Mannoprotein production and wine haze reduction by wine yeast strains

by

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Declaration

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Summary

Wine protein haze formation is a major challenge for wine makers, and several wine clarifying agents such as bentonite are used in the industry to protect wine from this occurrence. However, clarifying agents may have an undesirable impact on wine quality. Yeast mannoproteins have been shown to possess haze-protective properties, while also positively impacting on the sensorial properties of the product. However, while such mannoproteins are released into the wine during the wine making process, the amounts are low and therefore of limited oenological significance. However, and although commercial wine yeast strains display significant genotypic and phenotypic diversity, no broader assessment of haze protective activity and of mannoproteins release by different wine yeast strains has been undertaken.

In this study, several yeast strains were screened for their impact on wine haze formation in Chardonnay must and in a grape juice model system. The data show that strains of the species *Saccharomyces paradoxus* possess better haze protective properties than the common *Saccharomyces cerevisiae* wine yeast strains. Differences in the nature of the proteins released by these two species were investigated, and indicated that several mannoproteins were released at significantly higher levels by *S. paradoxus*, and that some of these proteins might indeed contribute to the haze-protective activity.

A further exploration of yeast cell wall properties indicated that the cell walls of haze-protective *S. paradoxus* strains contained higher levels of chitin than non-haze protective strains. Grape chitinases are likely to be primarily responsible for wine haze formation, and the data clearly demonstrate that these enzymes are able to bind to the yeast cell walls, and that strains with higher amounts of chitin in the cell wall will bind more chitinases. This finding suggests that the haze-protective nature of the strains is at least in part linked to the chitin levels of the strains.

Furthermore, the impact of some genetic modifications in two wine strains (namely *S. cerevisiae* VIN13 and *S. paradoxus* RO88) suggests that several proteins contribute to wine haze protection. However, none of the mannoprotein-encoding flocculation genes, *FLO1*, *FLO5*, and *FLO11* showed any impact on this property.

Further studies are required to assess the full impact of the *S. paradoxus* strains on haze protection. In particular, the possible use of such strains as starter cultures or the use of *S. paradoxus* yeast hulls as clarifying agent needs to be further explored.

Opsomming

Wyn proteïen-waas vorming is 'n groot uitdaging vir wynmakers en verskeie wyn verhelderings agente soos bentoniet word in die wynbedryf gebruik om wyn te beskerm teen die vorming van waas. Hierdie verheldering agente het egter 'n ongewenste impak op wynkwaliteit. Gis mannoproteïene is uitgewys as proteïene met moontlike waas-beskermende eienskappe wat ook 'n positiewe uitwerking op die sensoriese eienskappe van die produk het. Al word hierdie mannoproteïene egter vrygestel in die wyn tydens die wynmaak proses, is die hoeveelhede oor die algemeen te laag om van wynkundige belang te wees. Verder, ten spyte van die beduidende genotipiese en fenotipiese diversiteit van kommersiële wyngisrasse is daar nog geen breër assessering van die waas beskermende aktiwiteit van mannoproteïene, vrygestel deur verskillende rasse, tot dusver onderneem nie.

In hierdie studie is verskeie gisrasse gekeur vir hul impak op wyn waas-vorming in Chardonnay mos en ook in 'n model druiwesap. Die data wys dat rasse van die spesie *Saccharomyces paradoxus* besit beter waas beskermende eienskappe as die algemene *Saccharomyces cerevisiae* wyngisrasse. Verskille in die aard van die proteïene wat vrygestel is deur hierdie twee spesies is ondersoek, en dit is aangedui aangedui dat verskeie mannoproteins vrygestel aan aansienlik hoër vlakke deur S. Paradoxus. Dit is ook aangedui dat sommige van hierdie proteïene wel bydra tot die waas-beskermende aktiwiteit.

'n Verdere verkenning van gis selwand eienskappe het aangedui dat die selwande van waas-beskermende rasse van *S. paradoxus* hoër vlakke chitien as nie-waas beskermende stamme bevat. Druiwe chitinases is waarskynlik hoofsaaklik verantwoordelik vir wyn waas vorming, en die data toon duidelik dat hierdie ensieme in staat is om te bind aan die gis selwande, en dat die stamme met hoër vlakke chitien in die selwand meer chitinases sal bind. Hierdie bevinding dui daarop dat die waas-beskermende aard van die stamme ten minste gedeeltelik gekoppel is aan die chitien vlakke van die stamme.

Die impak van sekere genetiese modifikasies in twee verskillende gisrasse, naamlik die *S. cerevisiae* ras VIN13 en die *S. paradoxus* ras RO88, dui verder daarop dat verskeie proteïene dra by tot die beskerming teen wyn waas. Geeneen van die mannoprotein-koderende flokkulasie gene, *FLO1*, *FLO5* en *FLO11* het egter 'n impak op hierdie eienskap nie.

Verdere studies is nodig om die volle impak van die *S. paradoxus* rasse op waas beskerming te assesseer. In die besonder, die moontlike gebruik van sulke rasse as 'n inkolasie kultuur of die gebruik van *S. paradoxus* gis doppe as verheldering agent moet verder ondersoek word.

This dissertation is dedicated to my missing uncle Nelson Khumbulani Ndlovu and my family.

Biographical sketch

Thulile Ndlovu was born in Bulawayo, Zimbabwe on 19 August 1982. After doing Advanced level in Biology, Chemistry and Mathematics at Mzilikazi High school, she pursued a Bachelor of Science Honours degree in Applied Biology and Biochemistry at the National University of Science and Technology. She then went on further to pursue her Master's degree in Biochemistry at the University of Fort Hare. Intensified by her interest in Biotechnology and its application in solving real life problems, Thulile enrolled for a Doctor of Philosophy (PhD) study under the supervision of Prof Florian Bauer and Dr Divol Benoit at the Institute for Wine Biotechnology, Stellenbosch University.

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Preface

This dissertation is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the style of the journal Yeast.

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In a quest to understand and reduce wine protein haze: A review

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Contents

Chapter 1

Introduction and project aims

1.1 Introduction

Wine clarity refers to the absence or presence of suspended particles or sediments in wine. In white wine, clarity is of prime importance for consumers as a bottle showing haziness is likely to be rejected (Dupin *et al.,* 2000; Lomolino and Curioni, 2007), despite the fact that most hazes do not affect the olfactory and gustatory characteristics of the wine (Ferreira *et al*., 2004; Lomolino and Curioni, 2007). Indeed, light passing through a wine with great clarity appears sharp and brilliant, and clarity is therefore commonly used as a measure of quality. To control haze formation, wine makers perform a heat test (80ºC for six hours) before bottling to assess protein stability in white wines (Pocock and Waters, 2006; Mesquita *et al.,* 2001). The test provides an approximate haze formation potential, which is then used to determine the amount of clarifying agent to be added to prevent any haze formation during bottling and shelf-life of the wine. However, there is no standard protocol set out for carrying out the heat test as several researchers and the wine industry use different heating times, temperatures and spectrometric wavelength (Gonzalez-Ramos *et al.,* 2009; Batista *et al.,* 2009; Versari *et al.,* 2011). There is also no full agreement on how best to estimate the amount of clarifying agent to be added into wines (Pocock and Waters, 2006).

Wine must not only be clear at the time of bottling but also retain its clarity during aging and storage for an indefinite period, regardless of the storage conditions. Microbial problems and tartrate precipitations may result in a hazy wine, but the most common cause of haze is protein instability. The source of most of the macromolecules such as proteins in wine are the grapes themselves, but fermenting yeast and bacteria also produce a certain number of macromolecules, as do contaminating organisms such as *Botrytis cinerea* which is present in must when infected grape berries are used. Proteins are a relatively minor constituent of wine, ranging from undetectable levels to over 500 mg/l (Feuillat *et al.,* 2003). However, wine proteins can have significant positive and negative effects on wine quality. In particular, highly undesirable heat-induced protein hazes have been attributed to grape pathogenesis-related (PR) proteins that are present in the grape must and that survive the winemaking process

(Ferreira *et al.* 2002, Falconer *et al.,* 2010; Marangon *et al*., 2011). Although the PR proteins appear to be the major constituent of haze, data suggest that other wine components are involved in wine protein haze formation (Mesquita *et al.,* 2001; Pocock *et al.,* 2007). Furthermore, no correlation between the total amount of protein present in wine and protein instability has been observed (Esteruelas *et al.,* 2009). Polyphenols may be important in wine haze formation in the presence of other wine components as observed in a multi-factorial study carried out by Fenchak *et al.* (2002) who demonstrated that the interaction between pH, protein and polyphenol is important for haze formation. Common wine anions (e.g. acetate, chloride, citrate, phosphate, and tartrate), and cations (e.g. ferric phosphate and copper sulphide), when added at typical white wine concentrations were not found to be essential for protein haze formation (Pocock *et al.,* 2007). Nevertheless, the precise mechanism of wine haze formation still remains to be elucidated although some hypotheses have been put forward (Brown *et al.,* 2007; Batista *et al.,* 2009) and will be discussed in more detail in the literature review.

To avoid haze formation and improve wine clarity, several fining agents have been or continue to be commonly used in the wine industry. These include milk and casein, gelatin, isinglass, albumin and egg white. Blood by-products such as fresh cattle blood and dried blood were also used for many years for fining young red and white wines but are now illegal since these products could be responsible for human allergies that have been reported (Vincezi *et al.,* 2005; Cereda *et al.,* 2010). Today, bentonite montmorillonite clay is the most commonly used clarifying and wine stabilizing agent (Pocock and Waters, 2006). However, bentonite results in the loss of wine volume and removes some wine aroma components, thus potentially lowering wine quality. Moreover due to the large doses of bentonite being employed in industry ranging from 100 to 200 g/hl, bentonite poses sustainability and environmental issues as it is not recyclable (Waters *et al.,* 2005). Hence, new alternative better methods of protein stabilization are still sought after.

