decrease in residual sugars occurred, this agrees with findings reported in other studies (Ingledew & Kunkee, 1985; Mendes Ferreira *et al.*, 2004). Fermentation proceeded to dryness (< 4 g/L sugar), for the 200 g/L sugar treatments, containing higher nitrogen levels (250 and 400 mg/L) at both fermentation temperatures. When the initial sugar level was raised to 240 g/L, the fermentations containing 250 or 400 mg/L nitrogen were only able to reach dryness when fermented at 20°C. It is likely that the combination of high sugar and low temperature gave rise to a sluggish fermentation that possibly required more than 21 days to ferment to dryness.

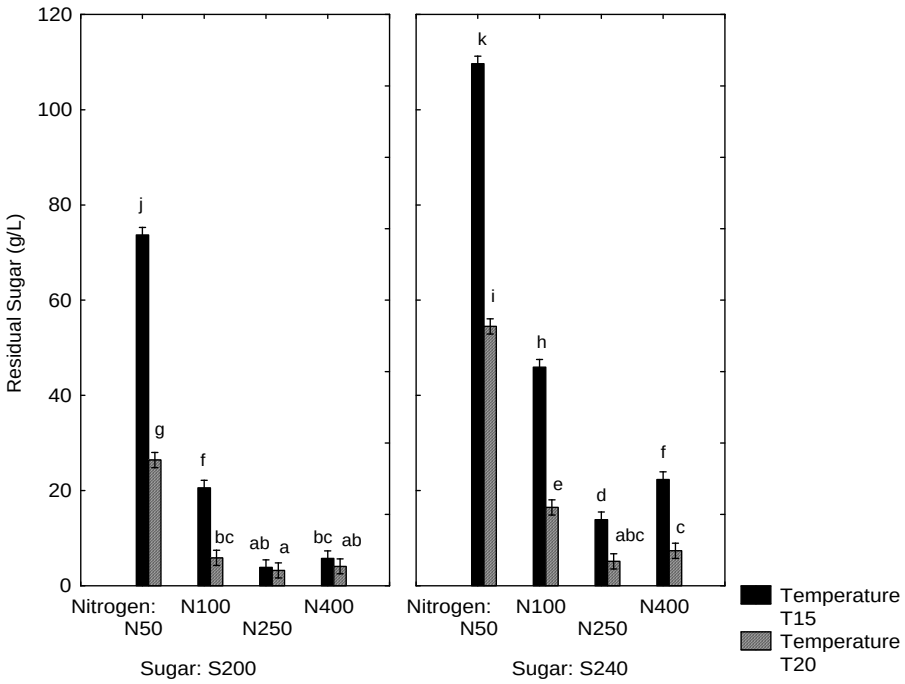

Figure 11: Influence of the nitrogen (50, 100, 250 or 400 mg/L), sugar (200 or 240 g/L), and temperature (15°C or 20°C) treatments on the total residual sugar levels. The data for all the strains within each specific treatment were combined. Error bars indicate 95% confidence intervals for the means. Letters indicate significant differences on a 5% ($p<0.05$) significance level.

3.3.2.2 Impact of treatments on the sugar consumption of individual yeast strains

The yeast strains were unable to ferment to dryness under the nitrogen deficient conditions of 50 mg/L nitrogen (Figure 12). However, the level of residual sugar reached by VIN7 was significantly lower than the other strains when nitrogen was limiting (50 and 100 mg/L) making it the most effective strain in terms of sugar consumption; this is also reflected in its high total weight loss (Figure 8).

The increase in nitrogen aided the fermentation kinetics (EC50, hill-slope and top value) and in so doing it also promoted sugar metabolism. The strains AWRI796, NT50, and NT116 had significantly higher levels of residual sugars when fermenting must containing 400 mg/L nitrogen compared to 250 mg/L. Most strains (228, AlchemyI, AlchemyII, EC1118, N96, NT112, NT202, NT45, PR7, VIN2000, VIN13, and WE14) reduced the sugar concentration to similar levels at the 250 and the 400 mg/L nitrogen levels.

The strains VIN7, EC1118, and NT45 display a similar degree of sugar consumption at 100 mg/L as other strains fermenting 400 and 250 mg/L nitrogen. At all nitrogen levels 228, AWRI796 and WE372 were among the least effective sugar consumers, and VIN7, NT45 and VIN2000 were among the most effective. It has been proposed that nitrogen excess induces rapid yeast growth and a consequential spike in ethanol; which cells are unable to respond to rapidly and consequently die (Chaney *et al.*, 2006). This may explain the relatively poor sugar consumption at higher nitrogen levels, but it does not explain the increase in fermentation lag phase and the decrease in the maximal fermentation rate, as the ethanol concentration should be too low at that juncture.

Fermentation at 20°C enhanced sugar consumption when compared to the 15°C fermentations (Figure 13). At both fermentation temperatures VIN7, EC1118, NT45 and VIN2000 were among the most efficient sugar consumers. The yeast strains 228, and WE372 were among the poorest sugar consumers, where their sugar consumption at 20°C was comparable to other strains fermenting at 15°C. At 15°C, VIN7, and N96 consumed sugars more readily than the other strains tested (Figure 13). This is confirmed by their slightly superior total weight loss (Figure 9).

The higher initial sugar content resulted in a higher residual sugar level at the end of fermentation (data not shown). In response to the initial sugar content, VIN7, EC1118, NT45 and VIN2000 were among the most efficient sugar consumers; whereas the strains 228 and WE372 were consistently among the poorest sugar consumers.

These results highlight the importance of the selection an appropriate yeast starter culture to conduct a fermentation of a specific grape must, as the stresses evaluated had dramatic consequences on the sugar consumption.

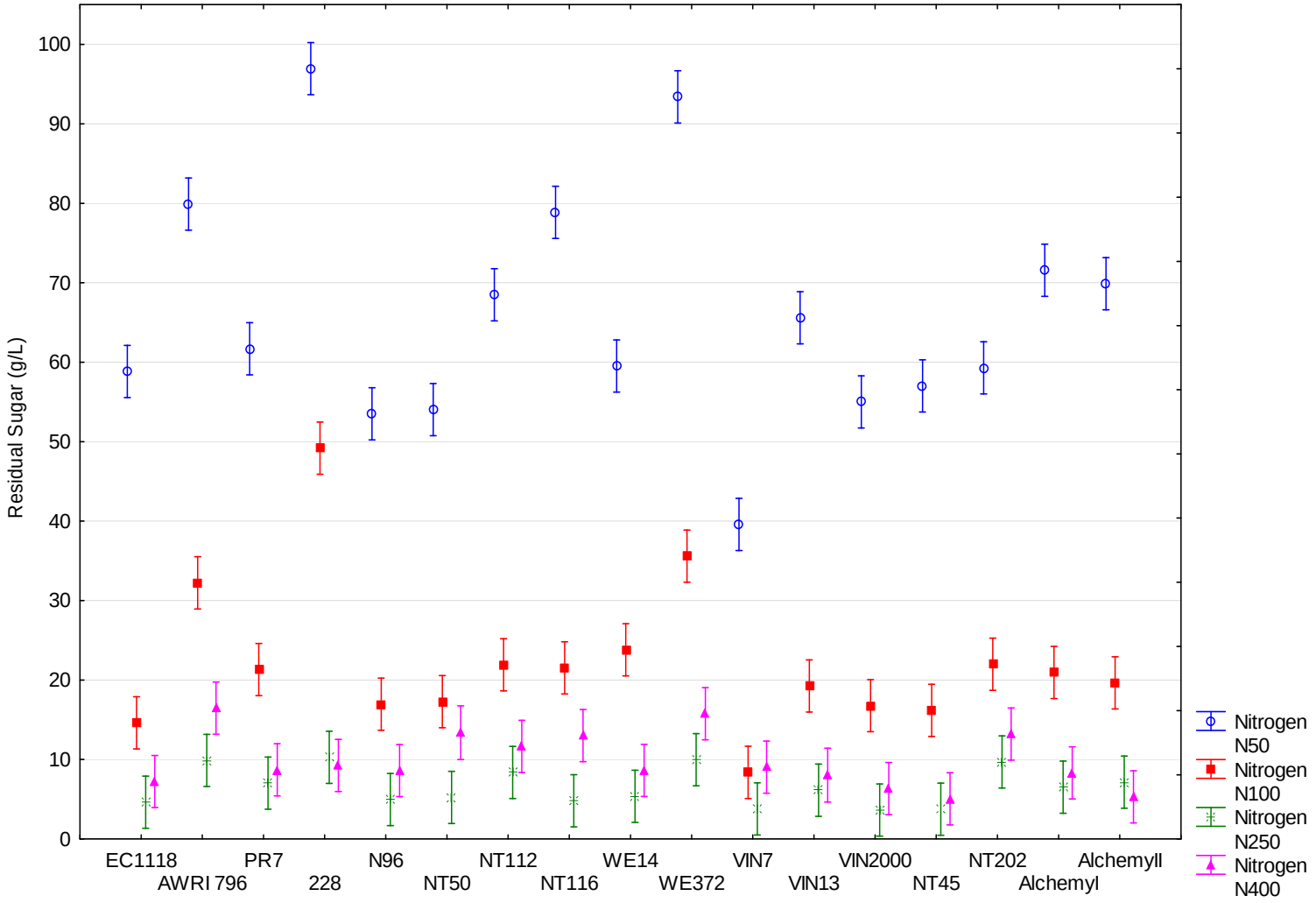

Figure 12: Influence of the different nitrogen levels (50, 100, 250 or 400 mg/L) on the total residual sugar levels of 17 commercial cultures. The temperature and sugar data were combined, resulting in a single value for each strain at the nitrogen levels evaluated. Error bars indicate 95% confidence intervals for the means. Letters indicate significant differences on a 5% ($p<0.05$) significance level.