The presence of mannoproteins in wines has attracted the attention of oenologists due to their positive attributes and impact on wine quality. Mannoproteins are thought to be responsible for

the reduction of visible protein haze formation in white wine (Dupin *et al.,* 2000; Gonzalez-Ramos *et al.,* 2008). They have a positive effect on the sensorial quality of red wines (Feuillat, 2003; Vidal *et al.,* 2004), increase colour stability (Guadalupe and Ayestaran, 2008), inhibit tannin aggregation (Riou, *et al.,* 2002), stimulate malolactic fermentation (Guilloux-Benatier *et al.,* 1995), improve tartrate stability in wine and interact with wine volatile compounds (Chalier *et al.*, 2007), among other qualities. There is however a paucity of data on the exact nature and on the mode of action of these mannoproteins. Nonetheless, Waters *et al.* (1994a) established that the addition of haze-protective mannoproteins did not prevent the proteins in wine from precipitating, but rather caused a diminution of haze particle size. The amount of mannoproteins released by yeast during wine making is usually too low to be of much oenological significance (Feillat *et al.,* 2003), but it has been demonstrated that aging wine on the yeast lees may lead to an increase in yeast mannoproteins (Dupin *et al.*, 2000; Fusi *et al.,* 2010). Industrial strains releasing high quantities of mannoproteins into wine during fermentation would therefore be of interest.

Several specific yeast mannoproteins have been shown to reduce wine haze formation, and include haze protection factors 1 and 2 (Brown *et al.,* 2007) and yeast invertase (Dupin *et al.,* 2000). Other proteins with such impacts include grape arabinogalactan-protein (Waters *et al.,* 1994b), and an apple arabinogalactan-protein (Pellerin *et al.,* 1994). However, the yeast proteins identified thus far only account for a fraction of the total yeast-derived haze-protective activity, and, according to our knowledge, no other studies have been undertaken to determine which of the other yeast parietal mannoproteins may contribute to haze protection. Determining the nature and identities of mannoproteins responsible for wine haze reduction will enable the development of new techniques or ways to increase the levels of these haze protective factors to oenological and commercial significant levels.

Several researchers have explored ways to increase the amount of mannoproteins released by yeast during fermentation by either manipulating cell wall regulatory processes or by creating thermo-sensitive mutants which will subsequently autolyse (Brown *et al.,* 2007; Gonzalez-

Ramos *et al.,* 2008). The physiological role of most yeast cell wall Pir- and GPI-anchored mannoproteins is unknown and gene disruptions leading to depletion of different proteins do not affect major functions of the wall (Klis *et al*., 2002; Vestrepen *et al.,* 2006).

In contrast to the intracellular processing steps, which have been studied in considerable detail, the extracellular steps leading to integration in the cell wall on glycoprotein arrival at the cell surface are mostly unknown. The glycan-processing enzymes and the cell wall cross-linking enzymes are with a few exceptions largely unknown or incompletely characterized. In light of this, this work investigated the effect of deletions involving genes related to cell wall biogenesis on the release of mannoproteins and consequently their impact on wine haze formation. The non-reducing ends of the β-1,3-glucan side chains are believed to function as acceptor sites for β-1,6-glucan and chitin, whereas the reducing end of the β-1,3-glucan molecules are thought to be involved in the linkage to Pir-CWP (Klis *et al.,* 2002; Aguilar-Uscanga and François, 2003). As a consequence, a lack of β-glucans in the yeast cell wall might result in less covalent linkage between the three cell-wall compounds, resulting in a more permeable and digestible cell wall resulting in increased quantities of mannoproteins released (Feuillat, 2003; Palmisano *et al.,* 2010). Overexpression and/ or deletion of some of the cell wall genes may lead to an increase in mannoproteins released in wine and as a result, ‘’purer’’ clarified wine. Considering the ever-growing interest in the selection and development of wine yeast strains able to release mannoproteins more efficiently than currently available strains, genetic determinants of mannoprotein release such as cell wall biogenesis genes in *S. cerevisiae* needs to be investigated.

Despite the known fact that mannoproteins are involved in wine haze reduction, Brown *et al*. (2007) observed that in isolation, deletion and overexpression of the haze protection factor genes could not conclusively confirm that the HPF gene products are the haze protective factors. In addition, in view of the fact that some mannoproteins have been implicated in increasing wine haze formation, investigating the possibility of other yeast cell wall components involvement in wine haze reduction is warranted. Therefore based on our preliminary findings in

this study, where no differences in total protein and mannoprotein being released by haze protecting and non-haze protecting strains were observed, further investigation of other possible yeast cell wall factors besides mannoproteins were further explored.

1.2 Scope and aims of the study

This study sought biological alternative or complementary approaches to the use of bentonite with less environmental impact and without negative impact on wine quality. The main aims of the study were to investigate:

- The ability of different wine yeast strains to protect wine from haze;
- The identification of mechanisms that may be responsible for the observed differences in haze protecting and non-haze protecting strains.

These aims were achieved through the following objectives:

Objective 1

The development of a reproducible haze assay by comparing several methodologies described in the literature and identification of differences in haze protective activities of different yeast strains

Objective 2

The profiling of exoproteomes of wine yeast strains with diverging wine haze protective activities and identification of individual proteins that may contribute to these differences;

Objective 3

The investigation of other cell wall properties that may contribute to protein haze reduction;

Objective 4

The evaluation of cell wall chitin as a contributor to haze protective activities in *Saccharomyces paradoxus* strains;

Objective 5

The evaluation of the effect of certain yeast genetic modification on wine haze formation.

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Chapter 2

Literature review

In a quest to understand and reduce wine protein haze: A review

In a quest to understand and reduce wine protein haze: A review

2. Summary

Heat induced protein haze is a continuous problem for the global wine industry. Despite extensive research that has been carried out for decades, the mechanism of haze formation remains largely unknown, although several mechanisms have been suggested. It is now widely accepted that protein haze formation in wines is a multi-factorial process being affected by physical and chemical factors such as heat, pH, polyphenols, organic acids, alcohol levels, as well as the concentration of proteins and of other non-proteinaceous wine components.

Wine haze is the result of the aggregation of wine proteins and of other wine components during wine storage, resulting in the formation of light-dispersing particles that are visually detected as haze. The most popular method used to prevent this unattractive haze includes the use of bentonite, in spite of its potentially negative impact on wine quality and volume. Other promising techniques for haze reduction have been explored and applied, and their impact on wine quality in some cases still needs to be evaluated. A potential substitute or complementary method for bentonite is the use of yeast parietal mannoproteins from *Saccharomyces cerevisiae* that have been demonstrated to reduce or attenuate wine haze. However, further studies are needed to explore the diversity of yeast mannoproteins and their effects on wine quality.

Low quantities of such haze-protecting mannoproteins are released during alcoholic fermentation by yeast and also during autolysis when wine is aged on lees. Considering the promising application of mannoproteins in wine making, genetic improvement of yeast strains to release larger quantities of mannoproteins would be beneficial. There is also a great need to further explore how the underlying molecular processes, cell wall composition and cell wall regulatory processes influence the release of mannoproteins. This review seeks to critically evaluate the studies that have been published to date in order to understand the phenomenon and provide novel and better solutions to address this problem.

2.1 Introduction

White wine clarity is of key importance for the winemaker as a bottle showing haziness is likely to be rejected by the consumer (Dupin *et al.,* 2000a; b; Lomolino and Curioni, 2007), regardless of the fact that most hazes do not affect the olfactory and gustatory characteristics of the wine (Ferreira *et al.,* 2004; Lomolino and Curioni, 2007). While the main form of wine haze is caused

by protein instability, in particular of certain grape proteins (Ferreira *et al*., 2002; Laborde *et al.,* 2006), turbidity in bottled wines (Figure 2.1) can be caused by several factors such as polysaccharide precipitation (Mesquita *et al.,* 2001), interactions between polyphenols and proteins, crystallization of tartrates, and the growth of microorganisms such as yeast and bacteria (Waters *et al.,* 1994; Marangon *et al.,* 2010a). Occurrence of haze may furthermore depend on specific chemical and physical properties of the wine such as pH (Batista *et al.,* 2010) and the alcohol level (Mesquita *et al*., 2001), as well as environmental factors associated with the processing and storage of wine, including temperature (Mesquita *et al.,* 2001; Marangon *et al*., 2010b).

Figure 2.1: A glass of hazy wine precipitation of unstable protein on the left and on the right, a glass of white wine clarified and stabilized

Wine haze is a more common problem with white than with red wines (Fenchak *et al.,* 2002). To explain the low levels of observed haze in red wines regardless of such wines having high levels of proteins, and phenolic compounds such as tannins, Fleet and Siebert (2005) assessed human visual perception of turbidity. Not unexpectedly, thresholds in darker liquids were higher than those in the clear and pale liquids, indicating that it is more difficult to observe turbidity in darker liquids. This observation certainly explains why haze is a more serious issue in white wines than in red wines.

However, of all factors mentioned above, wine protein haze is by far the most common. Wines contain varying amounts of different nitrogenous substances, including peptides and proteins. The sources of these macromolecules are the major wine relevant organisms, the grape vine, yeast and bacteria, but proteins may also originate from certain spoilage organisms such as *Botrytis cinerea*. Protein haze has mostly been linked to a group of proteins present in high levels in wine referred to as grape pathogenesis-related proteins (PR) which include different chitinases [poly(1,4-N-acetyl-ß-D-glycosaminide) glycanohydrolase, EC 3.2.1.14] and thaumatin-like proteins. These proteins are stable at wine pH and unaffected by proteolysis (Waters *et al.* 1998; Marangon *et al.,* 2010a; c). Under certain conditions, these PR proteins can aggregate forming large particles seen as haze or sediments. Other proteins such as β-1.3 glucanase and ripening-related protein Grip22 (Estereulas *et al.,* 2009) have recently been found in haze. Current research is exploring the mechanisms of precipitation of haze active proteins with the aim of understanding the unfolding behavior of these proteins under wine conditions (Marangon *et al.,* 2010a; c).