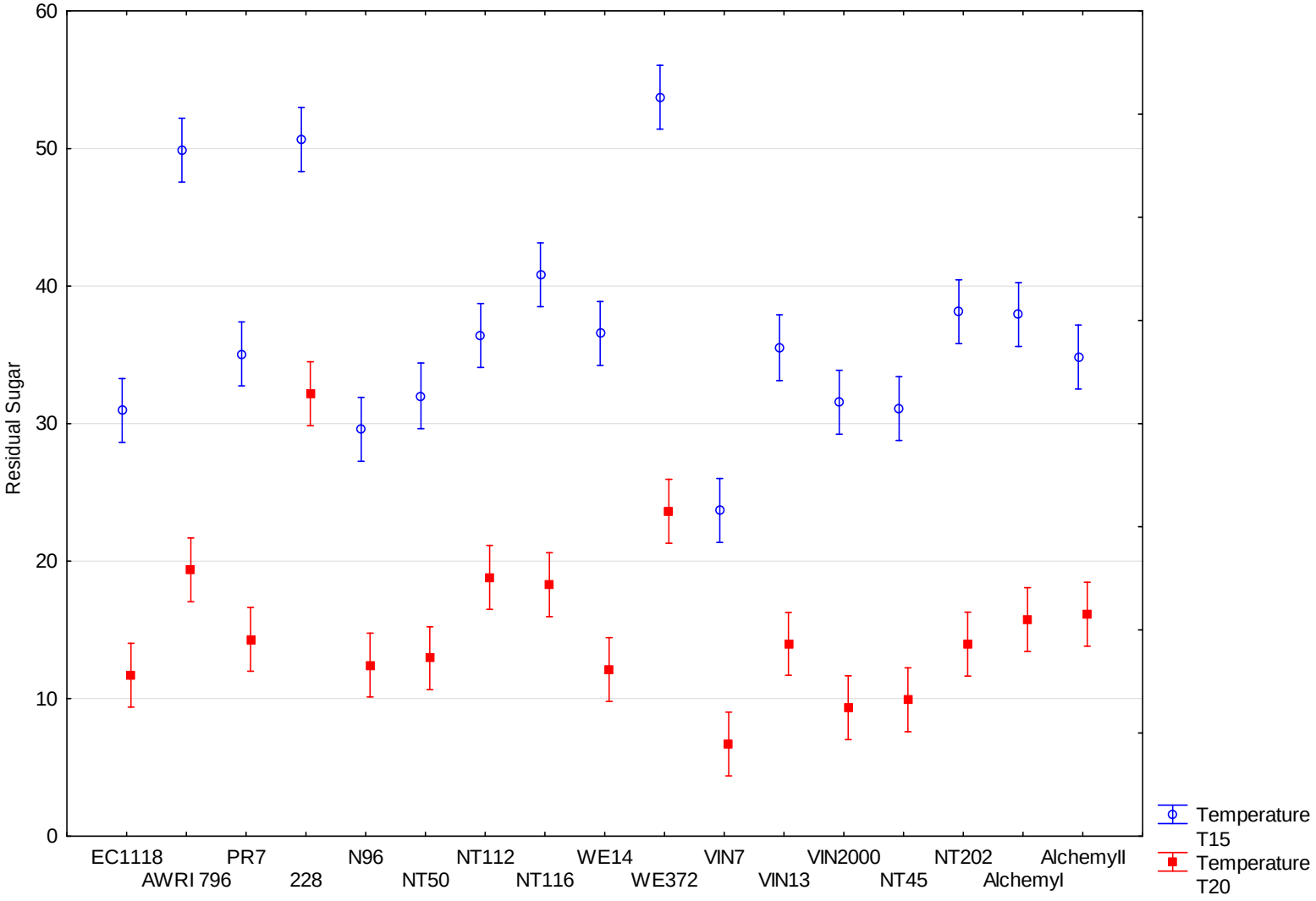

Figure 13: Influence of the fermentation temperature (15°C or 20°C) on the total residual sugar levels of 17 commercial cultures. The temperature and sugar data were pooled, providing a single value for each strain the evaluated temperatures. Error bars indicate 95% confidence intervals for the means. Letters indicate significant differences on a 5% ($p<0.05$) significance level.

Based on the results, VIN7 is the strain which most effectively metabolized sugars despite the prevailing fermentation stresses. However, its long lag phase may create an opportunity for unwanted *non-Saccharomyces* strains to dominate the fermentation for a longer period, and even more so at low temperatures where *non-Saccharomyces* strains would be somewhat protected from ethanol toxicity (Gao & Fleet, 1988; Heard & Fleet, 1988).

3.3.3 Influence of treatments on yeast dry weight

3.3.3.1 Impact of treatments on yeast dry weight production

At both temperatures, low nitrogen content has a greater impact on biomass production than the initial sugar content (Figure 13); as illustrated by a similar dry weight at 100 and 50 mg/L nitrogen irrespective of the initial sugar content.

As the nitrogen content increases, the higher initial sugar levels negatively impacts biomass production. At 15°C, the biomass production has a similar pattern for both sugar concentrations, showing an increase in biomass up to 250 mg/L nitrogen, which was followed by a decrease in biomass when nitrogen was raised to 400 mg/L. A similar pattern for biomass production is seen at 20°C when the initial sugar is 240 g/L; however for the 200 g/L sugar fermentations the biomass continues to increase at 400 mg/L nitrogen.

Biomass and intracellular activity are crucial in determining the fermentation rate; they are both affected by the nitrogen status of the must (Varela *et al.*, 2004). The low nitrogen fermentation treatments cause a low amount of biomass to be produced (Figure 13), resulting in a long lag phase (Figure 1), a low maximum fermentation rate (Figure 4), and ultimately high residual sugar levels (Figure 10).

3.3.3.2 Impact of treatments on the dry weight production of individual yeast strains

At 50 mg/L nitrogen, most strains produced a similar amount of biomass (Figure 15). For both 50 and 100 mg/L, WE372 and AWRI796 produce significantly more biomass than WE14, VIN7 and NT50. At 250 mg/L NT116, 228, WE372 were among the high biomass producers, and at 400 mg/L VIN13, VIN2000 and 228 were among the high biomass producers. The biomass increased with the increasing nitrogen levels (Beltran *et al.*, 2005) until the 400 mg/L level was reached. The dry weight for the 400 mg/L treatments were statistically similar (AlchemyII, N96, NT112, NT45, PR7, VIN2000, and VIN13), or significantly greater, (228, AlchemyI and VIN7, and significantly less, for AWRI796, EC1118, NT116, NT202, NT50, WE14, WE372) than that of the 250 mg/L treatments. Even though VIN7

produced more biomass in response to 400 mg/L compared to 250 mg/L nitrogen, its maximum biomass was significantly less than that of the high biomass producing strains.

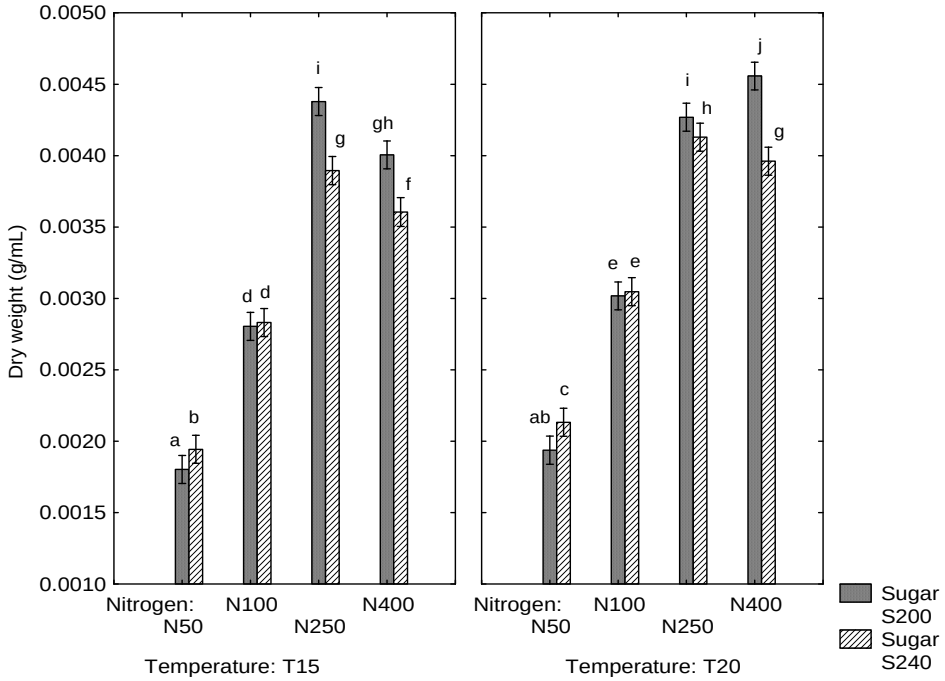

Figure 14: Influence of the nitrogen (50, 100, 250 or 400 mg/L), sugar (200 or 240 g/L), and temperature (15°C or 20°C) treatments on (dry weight (yeast biomass). The data for the yeast strain within a specific treatment were combined. Error bars indicate 95% confidence intervals for the means. Letters indicate significant differences on a 5% ($p<0.05$) significance level.

In response to fermentation temperature, AWRI796, 228, NT116, WE372, NT202 and AlchemyI, AlchemyII, VIN13 and VIN7 produced significantly more biomass at 20°C compared to 15°C, whereas the other strains had no significant change in their biomass production (Figure 16). Regardless of the fermentation temperature, WE372 and 228 were the highest biomass producing strains, while VIN7, NT45, and WE14 produced the lowest biomass.

The relationship between the initial sugar levels and the yeasts strains evaluated was not significant (data not shown).

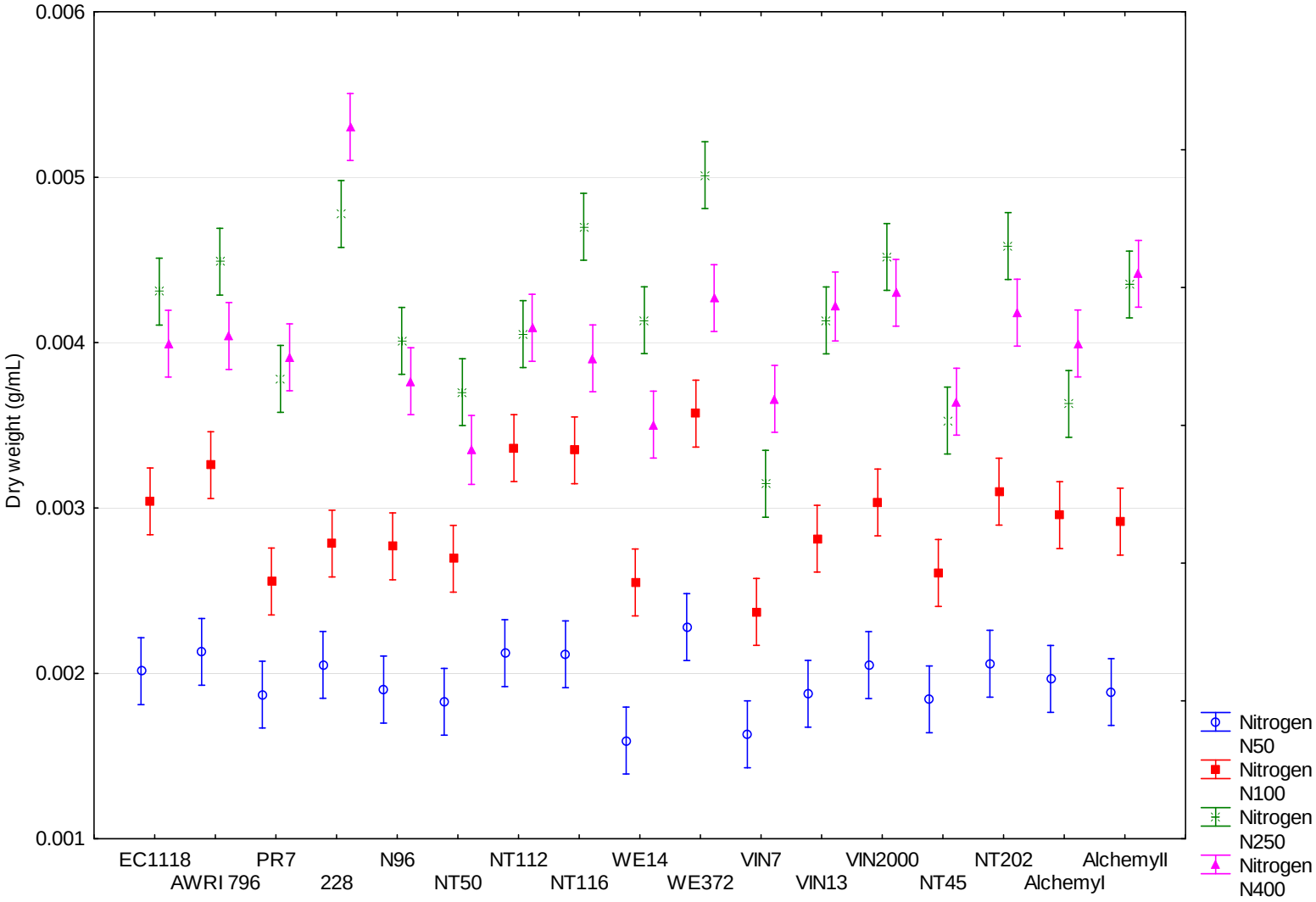

Figure 15: Influence of the nitrogen (50, 100, 250 or 400 mg/L) on the dry weight (biomass) of 17 commercial yeast cultures. The sugar and temperature data were pooled. Error bars indicate 95% confidence intervals for the means. Letters indicate significant differences on a 5% ($p<0.05$) significance level.

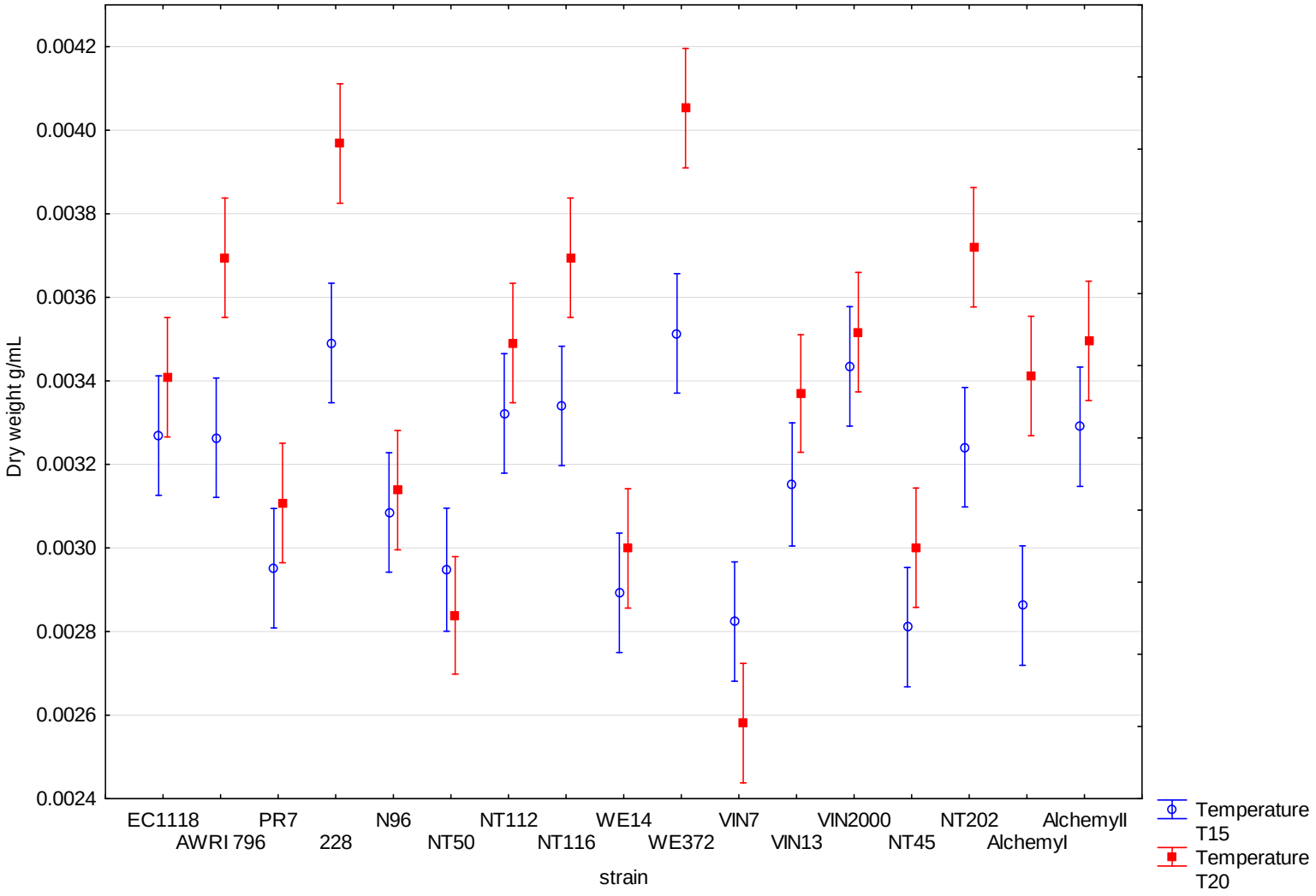

Figure 16: Influence of fermentation temperature (15°C or 20°C) on the dry weight (biomass) of 17 commercial yeast cultures. The nitrogen and sugar data was pooled. Error bars indicate 95% confidence intervals for the means. Letters indicate significant differences on a 5% ($p<0.05$) significance level.

3.4 Conclusion

This study confirms that the fermentation performances of commercial yeast cultures are significantly and differently affected by temperature (Fleet & Heard, 1993), initial nitrogen (Jiranek *et al.*, 1991; Manginot *et al.*, 1998; Taillandier *et al.*, 2007) and sugar levels (Erasmus *et al.*, 2004), when fermenting a defined synthetic grape must.

From the results obtained in this study it is clear that, under the conditions tested, the relationship between initial nitrogen level and fermentation performance is not purely linear, but is influenced greatly by parameters such as initial sugar, fermentation temperature, and yeast strain. As previously reported, when the nitrogen is limiting, the fermentation rate, sugar consumption and biomass production are all negatively affected (Henschke & Jiranek, 1993; Blateyron & Sablayrolles, 2001). The 250 mg/L nitrogen treatment showed an improved overall performance in terms of fermentation onset, maximal fermentation rate, total weight loss, sugar consumption, and biomass production compared to the fermentations supplemented with 100 or 400 mg/L nitrogen. This highlights the importance of measuring the YAN content of the grape must prior to the addition of DAP, or complex nutrients, as its addition in excess may hinder fermentation efficiency and completion (Taillandier *et al.*, 2007).

When experiencing hyperosmotic stress, yeast growth stops and only resumes once the accumulation of glycerol has resulted in sufficient water uptake to restore cell size to a critical level (Hohmann, 1997; Mager & Siderius, 2002). Consequently, the higher the initial sugar concentration the longer the lag phase (EC50) before fermentation starts, due to the cessation of growth while the cell adapts to the prevailing osmotic conditions. This is in agreement with our results , as the higher initial sugar content generally resulted in an increase in the EC50 value (fermentation onset), a decrease in the initial fermentation rate (hill-slope), an increase in the top value (total weight lost), and an increase in the residual sugar levels.

Temperature plays an influential role in yeast growth and metabolism (Fleet & Heard, 1993). *S. cerevisiae* has an optimum growth temperature of 25°C; therefore fermentations at lower temperature would be slower and also longer. It is not surprising then that an increase in temperature (20°C) significantly influenced the fermentation rates and fermentation completeness, at all nitrogen levels. It is interesting that at low nitrogen levels (50 mg/L and 100 mg/L), an increase in temperature (20°C) is able to overcome the nitrogen deficiency to a certain extent and even more so when the initial sugar concentration (200 g/L) is low. There seemed to be a correlation between the fermentation rate, fermentation completeness and biomass formation at each of the sets of conditions tested for each strain. Some strains (e.g. 228, WE372 and AWRI796) appear to be more sensitive to nitrogen limitation, especially under lower temperatures or high sugar conditions than others (VIN7), pointing to the latter strain being more nitrogen efficient and stress resistant.

When the fermentation conditions were “more stressful” the selection of the appropriate commercial strain made a significant contribution to fermentation onset, rate and sugar consumption. Thus the use of a strain adept to fermenting a characterised grape must to dryness may reduce the risk of problematic fermentations.

3.5 Acknowledgements

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Chapter 4

Research results

Impact of environmental stress on aroma production during wine fermentation

Chapter 4

Impact of environmental stress on aroma production during wine fermentation

Abstract

Wine character and style are mostly defined by the flavour of individual wines and are of crucial importance to the wine consumers (Swiegers et al. 2005). While the perception of wine flavour, which includes aroma, taste and mouth-feel, is somewhat subjective, most wines are made to reflect or to correspond to a specific flavour profile. Winemakers have a number of important tools to try to achieve specific wine styles, one of which is the choice of a specific yeast strains. Yeast strains contribute to wine flavour by the production of many volatile metabolites including esters, higher alcohols, carbonyl compounds, volatile fatty acids, and sulphur containing compounds and different yeast strains are known to produce significantly different flavour profiles. However, the interaction of yeast with specific grape juices and their responses to changing environmental conditions remains largely unexplored. During fermentation yeast strains continually experience stresses that may impact yeast viability, growth, and fermentation performance. These consequences of stress have been well-studied under laboratory conditions, but current literature provides few insights on the impact of environmental stresses during wine fermentation on wine quality, and in particular on aroma production. This is the first study to investigate the impact of two common fermentation associated stresses, hyperosmotic and temperature stress, on fermentation performance and on the production of aroma compounds in synthetic grape must. The results demonstrate that the stress conditions resulted in a number of significant changes to the aroma profile. Furthermore, the changes observed differed for each strain and each stress treatment. This implies that environmental stress causes numerous changes to the aroma profiles in a manner which is not conserved among the five wine strains tested.

Key words: Wine aroma, hyperosmotic stress, temperature stress, synthetic grape must fermentation

4.1 Introduction

Wine yeast strains mediate the conversion of sugar to ethanol and CO_2. This conversion proceeds despite continuous exposure to various forms of stress (Bisson 1999). The stresses may include temperature changes, hyperosmotic stress, ethanol stress, vitamin, mineral, nitrogen, and oxygen deficiencies, and a low pH, among others (Alexandre and Charpentier 1998, Attfield 1997, Bauer and Pretorius 2000, Bisson 1999, Malherbe et al. 2007). There is evidence that such stresses are also often associated with problematic (stuck and sluggish) fermentations (Bisson 1999, Gibson et al. 2007, Malherbe et al. 2007).