Despite the increasing knowledge of wine soluble protein composition, the mechanisms of haze formation in wines still require further investigation. Thorough knowledge of the mechanisms involved in haze formation is essential in order to be in a position to control and prevent protein hazes while avoiding excessive and detrimental wine treatments. To prevent protein haze formation, bentonite is usually employed in order to lower the concentration of wine proteins. Such treatments are applied despite the potentially negative impact on wine volume and quality. Indeed, because of bentonite's considerable swelling and poor settling characteristics, it is estimated that 3% to 10% of the wine volume is taken up by the bentonite lees (Tattersall *et al.,* 1997). Bentonite is also not recyclable which is not without problems regarding sustainability. In the year 2000, it was estimated that bentonite fining cost the world wine industry amounts ranging from U.S. $300-500 million annually (Høj *et al.,* 2000). It is therefore imperative to investigate alternative methods to be used in improving wine clarity (Waters *et al.,* 2005).

Yeast mannoproteins are released during fermentation and also in wine aged on lees. The presence of these mannoproteins in wines has of late attracted interest of enologists due to the positive impacts that have been attributed to these compounds, including the ability of some of these proteins to reduce haze formation (Dupin *et al.,* 2000a; b; Brown *et al.,* 2007). However, the mechanism of action of these proteoglycans in wine haze reduction still remains to be further elucidated. There is also a paucity of data regarding which yeast mannoproteins are specifically responsible for haze diminution. Furthermore, developing yeast strains having the ability to hydrolyze haze causing molecules could be explored.

2.2 Proposed mechanisms of protein haze formation

The mechanism responsible for protein haze formation in wines is not yet fully understood. Several hypotheses have however been put forward. Grape pathogenesis related proteins (PR) are thought to normally exist as globular entities soluble in wine (Kwon, 2004; Pocock *et al.,* 2007). However, the PR proteins responsible for haze are presumed to be tightly coiled containing between six and eight disulphide bridges. The mechanism of haze formation proposed by Pocock *et al.* (2007) and Marangon *et al.* (2010a) consists of two steps (Figure 2.2). PR proteins are uncoiled or denatured in the first step of the process of haze formation and this process is thought to be accelerated by heat, phenolic compounds, metal ions or sulphate ions (referred in earlier papers as factor X) through cross-linking with the denatured protein. The second stage involves the aggregation of denatured proteins which results in haze particles being formed.

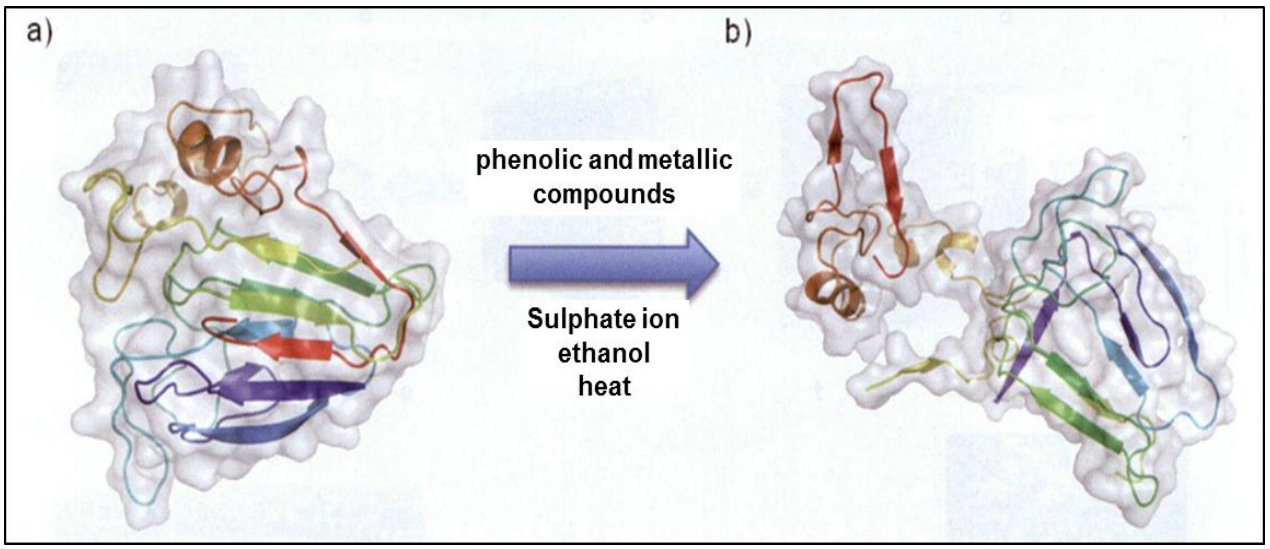

Figure 2.2: (a) Tertiary structure model of grape thaumatin-like proteins' secondary peptide backbone structure showed as the coloured strip in the native proteins and (b) the unfolding of protein after heat denaturation in wine like-solution (Modified from Marangon *et al*., 2010a)

Using circular dichroism, Falconer *et al*. (2010) investigated the thermal stability of thaumatin-like proteins, chitinase, and invertase isolated from Sauvignon Blanc and Semillon juice and their role in haze formation in wine. It was observed that chitinase unfolding follows three steps with an initial irreversible step from the native to an unfolded conformation, a reversible step between a collapsed and an unfolded non-native conformation, followed by irreversible aggregation associated with visible haze formation. Using model experiments, Marangon *et al.* (2010a) observed that haze proteins can unfold within minutes at temperatures above 60°C, and unfolding appears to be the prerequisite for haze formation. The size of the aggregated protein particles is thought to be dependent on the presence of other wine solutes such as phenolic compounds and metal ions. This unfolding of haze active proteins can be exploited in order to successfully degrade the stable haze proteins by proteases during winemaking.

Batista *et al.* (2009) proposed that two mechanisms are responsible for wine haze, one occurring only at the higher pH values, that appears to result from isoelectric precipitation of the proteins and another prevailing at the lower pH values that depends on the presence of sulphate ions. Further analyzing the chemical nature of protein aggregation as a function of pH proved that neither of the two proposed mechanisms responsible for the wine haze is electrostatic in nature, lectin-mediated or divalent cation-dependent with both mechanisms

showing minimum turbidity at pH 7, but increased turbidity at lower and higher pH values. In a recent study by Marangon *et al.* (2010a) on haze, it was revealed that chitinases have a half-life of two years at 25^{o}C and the thaumatin-like proteins had a half-life of 300 years at the same temperature. These findings explain the delay in haze formation in wine soon after bottling and that protein unfolding is likely to be the rate-limiting step in haze formation. In addition to the research being carried out to determine wine haze constituents or mechanism of wine haze formation, individual or interactions of wine components in wine haze formation still needs to be demonstrated in order to explain the inadequacy of wine proteins alone to form haze.

2.3 Factors influencing wine haze formation

Haze formation clearly is a complex, multi-factorial process and results from the combination and interaction of multiple environmental and chemical parameters. These interactions between wine components result in the formation of light-dispersing particles that above certain dimensions is visually detected as haze.

2.3.1 Protein

Wine proteins play important roles in various technological and enological processes as they affect wine clarity and stability (Kwon, 2004; Vincezi *et al.,* 2011), but contribute minimally to its nutritive value (Ferreira *et al.,* 2001; Batista *et al.,* 2009). Some peptides however, have been shown to exhibit surfactant and sensory properties that can influence the organoleptic characteristics of wine (Moreno-Arribas *et al.,* 2002). Wine proteins are present in very low concentrations (<500 mg/l total) in wine and vary significantly depending on cultivar, region, vintage, and viticultural and enological practices (Weiss *et al.,* 1998; Ferreira *et al.,* 2000; 2002; Waters *et al.,* 2005). Proteins with a molecular weight of between 18 and 26 kDa make up most of the dry weight natural protein precipitate in wine haze (Waters *et al.,* 1998; Esteruelas *et al.,* 2009) but some proteins of 14, 41, 53 and 69 kDa have been shown to be present with an isoelectric point between 4.2 and 5.0 (Esteruelas *et al.,* 2009). Direct protein analysis of the natural precipitate's composition by the Bradford dye-binding assay revealed that the proportion

of protein was only 10.3% (w/w), while phenolic compounds and polysaccharides represented 7.2% and 4.4%, respectively (Esteruelas *et al.,* 2009).

Several methods have been employed to study wine proteins, including dialysis, ultrafiltration, precipitation, exclusion chromatography, one or two-dimensional electrophoresis, capillary electrophoresis, isoelectric focusing, affinity and hydrophobic chromatography, immunodetection, high-performance liquid chromatography (HPLC), and fast protein liquid chromatography. However, these compositional analyses are frequently hampered by the need to concentrate or desalt the samples before analysis (Kwon, 2004; Palmisano *et al.,* 2010; Branconi *et al.,* 2011). More than 80 proteins soluble in wine were identified by several studies (Table 2.1) using a number of techniques ranging from SDS PAGE, MALDI-TOF, nano-high performance liquid chromatograpry/tandem mass spectrometry and yeast and grape protein antibodies. Some of the identified proteins are of bacterial origin (Kwone, 2004; D'Amato *et al*., 2011), and were postulated to originate from the vineyard possibly due to natural infections and improper handling during harvest (Kwon, 2004). The protein list below (Table 2.1) is however not exhaustive.

The combination of 2D-PAGE for protein separation coupled with mass spectrometry (MS) for protein identification has provided researchers with the possibility to analyze simultaneously thousands of proteins in a single experiment (Branconi *et al.,* 2011). Intrinsic 2D-PAGE limitations, such as under-selection for certain protein categories, limited dynamic range, co-migration of multiple proteins, and need for many replicates has been overcome by the development of alternative gel-free approaches such as liquid-chromatography (LC)-based technologies coupled with MS (Branconi *et al.,* 2011; Palmisano *et al*., 2010).

Table 2.1: Yeast and grape proteins identified from wine

Protein identified (gene name)	Molecular weight (kDa)	Species	Reference
Yju1p (*Yju1*)	21.8	*S. cerevisiae*	Kwon, 2004
Extracellular matrix protein 33 precursor (*Ecm33*)	48.3	*S. cerevisiae*	Kwon, 2004; Wigand *et al*., 2009
Cell wall protein 11 precursor	23.2	*S. cerevisiae*	Wigand *et al.,* 2009
Endo-β 1,3 glucanase (*Bgl2, Exg2*)	34.1, 63.5	*S. cerevisiae*	Kwon, 2004
Gp38p (*Ypg1*)	37.3	*S. cerevisiae*	Kwon, 2004; D'Amato *et al.,* 2010
Protein TOS1 precursor (target of SBF (*Tos1*)	47.9	*S. cerevisiae*	Kwon, 2004; Wigand *et al.,* 2009
Mannan endo-1,4-β-mannosidase (*Man5B*)	41.4	*Aspergillus aculeatus*	D'Amato *et al.,* 2010
Aspergillopepsin B (*PepB*)	28.2	*Aspergillus fumigatus*	D'Amato *et al.,* 2010
Rhamnogalacturonase (*Rhg*)	46.5	*Aspergillus aculeatus*	D'Amato *et al.,* 2010
Glucan 1,4-alpha-glucosidase (*AgdA*)	67.1	*Aspergillus fumigatus*	D'Amato et al., 2010
Thioredoxin h (*Trx-H*)	45.8	*V. vinifera*	D'Amato et al., 2011
Ripening-related protein-like (*Grip22*)	27.4	*V. vinifera*	D'Amato et al., 2011
Thiredexin-2 (*Trx2*)	11.3	*S. cerevisiae*	D'Amato et al., 2011
Cell wall mannoprotein (*Pir1*)	34.8	*S. cerevisiae*	*D'Amato et al., 2011*
Glucan 1,3-β-glucosidase (*Bgl2*)	34.3	*S. cerevisiae*	D'Amato *et al.,* 2011
RNA polymerase I-specific transcription initiation factor (*Rrn5*)	41.9	*S. cerevisiae*	D'Amato *et al.,* 2011
Glyceraldehyde-3-phosphate dehydrogenase (*G3p1*)	35.8	*S. cerevisiae*	D'Amato *et al.,* 2011
Phosphoglycerate kinase (*Pgk*)	44.8	*S. cerevisiae*	D'Amato *et al.,* 2011
Putative glycosidase (*Crh1, Utr2*)	49.9, 52.7	*S. cerevisiae*	Kwon, 2004; Wigand *et al.,* 2009; Palmisano *et al.,* 2010; D'Amato *et al*., 2010; 2011
Acid phosphates (*Pho3*)	52.7	*S. cerevisiae*	Kwon, 2004
β-1,3 glucanosyltransferase (*Gas1*)	59.5	*S. cerevisiae*	Kwon, 2004
Invertase 4 precursor (*Suc4*)	60.5	*S. cerevisiae*	Kwon, 2004; Okuda, 2006; Cilindre *et al*., 2008; D'Amato et al., 211
Daughter cell specific secreted protein (*Dse4*)	121	*S. cerevisiae*	Kwon, 2004
Lacasse 2	63.4	*B. fuckeliana*	Kwon, 2004
Osmotin-like protein (*Olp*)	30	*V. vinifera*	Okuda, 2006; Cilindre *et al.*, 2008; D'Amato et al., 2011
Lipid transfer protein (*nsLTP*)	11.6	*V. vinifera*	Okuda, 2006; Wigand *et al.,* 2009; D'Amato et al., 2011
Cell wall protein precursor (*Cwp1*)	24.3	*S. cerevisiae*	Wigand *et al.,* 2009
Succinyl-co-A synthetase (*sucCD*)	41.2	*P. putida*	Kwon, 2004
Translation elongation factors (*Eef*)	77.1	*P. syringae pv. Syringae B728a*	Kwon, 2004

Table 2.1 (cont.)

Protein identified (gene name)	Molecular weight (kDa)	Species	Reference
Basic extracellular β-1,3 glucanase precursor	14.6	*V. vinifera*	Kwon, 2004
Putative thaumatin-like protein (*Tlp*)	20.1; 23.8	*V. vinifera*	Kwon, 2004; Okuda, 2006; Cilindre *et al.*, 2008; Wigand *et al.,* 2009; D'Amato et al., 2010; 2011
Wtl1p (*Wtl1*)	23.9	*V. vinifera*	Kwon, 2004
Vacuolar invertase (*Suc2*)	71.5	*V. vinifera*	Kwon, 2004; Wigand *et al*., 2009
Class IV endochitinase (*Chit*)	27.5	*V. vinifera*	Kwon, 2004; Palmisano *et al.,* 2010; D'Amato *et al*., 2011
Enolase (*Eno1p, Eno2p*)	46.8	*S. cerevisiae*	Insenser *et al.,* 2010; Branconi *et al.,* 2011; D'Amato *et al*., 2011
Hexokinase-2 (*Hxk2*)	53.9	*S. cerevisiae*	Branconi *et al.,* 2011
6-phosphofructokinase subunit beta (*Pfk2*)	104.6	*S. cerevisiae*	Branconi *et al.,* 2011
Phosphoglycerate kinase (*Pgk1*)	44.7	*S. cerevisiae*	Pardo *et al.,* 2000; Insenser *et al*., 2010; Branconi *et al.,* 2011
Pyruvate kinase (*Pyk1*)	54.5	*S. cerevisiae*	Insenser *et al*., 2010; Branconi *et al.,* 2011
Glyceraldehyde 3-phosphate dehydrogenase (*Tdh3*)	35.7	*S. cerevisiae*	Insenser *et al*., 2010; Branconi *et al.,* 2011
Alcohol dehydrogenase 1 (*Adh1*)	36.8	*S. cerevisiae*	Pardo *et al*., 2000; Insenser *et al*., 2010; Branconi *et al.,* 2011
Pyruvate decarboxylase (*Pdc1*)	61.5	*S. cerevisiae*	Pardo *et al*., 2000; Insenser *et al*., 2010; Branconi *et al.,* 2011
6-phosphogluconate dehydrogenase, decarboxylating 1 (*Gnd1*)	53.5	*S. cerevisiae*	Branconi *et al.,* 2011
ATP-dependent molecular chaperone HSP82 (*Hsp82*)	81.4	*S. cerevisiae*	Insenser *et al*., 2010; Branconi *et al.,* 2011
Heat shock protein SSA1 (*Ssa1*)	69.7	*S. cerevisiae*	Insenser *et al*., 2010; Branconi *et al.,* 2011
Heat shock protein SSA2 (*Ssa2*)	69.5	*S. cerevisiae*	Insenser *et al*., 2010; Branconi *et al.,* 2011
Heat shock protein SSB2 (*Ssb2*)	66.6	*S. cerevisiae*	Pardo *et al*., 2000; Branconi *et al.,* 2011
Heat shock protein homolog SSE1 (*Sse1*)	77.4	*S. cerevisiae*	Insenser *et al*., 2010; Branconi *et al.,* 2011
Superoxide dismutase [Cu-Zn] (*Sod1*)	15.9	*S. cerevisiae*	Insenser *et al*., 2010; Branconi *et al.,* 2011
Translation elongation factor 2 (*Eft1*)	93.3	*S. cerevisiae*	Branconi *et al.,* 2011
Glutamyl-tRNA synthetase (*Gus1*)	80.8	*S. cerevisiae*	Branconi *et al.,* 2011
60S ribosomal protein L15-A (*Rpl15a*)	24.4	*S. cerevisiae*	Branconi *et al.,* 2011
60S ribosomal protein L17-A (*Rpl17a*)	20.5	*S. cerevisiae*	Branconi *et al.,* 2011
60S ribosomal protein L27 (*Rpl27a*)	15.5	*S. cerevisiae*	Branconi *et al.,* 2011
60S ribosomal protein L2 (Fragment) (*Rpl2b*)	27.4	*S. cerevisiae*	Branconi *et al.,* 2011; D'Amato et al., 2011
Ribosomal protein L3 (*Rpl3*)	43.8	*S. cerevisiae*	Insenser *et al*., 2010; Branconi *et al.,* 2011
60S ribosomal protein L5 (*Rpl5*)	33.7	*S. cerevisiae*	Branconi *et al.,* 2011
60S ribosomal protein L7-A (*Rpl7a*)	27.6	*S. cerevisiae*	Branconi *et al.,* 2011
60S ribosomal protein L8-B (*Rpl8b*)	28.1	*S. cerevisiae*	Branconi *et al.,* 2011

Table 2.1 (cont.)

Protein identified (gene name)	Molecular weight (kDa)	Species	Reference
60S acidic ribosomal protein P0 (Fragment) (*Rpp0*)	33.8	*S. cerevisiae*	Branconi *et al.,* 2011
40S ribosomal protein S12 (*Rps12*)	15.5	*S. cerevisiae*	Branconi *et al.,* 2011
40S ribosomal protein S15 (*Rps15*)	16.0	*S. cerevisiae*	Branconi *et al.,* 2011
40S ribosomal protein S2 (*Rps2*)	27.5	*S. cerevisiae*	Branconi *et al.,* 2011
40S ribosomal protein S20 (*Rps20*)	13.9	*S. cerevisiae*	Branconi *et al.,* 2011
40S ribosomal protein S5 (*Rps5*)	25.0	*S. cerevisiae*	Branconi *et al.,* 2011
40S ribosomal protein S7-A (*Rps7a*)	21.6	*S. cerevisiae*	Branconi *et al.,* 2011
40S ribosomal protein S18 (*Rs18*)	17.1	*S. cerevisiae*	D'Amato et al., 2011
40S ribosomal protein S19-A (*Rs19A*)	15.9	*S. cerevisiae*	D'Amato et al., 2011
Eukaryotic initiation factor 4A (*Tif1, Tif2*)	44.7	*S. cerevisiae*	Branconi *et al.,* 2011
Eukaryotic translation initiation factor 5A-1 (*Tif51a*)	17.1	*S. cerevisiae*	Branconi *et al.,* 2011; Insenser *et al.,* 2010
Elongation factor 3A (*Yef3*)	116.0	*S. cerevisiae*	Branconi *et al.,* 2011
Actin (Fragment) (*Act1*)	41.7	*S. cerevisiae*	Insenser *et al*., 2010; Branconi *et al.,* 2011
Protein BMH1 (*Bmh1*)	30.1	*S. cerevisiae*	Pardo *et al.,* 2000; Branconi *et al.,* 2011
Glucan 1,3-beta-glucosidase I/II (*Exg1*)	51.3	*S. cerevisiae*	Insenser *et al.,* 2010; Branconi *et al.,* 2011
Cell wall mannoproteins PST1 (*Pst1*)	45.8	*S. cerevisiae*	Insenser *et al.,* 2010; Branconi *et al.,* 2011
Fatty acid synthase subunit alpha (*Fas2*)	20.7	*S. cerevisiae*	Branconi *et al.,* 2011
Plasma membrane ATPase 1 (*Pma1*)	99.6	*S. cerevisiae*	Branconi *et al.,* 2011
Ribonucleoside-diphosphate reductase small chain 2 (*Rnr4*)	40.0	*S. cerevisiae*	Branconi *et al.,* 2011