The high initial sugar content of grape must results in hyperosmotic stress, which the cell counteracts through the accumulation of intracellular glycerol (Hohmann 1997, Mager and Siderius 2002, Tamás and Hohmann 2003). Low temperature decreases and high temperature increases membrane permeability, which disrupts transport systems into and out of the cell (Beales 2004, Bisson 1999).

Yeast strains differ in their abilities to sense and respond to stress. Most of these stresses have been extensively studied with regard to their impact on yeast growth and fermentation performance(Carrasco et al. 2001, Ivorra et al. 1999, Zuzuarregui and del Olmo 2004), yet literature provides few insights into the impact of fermentation-related stress applied during alcoholic fermentation on wine flavour.

Wine flavour is defined as the accumulative effect of smell, taste and mouth-feel (Francis and Newton 2005). It is the composite product of a combination of metabolites derived from the grapes, from microorganisms during fermentation, as well as from chemical processes during production and maturation (Rapp and Mandery 1986, Rapp and Versini 1996). The perceived flavour is the result of complex interactions between all the volatile and non-volatile compounds present in wine. Compounds present at levels above their detection thresholds, may mask or suppress the detection of others. However, when present below the detection threshold levels they may act synergistically with other compounds, and in so doing enhance wine complexity (Francis and Newton 2005). The volatile aromas produced *de novo* by wine yeast during fermentation include higher alcohols, esters and volatile fatty acids, among others (Rapp and Versini 1996). Higher alcohols are formed by the decarboxylation and reduction of α-keto acids, originating either from glycolysis or the Ehrlich pathway (Hazelwood et al. 2008, Rapp and Mandery 1986, Rapp and Versini 1996, Swiegers et al. 2005). Volatile fatty acids are produced as a result of acetyl-CoA decarboxylation and condensation reactions (Lambrechts and Pretorius 2000). Acetate esters are formed by enzyme catalysed condensation reactions between acetyl-CoA and higher alcohols or ethanol (Bell and Henschke 2005, Bisson and Karpel 2010, Lambrechts and Pretorius 2000), while ethyl esters are formed by the condensation

reaction between ethanol and either a fusel acyl-CoA or a fatty acyl-CoA (Bisson and Karpel 2010).

The metabolic pathways leading to the formation of these volatile aroma compounds are reasonably well mapped and established. However, the regulation of the metabolic flux through these networks is not well understood. Studies have shown that obvious factors such as the availability of precursors, fermentation conditions (Bisson and Karpel 2010, Henschke and Jiranek 1993, Lambrechts and Pretorius 2000, Rapp and Versini 1996, Saerens et al. 2008, Swiegers et al. 2005, Vilanova et al. 2007) and the genetic make-up of individual strains (Rossouw et al. 2008, Soles et al. 1982) play an important role in the modulation of aroma production. However, little data is available regarding the impact of environmental factors and in particular of environmental stress on the production of such metabolites.

Here we investigate the impact of two of the most common stresses experienced during alcoholic fermentation, temperature shifts and hyperosmotic stress, on the aroma production capacity of several wine yeast strains. To our knowledge, this is the first study investigating the impact of hyperosmotic and temperature stresses on fermentation performance and the production of fermentation derived volatile aroma compounds in synthetic grape must. This preliminary study provides information on how the exposure to stress impacts wine aroma and whether the observed changes are conserved among different commercial *Saccharomyces* yeast strains.

4.2 Materials and Methods

4.2.1 Yeast strains and growth conditions

The synthetic grape must was as described previously by Henschke and Jiranek (1993), and contained 100 g/L glucose and 100 g/L fructose. The vitamins, minerals, anaerobic factors (Henschke and Jiranek 1993) as well as the nitrogen sources, amino acids and ammonium chloride, (Bely et al. 1990) were filter sterilized and added to the autoclaved carbon, acid and salt sources (Henschke and Jiranek 1993). The nitrogen sources (Bely et al. 1990), were proportionally decreased to reach desired level of 250 mg/L nitrogen.

The yeast strains used in this study are listed in Table 1. Strains were stored at -80°C, and were streaked out onto YPD plates, which were subsequently incubated at 30°C. A single colony was used to inoculate 5 mL synthetic grape must; which was aerobically incubated at 30°C overnight. These cultures were used to inoculate the fermentations at an initial OD_{600} of 0.1, corresponding to approximately 1 x 10^6 cells/mL.

Table 1. List of *Saccharomyces* wine yeast strains used in this study

Commercial Name	Commercial Source	Strain
Lalvin EC1118	Lallemand Inc. Montréal, Canada	*Saccharomyces cerevisiae bayanus*
IWBT 285[1] (Cross Evolution™)		*S. cerevisiae* hybrid
NT 50	Anchor Yeast, Cape Town, South Africa	*S. cerevisiae* hybrid
VIN 7		*S. kudriavzevii* and *S. cerevisiae* hybrid[2]
VIN 13		*S. cerevisiae* hybrid

[1] Cross Evolution will be referred to by its IWBT name 285 from this point forward.

[2]Bradbury et al. (2006)

4.2.2 Fermentation conditions

The fermentations were performed in 100 mL glass bottles, containing 70 mL of synthetic grape must. The bottles were sealed with rubber stoppers and a CO_2 outlet. Fermentations were conducted in triplicate at 20°C, unless stated otherwise.

To induce hyperosmotic stress, sorbitol was added to a final concentration of 0.3 M (S1) or 0.5 M (S2) upon inoculation. To evaluate the impact of temperature stress a set of fermentations was moved to 8°C (T8) or 37°C (T37) for 16 hours on day two (D2) or day eight (D8) of the fermentation and subsequently returned to 20°C. Fermentations which were not subjected to any additional stress, served as the control data set.

The fermentations were weighed to monitor fermentation progress for 21 days, which was expressed as CO_2 weight loss.

4.2.3 Chemical Analysis

4.2.3.1 Residual glucose and fructose, and glycerol

Synthetic wine samples were scanned using the Winescan FT120 instrument (FOSS Analytical A/S software version 2.2.1) equipped with a purpose-built Michelson interferometer (FOSS Analytical A/S, Hillerød, Denmark) to generate Fourier transform mid infrared (FT-MIR) spectra. Quantified chemical data for residual glucose, fructose, glycerol, and volatile acid levels were predicted from infrared spectra by commercial calibrations or in-house adjustments using the Winescan FT120 2001 version 2.2.1 software.

4.2.3.2 Aroma compounds

For the extraction of the volatile aroma compounds from the samples, the protocol described by Louw et al. (2010) was followed with a few minor modifications. The internal standard 4-methyl-2-pentanol (100 µL) and 1 mL diethyl ether were added to a 5 mL sample of wine. The wine/ether mixture was subjected to 5 minutes of sonication. The wine/ether mixture was centrifuged at 4000 g for 3 minutes, Na_2SO_4 was added to the mixture and the centrifugation step was repeated. The ether layer was removed and dried on additional Na_2SO_4.

The volatile higher alcohols, esters, fatty acids and carbonyl compounds (Table 2) were quantified in duplicate using a Hewlett Packard 6890 Plus gas chromatograph (Little Falls, USA) with a split/splitless injector and a flame ionization detector. The protocol described by Malherbe (2011) was followed with a few modifications. The separation of compounds was achieved using a DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions 20 m length x 0.1 mm inside diameter x 0.2 µm film thickness. The initial oven temperature was maintained at 33°C for 8 minutes after which the temperature was increased by 12°C /minute until 240 was reached. This temperature was held for 5 minutes. A 1 µl sample was injected when the oven temperature reached 250°C. The split ratio 10:1 and the split flow rate was 36.7 cm/s. The column flow rate was 6.6 mL/min using hydrogen as the carrier gas. The detector temperature was 230°C. After each sample, oven temperature was maintained at 250°C, with a column flow rate of 30 mL/min to clean the column of all contaminants with high boiling points. After 22 injections the column was cleaned both thermally and chemically by a hexane injection at an oven temperature of 250 (Louw et al. 2010).

4.2.4 Statistical Analysis

Multivariate statistical analysis was performed using The Unscrambler software (version 9.2, CAMO ASA, Norway). Principal component analysis (PCA) was used to illustrate and evaluate the distribution of stress treatments relative to each other based on their individual volatile aroma profiles.

Additionally, fermentation data for stressed treatments were compared to that of the 'unstressed' control by creating pair-wise comparisons for each strain. These graphs were visualised using Cytoscape (version 2.8.2, http://www.cytoscape.org). These graphs only include information that differed significantly from the control; thus any omission was deemed as statistically similar to its control. In all cases a significance level of 5% ($p<0.05$) was used.

A blue node (ellipse) or edge (line) indicates a reduction compared to the control, whereas a red node or edge denotes an increase. The colour intensity of the node or edge is an indication of the magnitude of the fold change.

Table 2. The 39 volatile aroma compounds identified and quantified using GC-FID

Esters	Higher Alcohols	Volatile Fatty Acids	Carbonyl compounds
Ethyl esters			
Ethyl decanoate	Hexanol	Acetic acid	Acetoin
Ethyl hexanoate	Butanol	Propionic acid	
Ethyl butyrate	Methanol	Isobutyric acid	
Ethyl octanoate	2-Phenylethanol	Butyric acid	
Ethyl lactate	Propanol	Isovaleric acid	
Ethyl propionate	Isobutanol	Valeric acid	
Ethyl-2-methyl propanoate	Isoamyl alcohol	Hexanoic acid	
Ethyl-2-methylbutyrate	Pentanol	Octanoic acid	
Ethyl isovalerate	4-Methyl-1-pentanol	Decanoic acid	
Ethyl-3-hydroxybutanoate	3-Methyl-1-pentanol		
Acetate esters	1-Ethoxy-1-propanol		
Ethyl acetate	1-Octen-3-ol		
Ethyl phenylacetate			
Hexyl acetate			
2-Phenylethyl acetate			
Diethyl succinate			
2-Methyl-propyl acetate			
Isoamylacetate			

4.3 Results

4.3.1 Fermentation performance

4.3.1.1 Fermentation rate

The stresses evaluated were relatively mild so as to mimic those found in industrial fermentations. Generally, the fermentation rates (expressed as weight loss by CO_2 evolution) of the yeast strains differed from each other, as did their response to both hyperosmotic and temperature stresses (Fig. 1 and 2). EC1118 (S1 and S2) and 285 (S2) displayed an increased fermentation speed when exposed to the hyperosmotic stress treatments when compared to their respective controls (Fig. 2 A and B). Similarly in response to temperature stress, VIN13 (8°C and 37°C) and EC1118 (37°C) displayed enhanced fermentation rates (Fig. 3 G, H and C).