Until recently, the chemical nature of proteins responsible for wine turbidity remained unclear (Batista *et al.,* 2009) as there were contradictory findings in literature. Hsu and Heatherbell (1987) and Hsu *et al.* (1987) observed that the lower pI and lower molecular mass proteins are the major and most important fractions contributing to protein instability in wines. The isoelectric points of proteins are an important parameter affecting both the solubility of proteins in wine and their ease of removal by clarifying agents. Dawes *et al.,* (1994) also confirmed that the isoelectric points of proteins is an important property affecting both the solubility of proteins in wine and their ease of removal by clarifying agents as the adsorptive capability of bentonite are dependent primarily on its cation exchange capacity. Recently, in a study by Batista and colleagues (2009), it was observed that haze formation could result from isoelectric precipitation of the proteins occurring only at higher pH values (pH 3.8). Mesquita *et al.* (2001) observed that wine proteins were increasingly heat-stable when the pH of the solution in which the proteins were dissolved increased from wine pH to 7.5, thus concluding that the pattern of protein instability with increasing temperature is typical of each wine and is not determined by the

proteins but maybe controlled and determined by a combination of non-protein factors. However earlier studies conducted by Waters (1991) and Waters *et al.,* (1992) revealed that all the major wine protein fractions are present in wine hazes and all have been shown to be heat unstable.

It is now believed that pathogenesis-related proteins (PR) specifically thaumatin-like proteins and chitinases from grape are most commonly responsible for wine haze formation (Esteruelas *et al.,* 2009; Batista *et al.,* 2009; Falconer *et al.,* 2010). This presumption was based on the observation that the PR proteins are wine pH stable and persist through the vinification process (Waters e*t al.,* 1996; 1998) and also based on a thermal unfolding study of grape thaumatin-like protein and chitinase. Contradicting these results are findings made by Fusi *et al.* (2010), who observed that all the wine proteins were responsible for haze formation. A direct correlation between protein concentration and haze instability of the investigated wine samples was also observed by the same authors. Mesquita *et al.* (2001) demonstrated that the addition of a protein of non-wine origin (bovine serum albumin) to a protein-free wine did not alter the typical pattern of haze formation of the wine.

Chitinases have been shown to play a major role in wine hazing as they are the most prone to precipitation and a linear correlation was found to occur between chitinases content in wine and haze formed (Marangon *et al.,* 2010c; Marangon *et al.,* 2011b). In a study by Marangon *et al.* (2011b), thaumatin-like proteins were detected in the insoluble fraction by SDS-PAGE analysis but had no measurable impact on turbidity using differential scanning calorimetry thus confirming that the chitinases are the most likely candidate causing haze formation in wine. However in a study by Esteruelas *et al.* (2009), besides thaumatin-like proteins described by other authors as present in haze (Waters *et al.,* 1996, 1998), β-(1.3) glucanase and ripening-related protein grip22 precursor were also found in haze of Sauvignon white wine.

Besides being stable under wine conditions, PR proteins are also resistant to yeast proteases (Waters *et al.,* 1998), and glycosylation is thought to confer additional stability to these proteins

(Helenius and Aebi, 2004). The glycosylation status of many wine proteins remains however unclear (Batista *et al.,* 2009). The stability of PR proteins and their resistance to proteolytic attack could be explained by the observation that grape proteins that cause haze in wine exist as tightly wound globular entities held together with disulfide bridges and contain few, if any, exposed loops for proteases to attack (Figure 2.2). Heat and ethanol can cause the unfolding of the PR proteins and thus making them accessible to proteolytic enzymes (Pocock *et al.,* 2003).

Wu and Lu (2004) put forward a hypothesis that haze active proteins are high in the amino acid proline. When gelatin, a proline-rich protein, was used by these authors in a model system with tannins, a reduction in the protein from solution was observed as compared to when bovine serum albumin (BSA) was used. It is assumed that proline prevents the formation of an alpha helix and favors a more open protein structure therefore facilitating access to polyphenols (Siebert *et al.,* 1996; 2006; 2009). Grape PR proteins are however not proline-rich proteins in comparison to hordein, the barley prolamin containing about 20 mo% proline used for beer brewing (Siebert, 2006). Furthermore, Waters *et al.* (1996) detected no proline rich proteins among other amino acids from major wine haze proteins using the pre-column derivatization technique.

2.3.2 Organic acids

Organic acids found in wine include tartaric, malic, citric, gluconic, lactic acids with malic and tartaric acids being the most abundant (Ribĕreau-Gayon *et al*., 2006). Their concentrations in wines are dependent among other factors on the variety, environmental conditions and microflora's metabolic events occurring during winemaking and storage. Organic acids are known to interact with wine components which include phenolic acids, free amino acids, pectic compounds, tannins and sulphate ions (discussed in 2.3.4) thus preventing their interaction with proteins (Vernhet *et al,* 1999a, b; Batista *et al.,* 2010). In a current study by Batista *et al.* (2010), it has been revealed that organic acids exhibit a stabilizing effect on the protein haze formation potential of wines. This effect has been attributed to electrostatic interactions that depend upon

the acid pKa, protein pI values and the medium pH. Gallic acid has for some time been suspected to interact with the wine proteins, leading to their precipitation (Waters *et al.,* 2005). However, this has been contradicted by findings made by Pocock *et al.* (2007) and Batista *et al.* (2010) who failed to detect haze in model wine solution in the presence of gallic acid.

2.3.3 Polyphenols

Phenolic compounds are secondary plant metabolites found in all fruits and vegetables and are thought to be involved in the defense of plants against invading pathogens (Friedman and Jurgens *et al.,* 2000). Wine protein reactivity with endogenous grape tannins has been extensively studied (Siebert, 2006; Fenchak *et al.,* 2002; Charlton *et al*., 2002; Laborde *et al.,* 2006). The interactions between proteins and polyphenols are thought to contribute to haze formation in beverages including beer and wine (reviewed by Siebert *et al.,* 1996; 2006; 2009) due to the formation of protein-polyphenol complexes. Phenolic compounds such as proanthocyanidins are known to interact with proteins over wide pH and temperature ranges. Sibert *et al.* (1996) observed a 7 fold increase in haze levels in a model solution containing same amounts of protein gliadin with a pI of about 8 and polyphenol when pH was raised from 3 to 4. The protein-polyphenol haze formation is to a greater extent affected by the ratio of haze-active protein to haze-active polyphenol, the largest amount of haze occurring when the numbers of polyphenol binding ends and protein binding sites are nearly equal (Siebert, 2009). Concurring with these results are findings by Marangon *et al*. (2010c) who demonstrated that haze formation in white wines is related to hydrophobic interactions occurring among proteins and tannins and these interactions are thought to occur on hydrophobic tannin-binding sites, whose exposition on the proteins can depend on both protein heating and reduction. Moreover Esteruelas *et al.* (2011) observed that several phenolic compounds were present in the protein haze obtained from Sauvignon Blanc white wine. The phenolic compounds included tyrosol, trans-p-coumaric, trans-caffeic, vanillic, protocatechuic, syringic, gallic, ferulic, shikimic acids, (+)-catechin, ethyl coumaric acid ester and quercetin. The same authors also detected cyanidin after acid hydrolysis indicating the presence of procyanidins.

Other polyphenols that may participate or even trigger the appearance of wine haze formation include anthocyanins and tannins (Esteruelas *et al.,* 2009; 2011). Proanthocyanidins, refer to a larger class of polyphenols, called flavanols which form the group of tannins while anthocyanin refers to polyphenols that have colour belonging to the flavonoids group. Yokotsuka *et al.* (1991) observed that tannins isolated from Riesling and Koshu wines interacted with isolated grape juice proteins to form a haze in a tartrate buffer solution while Waters *et al.* (1995) detected the presence of proanthocyanidins (0.02–4.9% w/w) in heat-induced and natural hazes isolated from various white wines. However, white wine has low levels of phenolic compounds and anthocyanins (Laborde *et al.,* 2006; Marzia *et al*., 2010). Haze is primarily a problem in white wines and this fact may reduce the importance of protein-phenolic compound interactions. On the other hand, the high tannin content present in red wines, may result in precipitation of most wine proteins before clarification thus reducing haze active proteins in bottled wines. The polyphenols may be important in the presence of other wine components as observed in a multi-factorial study carried out by Fenchak *et al.* (2002) who observed that the interaction between pH, protein and polyphenol is important for haze formation. Marangon *et al*. (2010a) hypothesized that variations among the hydrophobicity level of different protein classes, affected by variations in wine matrix conditions such as redox-reduction and temperature fluctuations during storage, are involved in protein hazing of white wines. Pocock *et al.* (2007) observed that the individual or combined addition of caffeic acid, caftaric acid, epicatechin, epigallocatechin-O-gallate, gallic acid, or ferulic acid at typical white wine concentrations did not generate protein haze but PVPP (polyvinylpolypyrolidone) fining of wines resulted in a reduction in protein haze. This revelation could mean that phenolic compounds may play a modulating role in haze formation but when added at typical white wine concentrations tannins are not essential for protein haze formation.

2.3.4 Other factors

Not much attention has been given to the effect of non-proteinaceous wine components on haze formation. A study on how protein, polyphenol, sucrose, and pectin, along with pH and

ionic strength interact to cause wine haze by Fenchak *et al.* (2002) revealed that polyphenol and protein, interactions as well as polyphenol and pectin interaction were all significant factors contributing to wine haze formation. Polyphenols have also been implicated in causing hazes not only in white wine but also in beer and apple juice. A non-proteinaceous wine component that was initially referred to as Factor X was shown to be required for visible protein haze to be formed in commercial white wine was later identified as sulfate ions (Pocock *et al.,* 2007). This conclusion was based on the ability to reconstitute protein haze upon heating artificial model wine solutions (500 mg/l thaumatin, 12% ethanol, 4 g/l tartaric acid, with no protein haze) to which candidate components were added in the identification of factor(s) X. Batista *et al.* (2009) also confirmed the importance of the sulfate ion in causing haze at lower pH values (pH 2.8).

Several polysaccharides have also been associated with beverage hazes and these include arabinans in red wine, beta-glucan, starch and mannan in beers (Siebert, 2009). There have been contradictory findings on metal ions such as copper and iron regarding their involvement in wine hazes formation. Pocock *et al.* (2007) found that anions, acetate, chloride, citrate, phosphate, tartrate, and the wine cations, $Fe^{2+/3+}$ and $Cu^{+/2+}$, when added at typical white wine concentrations were not essential for protein haze formation. However, Besse *et al.* (2000) initially observed a decrease in copper concentrations in wine after haze removal, implying that copper was part of the protein precipitate.

Despite the fact that ethanol can be an important factor in wine haze formation, not much research has been carried out to determine its precise contribution to wine haze formation (Waters *et al.,* 2005). Mesquita *et al.* (2001) showed that addition of ethanol to a protein-free wine did not alter the typical pattern of haze formation of the wine at high temperatures. However, in the same study it was observed that wine polysaccharides did affect the characteristic behavior of wine by increasing protein instability under moderately high temperatures (40 to 50°C).

2.4. Methods used to assess haze in wine

Protein haze in bottled wines is observed as an amorphous fluffy white suspension or sediment composed primarily of protein (Waters *et al*., 2005). As bentonite and other treatments may have a negative impact, it is important for the winemaker to be able to judge whether a wine is stable or not. Protein instability does not correlate well with total protein concentration because individual proteins behave differently (Esteruelas *et al.,* 2009) and protein-based essays are therefore of limited value. Furthermore, other wine-components play a role in protein instability (Pocock *et al.,* 2007). Different tests have been developed and are based upon different types of procedures, most of which artificially induce protein aggregation and precipitation (Reviewed by Waters *et al.,* 2005; Pocock and Waters, 2006). These tests are however empirical and thus do not necessarily reflect changes and destabilization phenomena liable to occur in real wine storage conditions.

The level of fining agent such as bentonite addition required for stabilization is also based on stability tests results (Waters *et al.,* 2005; Sauvage *et al.,* 2010). These tests sometimes provide different results, which creates uncertainty (Toland *et al.,* 1996; Sarmento *et al.,* 2000; Waters *et al.,* 2005), and may result in excessive use of clarifying agents.

2.4.1 Heat tests

Heat test involves heating wine to a specific temperature. After cooling, absorbance is measured using a spectrophotometer or nephelometer to determine the turbidity of the heated wine. Based on work undertaken by Pocock and Rankine (1973), most winemakers and researchers heat wines to 80ºC for six hours (Vincenzi *et al*., 2005; Batista *et al.,* 2009). Wine is said to be hazy when the calculated difference in absorbance reading before and after heating is greater than 0.02 (Waters *et al*., 2005). This test is however considered by some winemakers as being too severe resulting in wines being over-fined with bentonite (Pocock and Waters, 2006). Several studies use other heating temperatures and cooling times (Gonzalez-Ramos *et al*., 2006 Sauvage *et al.,* 2010; Lolimo and Curioni, 2007). Esteruelas *et al.* (2009)

demonstrated that a fast heat test at 90^{o}C for 1 hour imitates the natural precipitate in terms of its chemical composition and therefore is likely a more appropriate stability test to be used. Pocock and Waters (2006) suggested a least severe stability test, heating wine to 80ºC for 2 hours, an derived lower dosage rates of bentonite. The determination of the precise amount of fining agent required to confer heat stability while maintaining all the sensory attributes of a wine remains therefore a challenge.

2.4.2 Spectroscopic methods

Near infrared spectroscopy (NIR) has been previously used to monitor the fermentation processes successfully (McLeod *et al.,* 2009). The feasibility of utilizing infrared spectroscopy for the prediction of haze formation in white wines resulting from heat and colloidal stability tests has been explored (Versari *et al*., 2011). Chemometric techniques such as partial-least squares regression (PLS) analysis can then be used to mine information and generate predictive models based on the physical-chemical properties of wine contained in the infrared spectrum. However there is still need for further IR analysis on a large number of wines with the aim of building a 'global' PLS model that takes into account all the factors that can influence the composition of grapes and wines. Including the current knowledge, there is also need to explore further kinetics of heat-induced precipitation of different protein classes with the interaction of non-proteinaceous wine components at different storage temperatures as temperature besides other factors is known to affect mainly protein unfolding rates (Falconer *et al.,* 2010; Marangon *et al.,* 2010a).

2.5 Haze reduction

2.5.1 Use of adsorbents and chelating agents to clarify wine

Bentonite is used in the wine industry to adsorb proteins responsible for causing haze through a cation exchange process (Pocock and Waters, 2006). Wine proteins are positively charged at wine pH, and thus can be exchanged onto bentonite, which carries a net negative charge (Høj

et al., 2000, Ferreira *et al.,* 2002). Bentonite treatment results in loss of between 3 and 10 % of wine volume as bentonite lees (Brown *et al.,* 2007). As a result of mutual flocculation with positively charged hydrocolloids and adsorption, bentonite interacts not only with proteins, but also with other molecules such as some volatile compounds, including a few odor-active molecules that are directly removed by bentonite through adsorption (Lambri *et al.,* 2010). Bentonite therefore results in a loss of both volume and aromatic properties of wine. Another drawbacks of using bentonite are the fact that it is non-recyclable thus posing disposal problems and as a result may not be sustainable over the long run as large doses of about 100-200 g/hl^{-1} are often employed in the wine industry (Sauvage *et al.,* 2010). Finally, bentonite handling is also of concern for occupational health and safety issues.

Recovering wine from the bentonite lees by filtration results in loss of flavours, and the wine generally is of poor quality. However, using difference testing, Pocock *et al.* (2003) reported that bentonite fining of a Chardonnay and Semillon wine had no effect on wine aroma and palate. This contrasts with previous findings made by Miller *et al.* (1985) who demonstrated reduced concentration of aroma compounds after bentonite addition to juice, must or wine.

More recently Pollnitz *et al*. (2003) elegantly confirmed that aroma compounds can be absorbed by bentonite, as did Cabaroglu *et al*. (2003), although the latter study found no sensory effect of bentonite fining of Gewürztraminer wine. Martinez-Rodriguez and Polo (2003) concurred with these findings and also observed similarity in sparkling wines when bentonite was added to the tirage solution. Bentonite has been reported to also affect the foaming properties of sparkling wines (Martinez-Rodriguez and Polo, 2003; Vanrell *et al.,* 2007). Developing a viable alternative treatment to remove PR proteins from wine prior to bottling would therefore be beneficial.

A pilot study by Salazar *et al.* (2006) on application of continuous stabilization of white wine protein using a column packed with zirconia was studied and compared to the traditional bentonite treatment. The treated wine was protein stable and the polyphenols removed was less

than 10 %, and similar amounts were removed from the wine regardless of residence time, while 20.6 % of polyphenol was removed using bentonite. A similar study carried out by Marangon *et al.* (2011a) also confirmed that zirconia is an excellent candidate for protein adsorption from wines. The physicochemical and sensory properties of wine treated with bentonite were similar to those of wine treated with zirconia. Zirconia has however been also shown to remove some metal ions, reduce acidity and slightly lower wine aroma and flavor intensity (Marangon *et al.,* 2010c; Marangon *et al.,* 2011a). The other drawback of using zirconia for wine stabilization is that large quantities are required for reducing or completely removing wine proteins (25 g/l in 72 hours) (Marangon *et al.,* 2010c; Marangon *et al.,* 2011a).

In a study by Trela (2008), an alternative method of standard protein stabilization for a model wine containing bovine serum albumin was investigated using phytic acid, a form of phosphorus storage in plants. Treatments of model wine with increasing phytic acid at initial BSA concentrations of 1 g/l and 0.2 g/l resulted in significant BSA reductions or its complete removal without changing the pH. However, the effects on both the sensory perception and the long-term stability of wines treated with phytic acid needs to be established under actual winemaking conditions. These results have to be interpreted with caution as the behavior of wine haze active proteins may differ to that of BSA. However, phytic acid (PA), or myo-inositol hexakis (dihydrogen phosphate), is a strong chelating agent especially for polyvalent cations such as iron and calcium over a wide range of pH values (Vasca *et al.* 2002) and an antioxidant present in all seeds. With its chelating properties, phytic acid can be a potential fining agent replacing bentonite treatment in wine (Trela, 2008).

2.5.2 Grapevine plants and seeds

Wild-type varieties of *Vitis vinifera* are susceptible to fungal attack, which results in the accumulation of the PR proteins and other natural defense mechanisms. The resultant grapes are thought to produce wines with varying amounts of PR proteins and consequently different haze forming properties. This might suggest that the breeding of grape varietals with the

objective of improving wine clarity could be the solution to protein wine haze formation. There is also need to exploit further mechanisms of increasing grape plant defense without over-expressing the PR proteins (Ferreira *et al.,* 2004). However, some contradictory data have been reported concerning the effect of growing and harvesting conditions of grapes on haze – causing protein levels. Bezier *et al*. (2002) and Robert *et al.* (2002) observed increased PR proteins in leaves and berries of grapevines infected with pathogens such as *Botrytis cinerea* and *Uncinula necator.* Contradicting these results are the findings made by Cilindre *et al.* (2007 and 2008), who observed a reduction in thaumatin-like and osmotin-like protein levels in the juice and wine produced from *Botrytis*-infected grapes. The authors suggested that the reduction could be due to proteolytic degradation of grape PR proteins by *B. cinerea* enzymes. Substantiating these results are findings of Girbau and colleagues (2004) who observed a reduction in protein levels in juice when *B. cinerea* was grown in the medium. Further research needs to be explored to extract the proteolytic enzymes from *B. cinerea*, if these enzymes have the potential to replace bentonite fining for protein stabilization. The production of extracellular proteases by plant pathogenic fungi is well documented (Abidi *et al.,* 2008).