The severity (Gasch et al. 2000, Gasch and Werner-Washburne 2002) of the stress application also had a significant impact on the fermentation rate. This is illustrated by the similar fermentation rates of the S1 (0.3 M sorbitol) treatments and the control fermentations, and differences in the fermentation rates that only emerged when the level of hyperosmotic stress was increased to 0.5 M sorbitol (Fig. 1).

4.3.1.2 Residual glucose and fructose, and glycerol

The synthetic grape must was not fermented to dryness within the 21 days of the experiment and stress treatments generally did not lead to significant changes with regard to residual sugar levels. The few cases where the treatments differed significantly from the controls are illustrated in Fig. 2. Glucose and fructose consumption was significantly decreased in the case of VIN7_D2.T37 when compared to the VIN7 control. Exposure to 8°C on day 8 significantly enhanced the sugar (both glucose and fructose) consumption ability of VIN13 compared to the VIN13 control treatment (Fig. 3); which corresponds with its improved fermentation rate in Fig. 2h.

In response to hyperosmotic stress, all strains produced more glycerol in comparison to their controls (Fig. 4), but no increase in acetic acid was observed by measurement with GC-FID. This was confirmed by analysis with FT-MIR, which found that the volatile acidity levels (predominantly acetic acid) ranged from 0.5 to 0.6 g/L for the control fermentations, 1.4 to 1.6 g/L for S1 and 2 to 2.2 g/L for S2 treatments.

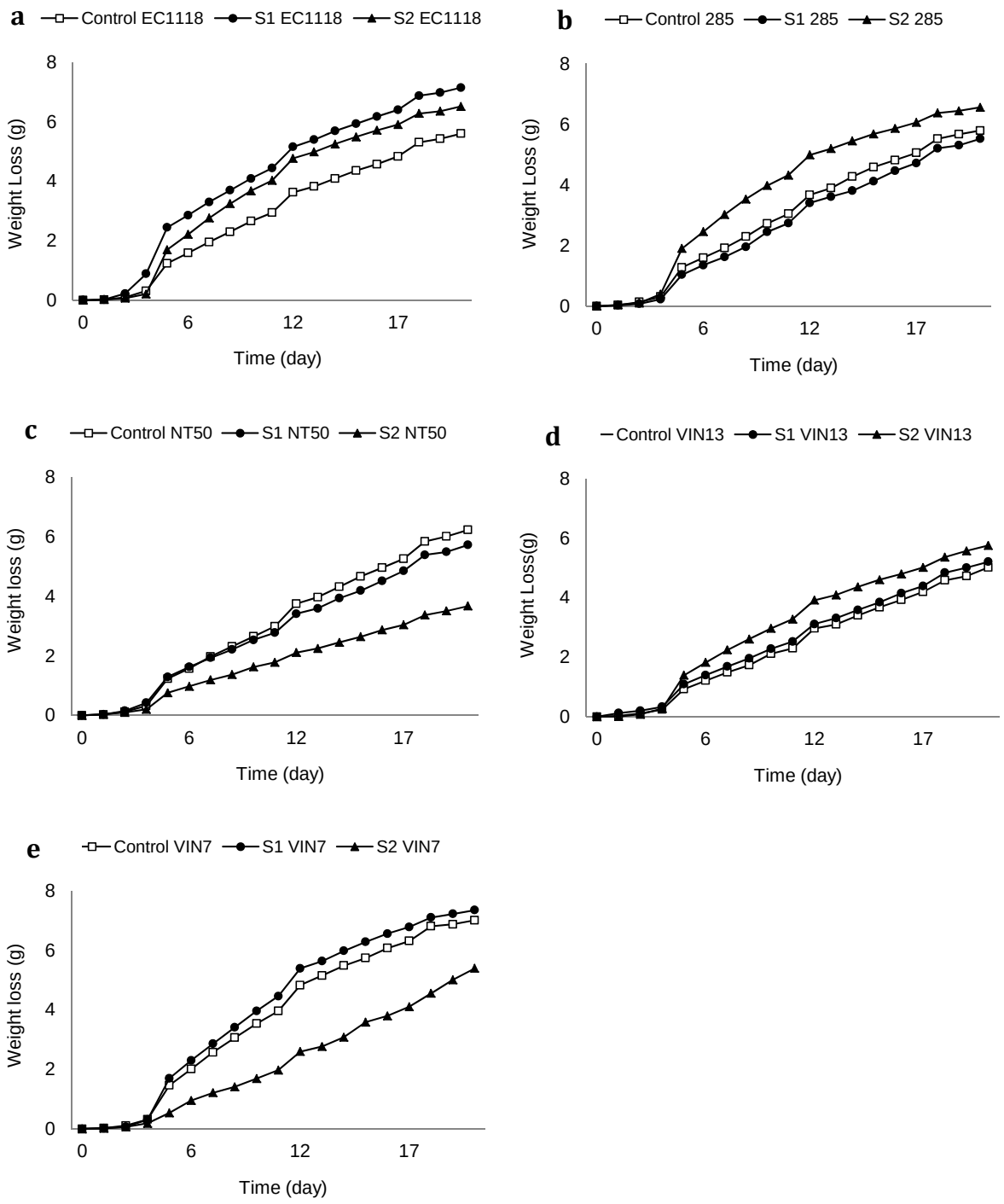
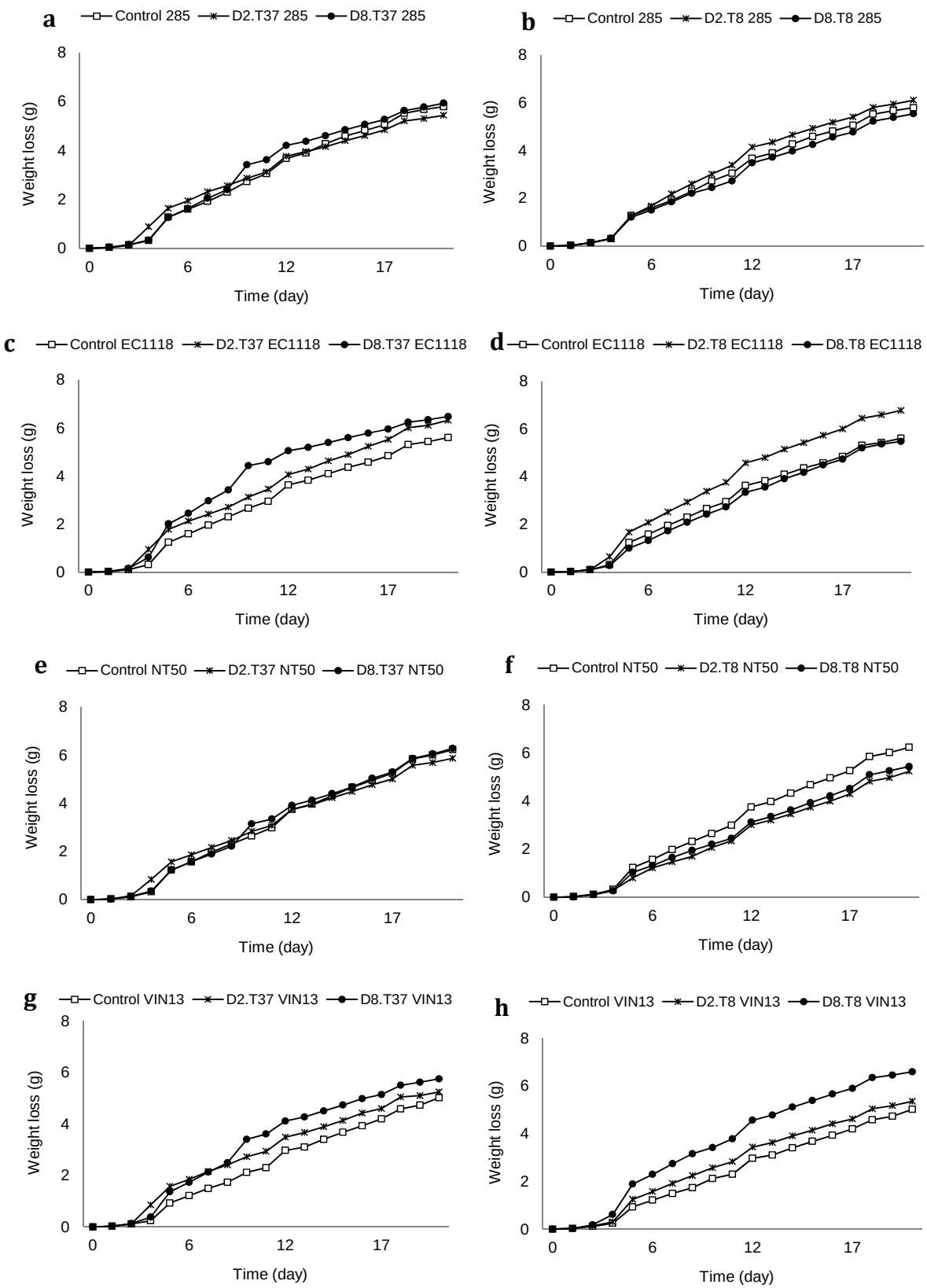


Figure 1 Fermentation rates of strains (a) EC1118, (b) 285, (c) NT50, (d) VIN13 and (e) VIN7 in response to hyperosmotic stress treatments, S1 and S2 compared to a control. Results are the average of triplicate fermentations (error bars are not shown for the clarity of the graphs)



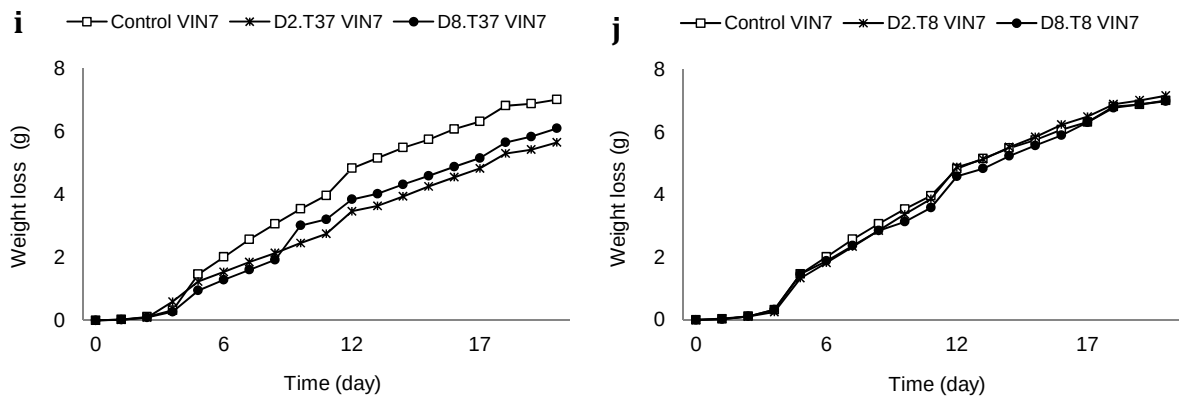


Figure 2 Fermentation rates of temperature stressed fermentations: (a) 285 control, D2.T37, D8.T37; (b) 285 control, D2.T8, D8.T8; (c) EC1118 control, D2.T37, D8.T37; (d) EC1118, D2.T8, D8.T8; (e) NT50 control, D2.T37, D8.T37; (f) NT50 control, D2.T8, D8.T8 (g) VIN13 control, D2.T37, D8.T37; (h) VIN13 control; (i) VIN7 control, D2.T37, D8.T37; (j) VIN7, D2.T8, D8.T8. Results are the average of triplicate fermentations; (error bars are not shown for the clarity of the graphs)