2.5.3 Enological processes

2.5.3.1 Ultrafiltration

A number of studies have investigated the effectiveness of ultrafiltration as a bentonite substitute for protein stabilization (Hsu *et al*. 1987; Flores *et al*. 1990). However, the presence of residual proteins in the filtrate, together with high set up and running costs, and possible loss of organoleptic compounds (Miller *et al*., 1985), has rendered ultrafiltration unattractive for commercial practice.

2.5.3.2 Protein and polysaccharide clarifying agents

The use of casein (egg albumin) and potassium caseinate (derived from bovine milk), by winemakers with the aim of reducing or eliminating wine haze is a cause of concern. These agents are potentially hazardous to consumers as they are known to be allergens (Cereda *et*

al., 2010). The use of gelatin has been discontinued despite the observation that when it is combined with bentonite and silica gel gelatin gives very good clarification. The use of bovine casein was of serious concern due to some cases of bovine spongiform encephalopathy (BSE, mad cow disease) with some chance of being transmitted to humans (Regulation CE 2087/97, Council of October 20, 1997). The hunt for a substitute for gelatin has been explored by comparing vegetable proteins such as gluten with gelatin fining treatments. Marchal *et al.* (2002 and 2003) observed that turbidity decreased by 86% for the gluten/bentonite fining and by 60% for the gelatin/bentonite fining. However gelatin may also be an allergen to other wine consumers.

Wine stabilization is thought to require the use of negatively charged clarifying agents to remove proteins. Due to their negative charge, polysaccharides agar such as carrageenan, and alginic acid extracted from seaweeds at low pH, can electrostatically bind and precipitate positively charged proteins from aqueous solutions. Cabello-Pasini *et al.* (2005) observed that proteins were flocculated by seaweed polysaccharides, tannins were not adsorbed by agar, carrageenan, or alginic acid and carrageenan adsorbed and precipitated most proteins at concentrations surpassing 400 mg/l. Under the same study, it was also observed that the protein fraction adsorbed by agar, carrageenan, alginic acid, and seaweeds in Chenin blanc wine was also similar to the protein fraction adsorbed by bentonite (Cabello-Pasini *et al.,* 2005), indicating that these polysaccharide might have a greater wine stabilization capacity without modifying the tannin composition of wines as compared to bentonite (Salazar *et al.,* 2006).

Addition of chitin [poly(N-acetyl-1,4-β-D-glucopyranosamine)] has been shown to remove wine protein involved in haze formation more specifically than bentonite by targeting the class IV chitinase of grape origin involved in white wine instability (Vincezi *et al.,* 2005). This is likely due to that class IV chitinases bear a chitin-binding domain which most likely acts as a specific interaction of these wine proteins with chitin (Graham and Sticklen, 1994). Vincezi *et al.* (2005) showed that the effect of chitin on white wine stabilization allowed a reduction of up to 80% of

the haze induced by the heat test, which corresponded to a reduction in wine protein content of less than 29 % while bentonite fining, although allowing a complete stabilization, resulted in the removal of almost all the proteins from wine. Other glycoproteins with haze protective activity in wine include a wine arabinogalactan-protein, gum arabic, and an apple arabinogalactan-protein (Pellerin *et al.*, 1994).

2.5.3.3 Proteolytic enzymes and heat

Despite their globular nature with few exposed loops accessible to yeast proteases (Tattersall *et al.* 1997), wine haze proteins are susceptible to *B. cineria* fungal peptidases, and some studies have been exploring the possible use of non-yeast peptidases to clarify wines. The principle of using proteases to prevent chill-haze in beer was already patented in 1978 (Patent 974373735). The use of enzymes targeting the proline-rich proteins was recently investigated using proline-specific, microbial endo-proteases to prevent chill-haze in beer (Lopez and Edens, 2005). However, proteases and heat treatments are rarely used during winemaking possibly due to the perception that heating under any condition is detrimental to wine quality. Moreover, the proteins that are responsible for haze formation are known to be difficult to degrade. Nevertheless, a study by Pocock *et al.* (2003) revealed that proteins can be degraded after being denatured at 90 ^{o}C with proteolytic enzymes, and that the sensory impact of heating and proteolytic enzyme treatment on wine was negligible.

2.5.3.4 Mannoproteins and aging wine on lees

Parietal mannoproteins (Figure 2.3) have attracted significant interest in the wine industry due to their reported ability to contribute to sensorial wine quality and to prevent haze formation. The production and release of mannoproteins to wine is yeast strain dependent (Feulliat, 2003; Giovani *et al*., 2010). However, it is generally considered that the quantity of mannoproteins produced by yeast under wine making conditions is too low to be of much commercial significance (Dupin *et al.,* 2000b; Feuillat, 2003). The concentration of mannoproteins released during alcoholic fermentation ranges from 100-150 mg/l (Chalier *et al.,* 2007). Dupin *et al.*

(2000b) demonstrated that in order to reduce wine haze by 40 %, 400 mg/l of invertase would be required. Thus alternative methods for mannoprotein production were investigated (Feulliat, 2003; Brown *et al.,* 2007). Genetically engineered wine yeast strains for increased mannoprotein release have been constructed taking advantage of the current knowledge concerning *S. cerevisiae* cell wall biology (Brown *et al.,* 2007; Gonzalez-Ramos *et al.,* 2009). With the aim of satisfying current consumer preferences winemakers can thus use oenological and biotechnological tools to increase mannoprotein levels, and as a consequence, the reduction in haze and improvement of wine quality can be achieved.

In studies by Moine-Ledoux and Dubourdieu (2002) and Fusi *et al.* (2010), the existence of a linear correlation between haze potential and both protein concentration and protein/glycoprotein ratio was observed. Despite the fact that wine components such as yeast mannoproteins could explain absence of haze formation in synthetic wine (Dupin *et al.,* 2000a; b; Gonzalez-Ramos *et al.,* 2008), there is no explanation for the absence of haze in model wine solutions containing alcohol, grape proteins and tartaric acid (no yeast mannoproteins). Moreover, attempts by Bayly and Berg (1967) to correlate the total wine protein contents to their sensitivity to protein haze failed. Wines aged on yeast lees have lower haze potential than wines aged without lees and this has been attributed to the protective effect of the mannoproteins released from yeast cell walls (Dupin *et al.,* 2000a; b). Invertase and haze protection factors (Hpf1p and Hpf2p) have been shown to lower haze formation by competing with grape-derived proteins for some unknown factors in wine required to form large light scattering protein aggregates responsible for haze (Dupin *et al.,* 2000a; b; Brown *et al.,* 2007). Overproduction of Hpf2p protein reduced turbidity by up to 40 % when it was added to wine (Brown *et al.,* 2007). Invertase and haze protection factors (Hpf1'p, Hpf1p and Hpf2p) are all glycosylated mannoproteins.

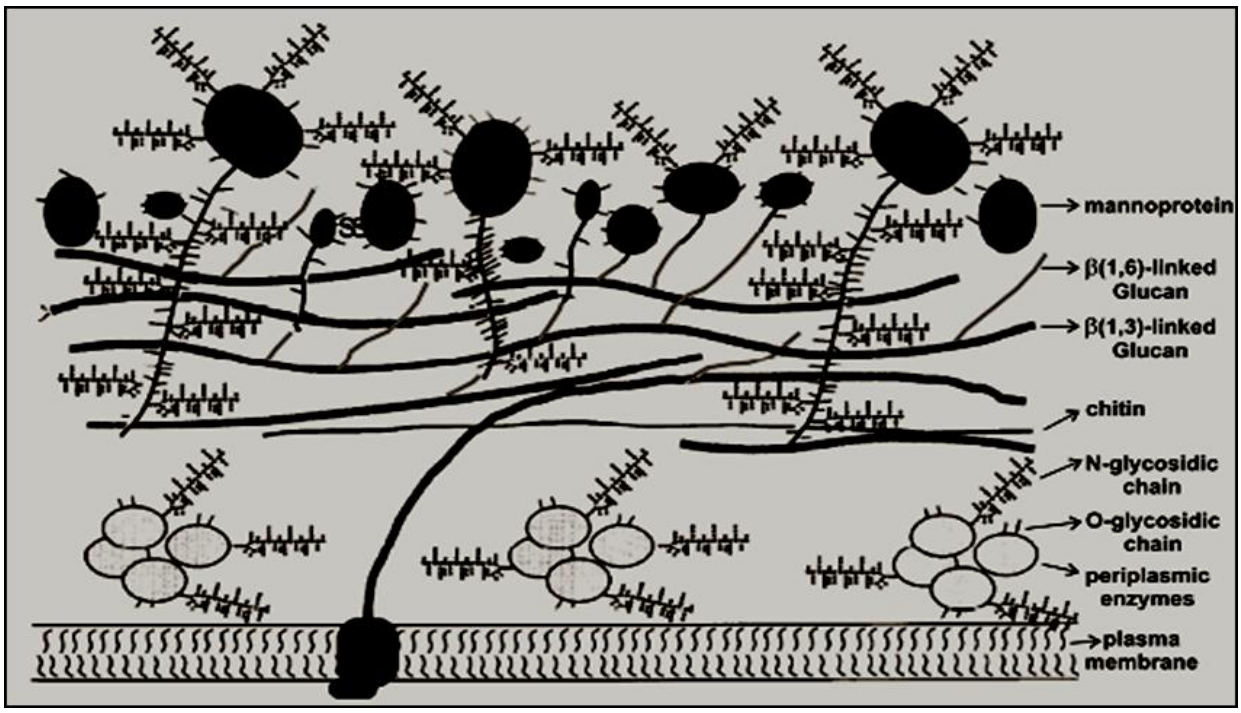

Figure 2.3: Yeast cell wall (Schreuder *et al.,* 1996). The cell wall is located outside the plasma membrane and consists of two layers. The inner layer is made of β-1,3-glucan and β-1,6-glucan layer cross linked with chitin and the outer layer consist of mannoproteins most of them covalently linked to the inner glucan layer. Periplasmic enzymes such as invertase are trapped between the plasma membrane and the inner skeletal layer.