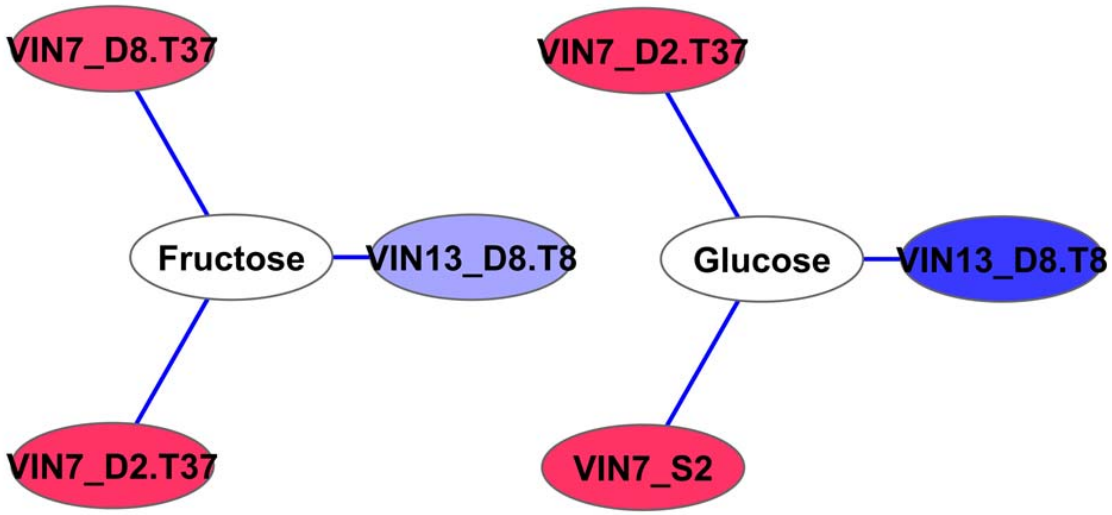


Figure 3 Residual glucose and fructose for each strain was compared its control. The graph depicts the strains and treatments which differed significantly ($p<0.05$) from their controls, as viewed by Cytoscape. A blue node (ellipse) indicates a reduction compared to the control and a red node an increase the colour intensity denotes the magnitude of the fold change observed

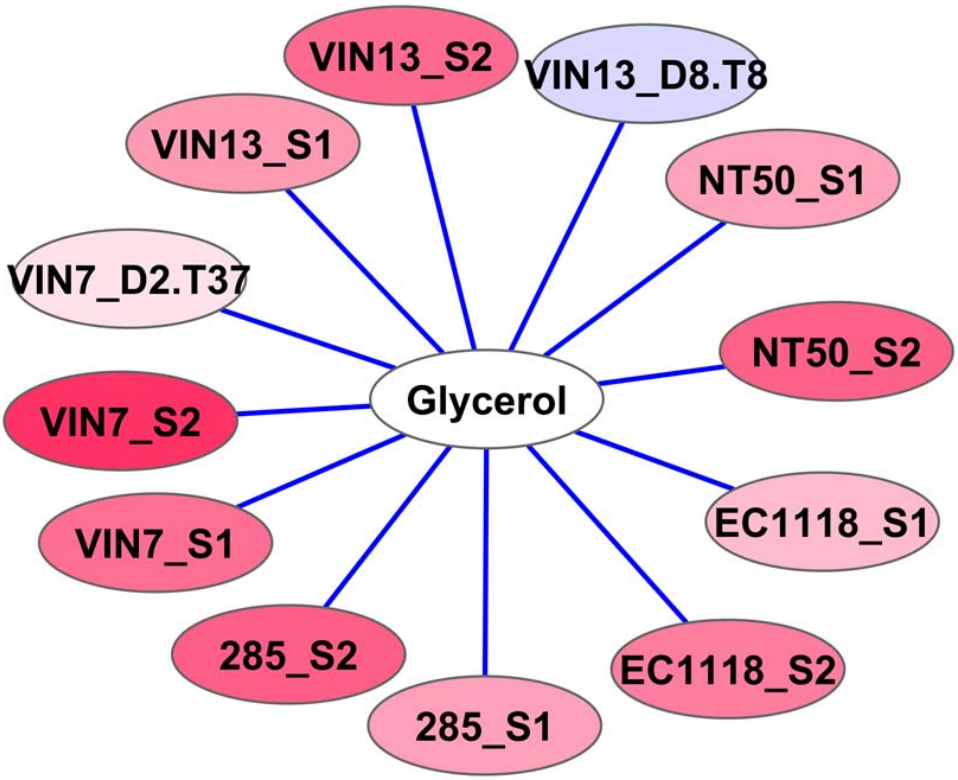


Figure 4 Glycerol levels for stress treatments significantly ($p<0.05$) different from its unstressed control. A blue node (ellipse) indicates a reduction compared to the control and a red node an increase the colour intensity denotes the magnitude of the fold change observed

4.3.2 Aroma compounds

Principal component analysis (PCA) was performed on the aroma data set in its entirety, using *The Unscrambler software* (version 9.2, CAMO ASA, Norway). PCA analysis was used to evaluate whether the changes could be attributed to a specific stress treatment, and whether the observed changes were conserved among the strains (Fig. 5). Individual compounds separated and dominated the multivariate space, and strongly influenced the model when comparing the aroma profiles of each strain within a treatment, as well as when comparing the aroma profile for one strain across all treatments (data not shown). In general, these influential compounds could be tentatively associated with individual strains: VIN7 associated with isoamyl alcohol, and propanol; 285 with isobutanol; EC1118 with decanoic acid and ethyl lactate; and NT50 with 1-octenol. The multivariate data analysis did therefore not allow for the comparison between individual stress treatments and corresponding controls in this study, as the model was dominated by individual aroma compounds.

Control	S1	S2	D2.T8	D2.T37	D8.T8	D8.T37

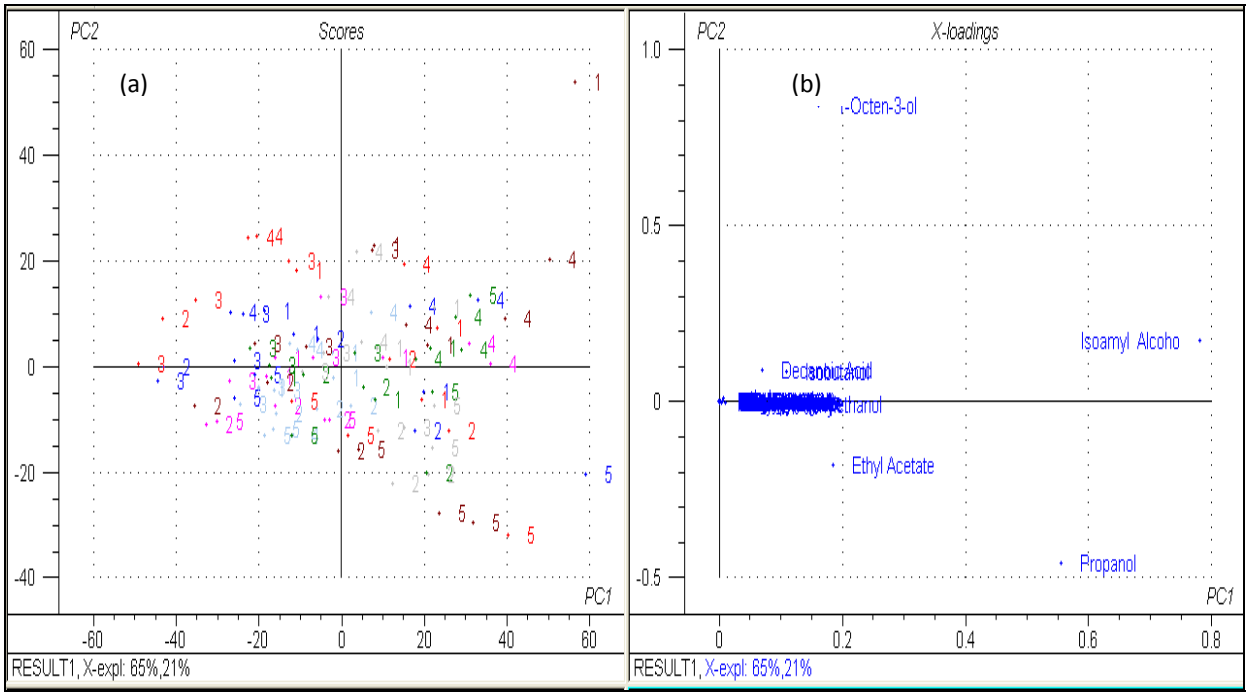


Figure 5 (a) PCA scores plot shows poor differentiation between colour coded treatments and numbered strains (285 = 1; EC1118 = 2; NT50 = 3; VIN7 = 4; and VIN13 = 5) using the GC – FID data. (b) Corresponding loadings for the PCA indicates which variables contribute significantly to the differentiation between treatments and numbered strains

Cytoscape was used to visualise pair-wise comparisons between the stress and control treatments. The data showed that the influence of exposure to low temperature on the volatile aroma profile is time and strain dependant (Fig. 6), generally showing an increase in compound levels on day two and a decrease on day eight. The only conserved change was the decrease in the levels of octanoic acid produced by EC1118, irrespective of when the stress was applied. Exposure to low temperature on day 2, increased the volatile fatty acid production by VIN13 (isovaleric, isobutyric, butyric and propionic acid), and VIN7 (isobutyric acid).

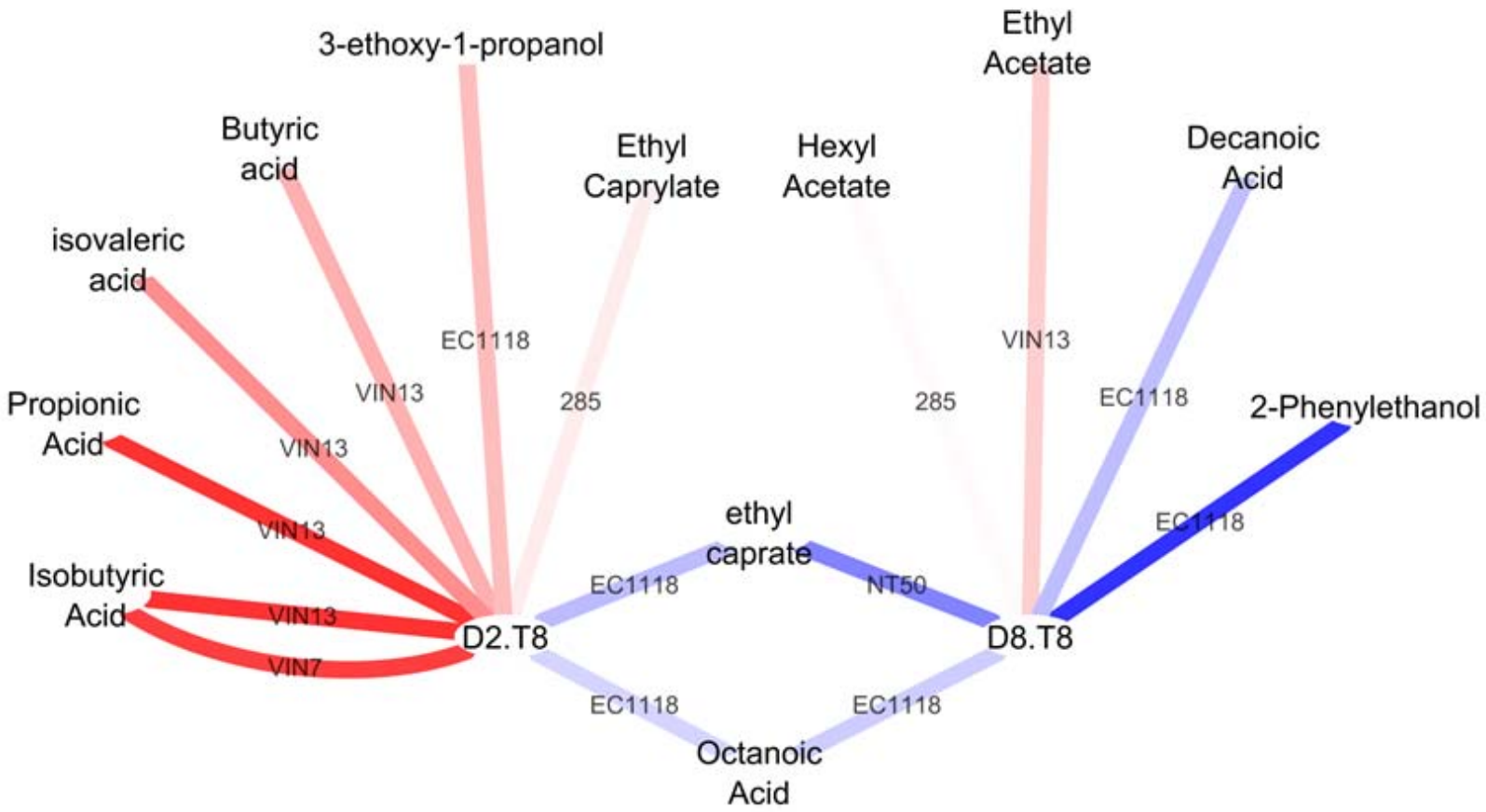


Figure 6 The significant changes ($p<0.05$) in the levels of the volatile aroma compounds due to low temperature stress, day eight at 8°C or day two at 8°C, when comparing the low temperature stressed fermentations to their controls. A blue edge (line) indicates a reduction compared to the control and a red edge (line) an increase the colour intensity denotes the magnitude of the fold change observed

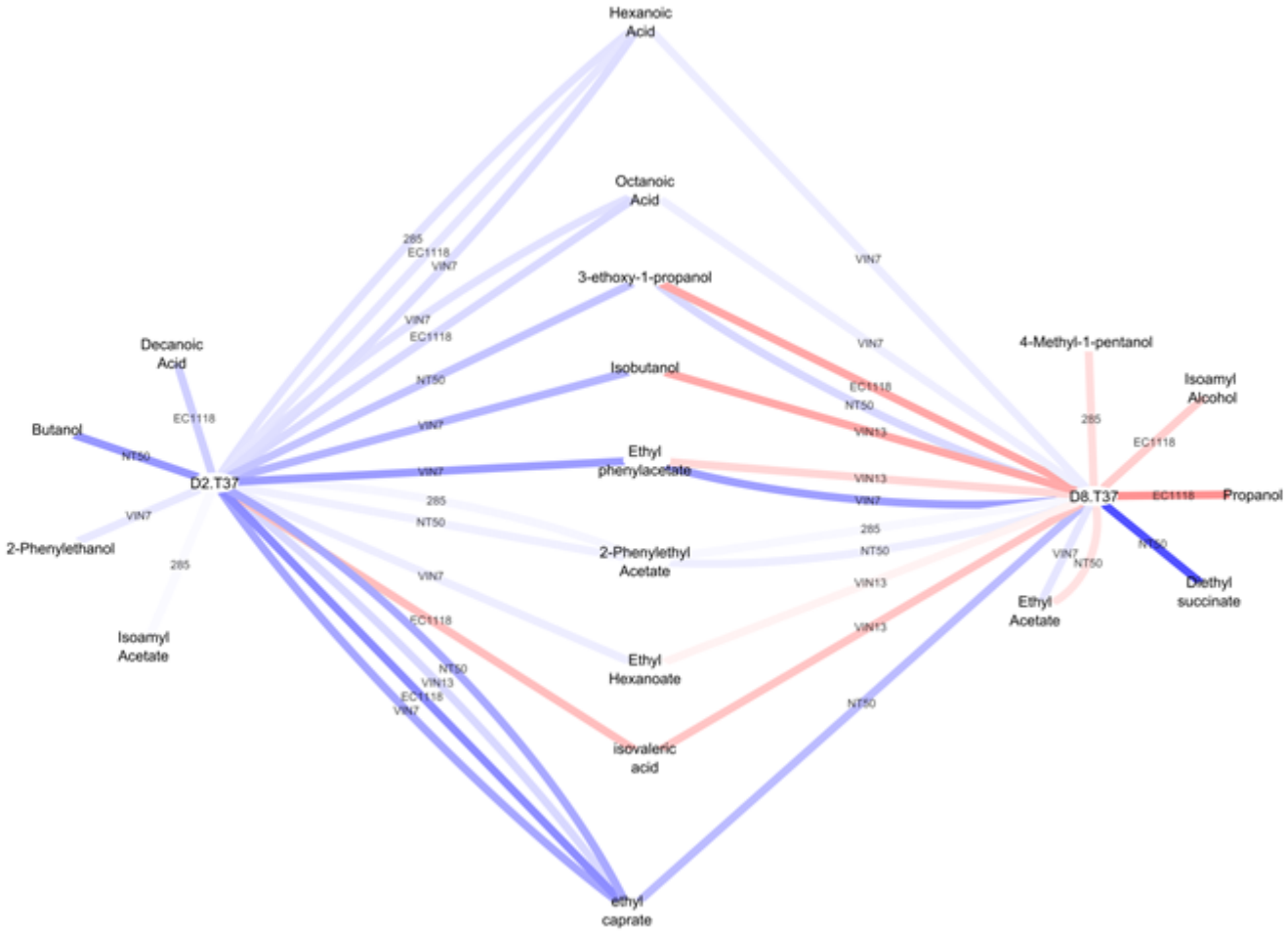


Figure 7 The significant changes ($p<0.05$) in the levels of volatile aroma compounds due to high temperature stress, day eight at 37°C and day two at 37°C when comparing the high temperature stressed fermentations to their controls. A blue edge (line) indicates a reduction compared to the control and a red edge an increase the colour intensity denotes the magnitude of the fold change observed

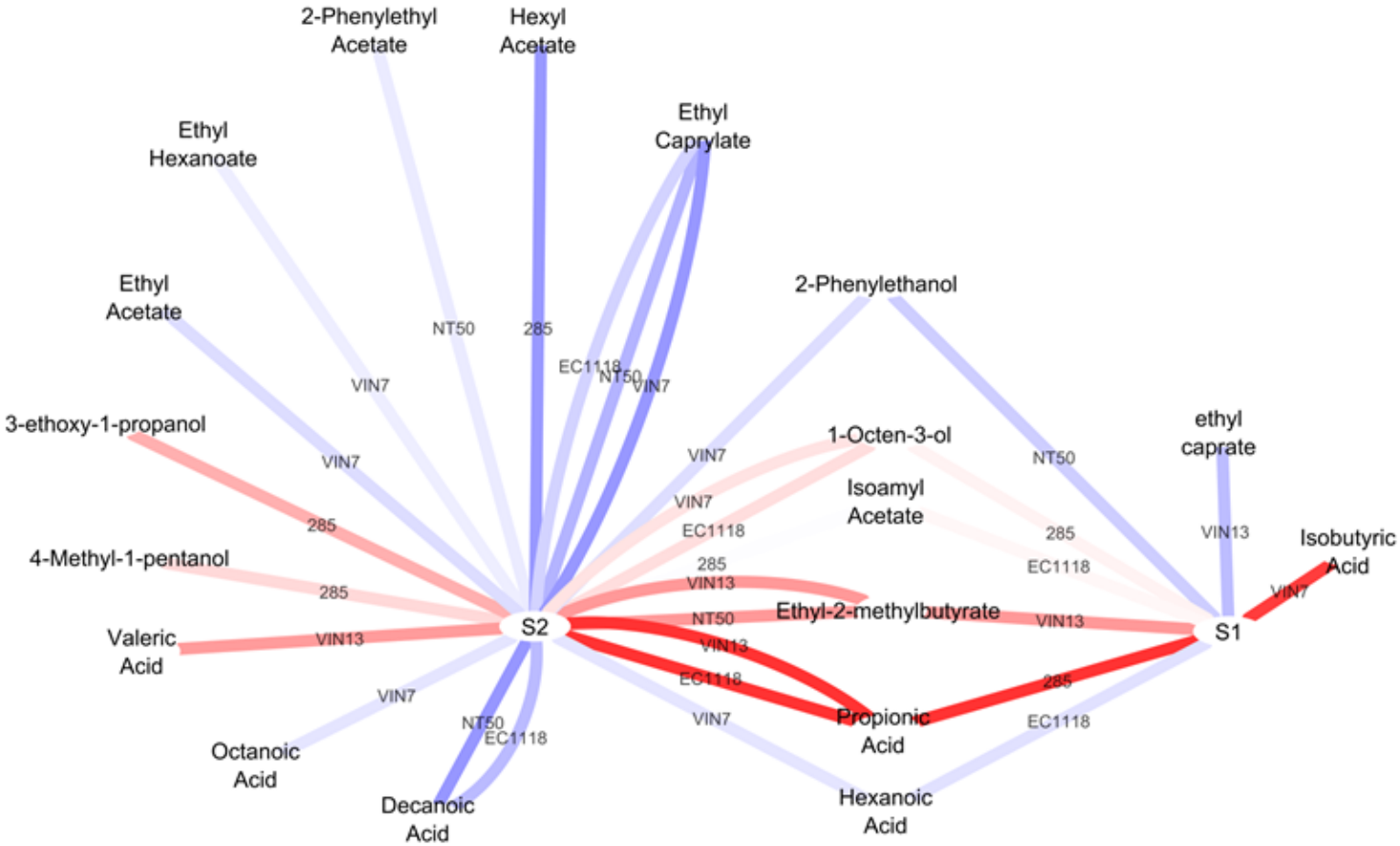


Figure 8 The significant changes ($p<0.05$) in the levels of the volatile aroma compounds due to hyperosmotic stress, treatments S1 (0.3 M) and S2 (0.5 M), when comparing the hyperosmotic stress treatments to their controls. A blue edge (line) indicates a reduction compared to the control and a red edge an increase the colour intensity denotes the magnitude of the fold change observed

Of the stresses evaluated, exposure to 37°C resulted in the greatest number of changes to the volatile aroma profile (Fig. 7). When the stress was applied on day 8, a mixture of increases and decreases was observed for each compound group. Conversely, exposure on day two resulted in a general decrease in the levels of the volatile aroma compounds. A few conserved changes were noted in response to high temperature. Ethyl caprate levels were reduced for all evaluated strains but 285. Octanoic acid decreased for EC1118 and VIN7, and hexanoic acid decreased for EC1118, VIN7 and 285. Furthermore, a few compounds displayed a same response (relative increase or decrease) to 37°C irrespective of when the stress was applied, namely; hexanoic acid (VIN7); octanoic acid (VIN7); ethyl-phenylacetate (VIN7); 2-phenylethyl acetate (NT50 and 285); and ethyl caprate (NT50).

The higher level of hyperosmotic stress (S2) brought about a larger number of changes in the volatile aroma composition compared to the lower level of hyperosmotic stress (S1) (Fig. 8). The S1 treatment resulted in inconsistent (increase and decrease) changes in the compounds groups (three acids, two esters, two higher alcohols). There appears to be no discernable pattern, as the relative changes usually occurred for a different strain each time. Even the compounds which were influenced by both hyperosmotic stress levels were in each case as a consequence of fermentations by different strains.

4.4 Discussion

The impact of environmental stress on yeast fermentation performance has been widely studied, but to our knowledge this is the first study to assess the impact of hyperosmotic and temperature stress on the volatile aroma profile produced by yeast during alcoholic fermentation.

The residual sugars measured at the end of fermentation served as an indicator of fermentation performance (Fig. 3), and most of the stress treatments applied here did not result in significant changes when compared to the unstressed controls. The only exceptions were VIN7 which did not adapt well to high temperature and hyperosmotic stress (S2), and VIN13 following exposure to 8°C on day 8. Other studies have reported on the inability of VIN7 to complete fermentations with high initial sugar concentrations (Erasmus et al. 2004, Erasmus and van Vuuren 2009).

Glycerol is produced as a compatible solute produced in response to hyperosmotic stress, whereby it increases the cells internal solute content promoting water uptake. Therefore it is not surprising that all strains displayed a relative increase in their glycerol levels proportional to the hyperosmotic stress applied.

Other investigations into the impact of heat shock on glycerol production reported an increase in glycerol levels in response to temperature shock, when temperature was increased from 18°C to 34°C (Berovic et al. 2007, Kukec et al. 2003). Berovič et al. (2007) reported that the longer the duration of the temperature shock (24 hours compared to 4 hours) the greater the amount of glycerol produced. The only temperature treatment to increase its glycerol concentration in our study was VIN7_D2.T37. However, Berovič et al. (2007) applied heat shock within the first 30 hours of fermentation, in our study the stress was only applied on day two and day eight of the fermentation. It is possible that timing of the stress application played a role in glycerol production in the case of the other strains, and that they would have been more responsive had the stress been applied earlier.

The changes in aroma compounds after exposure of fermentations to low temperature (8°C) are time and strain dependant (Fig. 6), as illustrated by an increase in the levels of four compounds (volatile fatty acids) for VIN13 on day two, compared to one ester (ethyl acetate) on day eight. Long chain fatty acids (C16 and C18) are vital components of the plasma membrane, where they aid transport of various compounds across the plasma membrane (Lambrechts and Pretorius 2000). In response to fermentation at low temperatures yeast strains are reported to modulate membrane fluidity by increasing the content of unsaturated fatty acids, or the production of medium chain fatty acids (Beales 2004, Beltran et al. 2008). This production of medium chain fatty acids may account for the increase in the production of volatile fatty acid by VIN13.

The only conserved change in response to 8°C was the decrease in the levels of octanoic acid produced by EC1118, irrespective of when the stress was applied. An intracellular accumulation of medium chain fatty acids may account for the observed decrease seen in octanoic and decanoic acid (day 8) for EC1118. At low temperatures the cell membrane becomes less permeable (Bisson 1999), further hindering the passive diffusion of hydrophobic medium chain fatty (C8 and C10) acids into the medium.

The fermentations stressed at 37°C displayed the greatest number of changes to the volatile aroma profiles (Fig. 7). When applied on day eight, a mixture of increases and decreases was observed for each compound group. Conversely, exposure on day two resulted in a general decrease in the levels of the volatile aroma compounds.

Yeast cells respond to stress in a graded manner (Gasch et al. 2000). Similarly, the lower level of hyperosmotic stress condition (S1) induced fewer significant changes compared to that of the higher hyperosmotic stress condition (S2) (Fig. 8). It has been suggested that redox balancing plays an important role in the regulation of the metabolic pathways responsible for the production of aroma compounds (Lambrechts and Pretorius 2000). When experiencing osmotic stress, cells respond by accumulating of glycerol, which results in a surplus of NAD^+. This redox imbalance is corrected by the oxidation of acetaldehyde to acetic acid with

subsequent regeneration of NADH. This is illustrated by the increase in volatile acidity in response to increasing levels of osmotic stress.

In recent studies, Jain et al. (2011) and Styger et al. (2011) reported that an excess of NADH promotes the production of higher alcohols by a lab yeast strain, in an attempt to reduce NADH to NAD^+. Also, this redox imbalance due to a surplus of NADH hinders the production of esters which requires NAD^+. If the reverse were true, the excess NAD^+ produced due to glycerol formation should result in a decrease in the formation of higher alcohols and an increase in ester production. This is generally not the case for this study, as esters and higher alcohols both increased and decreased subsequent to osmotic stress. This suggests that redox balancing only influences the production of aroma compounds to a limited extent under the fermentation conditions evaluated.

Literature also suggests that the increase in volatile fatty acids would display a corresponding increase in the levels of esters (Bisson and Karpel 2010); however no such trend was observed in this study.

Exposure to environmental stress caused a relatively small number of specific changes to individual compounds; however, as aroma compounds interact with each other, they may cause significant changes in the overall aroma profile of the resultant wine. This is the first study to investigate the influence of environmental stress on the volatile aroma profile in a synthetic grape must. The results demonstrate that the evaluated stresses caused significant changes in the levels of a number of aroma compounds. The changes observed also differed for each of the strains, and the fermentation conditions tested. Further analysis on the impact of environmental stress during grape must fermentation and the duration of the stress application would provide information regarding the extent to which environmental stress impacts the aroma profile of wine.

4.5 Acknowledgements

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Chapter 5

General discussion and conclusions

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5.1 Introduction

During the alcoholic fermentation of grape must, yeasts are subject to numerous environmental stresses (hyperosmotic stress, temperature changes, nutrient starvation, pH, etc.). Yeast strains unable to rapidly perceive and respond to stress are more likely to be involved in fermentations that proceed at a slow rate (sluggish fermentation) or result in high residual sugar levels (stuck fermentation) (Alexandre & Charpentier, 1998; Bisson, 1999; Gibson *et al.*, 2007; Malherbe *et al.*, 2007).

In chapter 3 the impact of hyperosmotic stress (imparted by the initial sugar concentration), nitrogen content and low temperature on the fermentation performance of commercially available yeast strains was investigated. The results show that the relationship between initial nitrogen supplementation and fermentation performance is not linear, and is significantly affected by initial sugar content, fermentation temperature, as well as the yeast strain used. Our results show the importance of investigating not only individual fermentation parameters affecting fermentation performance, but also the interactive effect they may have on each other. Nitrogen deficiencies are the most common cause of problematic fermentations. Of the nitrogen levels tested, 250 mg/L resulted in an improved overall performance in terms of fermentation onset, maximal fermentation rate, total weight loss, sugar consumption, and biomass production. Conversely, fermentations supplemented with 400 mg/L nitrogen showed no comparative improvement and in some cases fermentation performance and sugar consumption was reduced relative to the 250 mg/L nitrogen treatment. This illustrates the danger of excessive nitrogen supplementation in terms of fermentation performance; and further advocates determining the must characteristics prior to inoculation.

The effect of stress on the fermentation performance of yeast strains has been the focus of much study, but these studies do not address whether stress impacts wine quality; which is the basis of consumer liking. Wine quality is generally as a consequence of perceived flavour, being the sum of smell, taste and mouth-feel. Wine yeast strains produce volatile aromas (higher alcohols, esters and volatile fatty acids) during fermentation. The production of volatile aroma compounds is affected by the availability of precursors, fermentation conditions (Henschke & Jiranek, 1993; Rapp & Versini, 1996; Lambrechts & Pretorius, 2000; Swiegers *et al.*,

2005; Vilanova *et al.*, 2007; Saerens *et al.*, 2008; Bisson & Karpel, 2010) and the individual strains yeast strains used (Soles *et al.*, 1982; Rossouw *et al.*, 2008).

Generally, wine flavour is a significant component of consumer liking. Consumer liking is subjective and plays a major role when making the decision to purchase wine. To our knowledge, this is the first study to investigate the impact of hyperosmotic and temperature stresses on fermentation performance and the production of fermentation derived volatile aroma compounds. The volatile aroma compounds were quantified using a gas chromatograph and a flame ionization detector (GC-FID). The results in chapter 4 show that exposure to stress has the potential to significantly change the wine aroma profile. Somewhat unexpectedly, the observed changes were different for all the strains and stresses evaluated. Nevertheless, a more in-depth analysis of various fermentation stresses, concurrently and sequentially, may lead to specific guidelines of how individual yeast strains may perform on specific musts and fermentation conditions. It may also provide wine makers with tools to influence the aroma profile of a wine in a specific manner.

The data in chapter 3 could be adapted to provide winemakers with an indication of what the fermentation capacity of the specific strains would be if a set of fermentation and must stress conditions is expected. However, the performance of yeast strains fermenting synthetic grape must may vary when compared to “real” grape must. Nonetheless, the selection of the appropriate yeast strain and the nitrogen supplementation strategy may reduce the incidence of slow or incomplete fermentations. Future studies should further investigate the use of suitable strain selection as a potential strategy to avoid problematic fermentations in grape must, in addition to characterising the relationship between environmental stress and the volatile aroma profile.

5.2 Literature cited

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