Schimdt *et al.* (2009) demonstrated the importance of glycan outer chain length of Hpf2-Pp in haze prevention. Glycosylation is thought to have a direct role in haze protective activity by providing an “active” site for interaction with haze forming proteins or other wine components. It may also have an indirect role by maintaining stability of the mannoprotein peptide backbone. In a study by Poncet-Legrand *et al.* (2007), low molecular weight mannoproteins of about 51 kDa stabilized the wines while high molecular weight molecules of about 337 kDa had no impact in standard wine conditions (pH 3.4 buffers containing 2 g/l tartaric acid and 12 % ethanol). At high ionic strengths or low ethanol concentrations, the authors found that all mannoprotein fractions prevented tannin aggregation and precipitation. This was accredited to steric stabilization where medium and low molecular weight polymers were more efficient than high molecular weight polymers, and all polysaccharides tested prevented polyphenol precipitation when ionic strength was increased (demonstrated in Figure 2.4 below). In a study by Gaudalupe and Ayestaran (2008), mannoprotein addition did not modify the content and composition of either monomeric anthocyanins or other monomeric phenolics as similarly predicted for polysaccharides by

Poncet-Legrand *et al. (*2007). Poncet-Legrand *et al*. (2003) reported that mannoproteins prevent tannin aggregation and precipitation. However, Riou *et al.* (2002) observed that mannoproteins did not prevent initial tannin aggregation, but slowed down particle size evolution. In a separate study, reduction of wine haze was observed to be due to a N-glycosylated, 31.8 kDa mannoprotein that corresponds to a parietal invertase fragment of *Saccharomyces cerevisiae* (Moine-Ledoux and Dubourdieu, 2002). Further studies are needed to understand the molecular mechanism of yeast mannoproteins in preventing haze and the interaction of these mannoproteins with wine components in reducing haze.

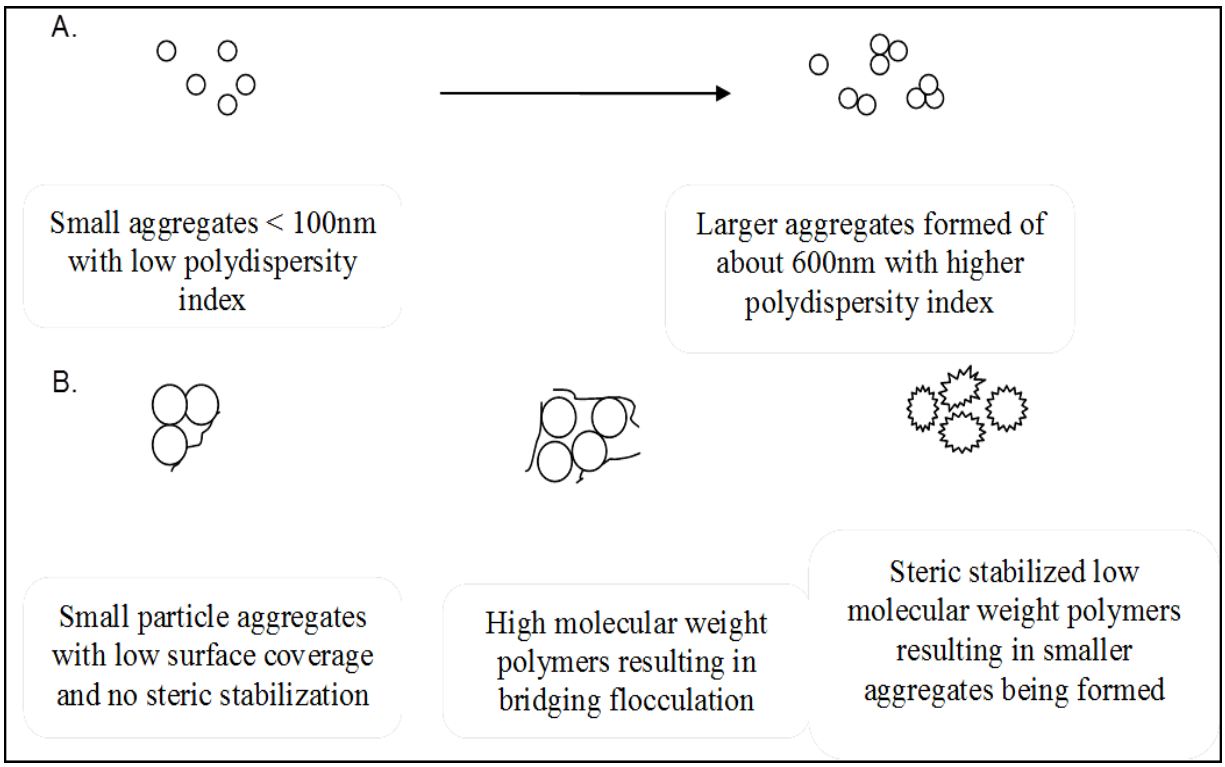

Figure 2.4: Scheme of (A) polyphenol particle self-aggregation and (B) steric stabilization or bridging flocculation of tannin particles by (Taken from: Poncet-Legrand *et al.,* 2007).

2.5.3.5 Yeast extract

Due to the encouraging results of using yeast mannoproteins for wine clarification mentioned afore, the use of additive methods based on yeast cell extracts have been attempted by researchers (Dupin *et al.,* 2000b; Lomolino and Curioni, 2007). The treatment of white wines with yeast mannoproteins from yeast wall degradation was approved in 2001 by the International Organization of Vine and wine (OIV) and amended in 2005 by the International

Code of Oenological Practices (resolution: Oeno 4/01; 15/05). Several methods for mannoprotein extraction from yeast cells have been investigated, including chemical (reducing or chelating agents and detergents), enzymatic, and physical techniques (temperature treatments) (Dupin *et al.,* 2000b; Lomolino and Curioni, 2007). The choice of extracting reagents and techniques, the sequence of extraction methods, and the use of either intact cells or purified cell walls as the starting material and the effect of the produced material on haze has been also studied. Mannoproteins loosely associated with the cell wall are extracted from the cell wall using sodium dodecyl sulphate (SDS), whereas those that are covalently-linked are solubilized by mild alkali such as dithiothreitol (DTE) or β-mercaptoethanol (Figure 2.5) for their extraction or glucanase digestion of the glucan layer (Pardo *et al.,* 1999; Dupin *et al.,* 2000b). The glucanase-extractable mannoproteins have three domains, signal peptide at the N-terminus, the middle serine and/or threonine domain and a putative glycosyl phosphatidyl-inositol (GPI) attachment signal (Verstrepen and Klis, 2006). Pir proteins are covalently attached to chitin or glucan but can also be extracted from the cell by a mild sodium hydroxide treatment (Kapteyn *et al.,* 1999).

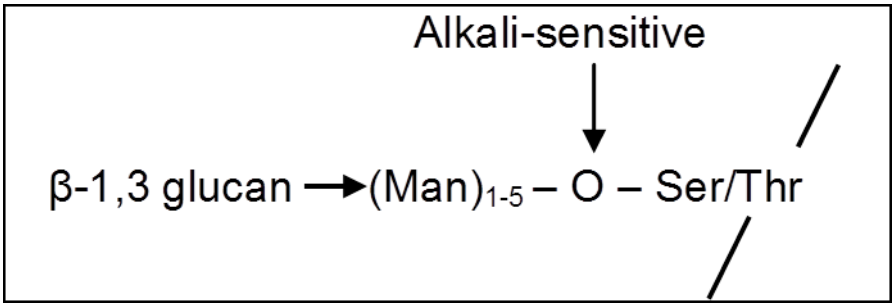

Figure 2.5: Hypothetical linkage between a Pir-CWP and a β1,3 glucan polymer. A β1,3 glucan molecule is linked with its reducing end to an oligomannoside, O-linked to a serine or threonine residue of a Pir-CWP and the glycopeptides linkage is alkali-sensitive (Klis *et al.,* 2002)

When these methods for mannoprotein extraction were compared, digestion with Zymolyase of cells pretreated with DTE (dithioerythritol) and EDTA (ethylenediaminetetraacetic acid) gave the greatest yields of active material (Dupin *et al.,* 2000a). Heat treatment of cells with SDS also released low quantities of active material. Dupin e*t al.* (2000a) also observed that the haze

protective material (HPM) was non-covalently linked to other cell wall components and is loosely associated with the cell wall.

Regardless of the availability of techniques used to extract mannoproteins from the yeast cell wall, the possibility of stabilizing white wines by using yeast extracts is thought to be rather unrealistic or economically inconvenient because of the large quantity of fresh yeast cells that is required (Moine-Ledoux and Dubourdieu, 2002). However, on the basis of the study carried out by Lomolino and Curioni (2007), it appears that better extraction methods can increase the yield. For example, the simple reduction step with DDT (dichlorodiphenyltrichloroethane) of the yeast cell wall components can yield an extract that when added at about 1 g/hl is able to reduce haze formation in wine by 20%. There are other commercial yeast cell wall preparation products now available in the market (Lallemand and Laffort) such as Mannostab® that can be used for wine clarification.

2.5.3.6 Genetic engineering

Currently numerous research laboratories worldwide have obtained engineered strains possessing a wide range of optimized or novel oenological properties, capable of satisfying the demanding nature of modern winemaking practice (Schuller and Casal, 2005). The yeast cell wall consist of about 42 Pir and GPI-anchored mannoproteins (Vestrepen *et al.,* 2006; Klis *et al*., 2002) but the physiological role of most mannoproteins is unknown. Indeed, disruptions of many of the genes encoding these proteins do not affect major functions of the wall.

S. cerevisiae deletion mutants (*mnn1*Δ*, mnn2*Δ*, mnn4*Δ, and *mnn5*Δ), defective in different aspects of glycan processing revealed that the altered mannoprotein glycosylation profile impacted on the physical properties of Hpf2-Pp and its capacity to provide protection against protein haze formation in white wine compared to Hpf2 (Schimdt *et al*., 2009).

In separate studies conducted by Gonzalez-Ramos *et al.* (2008; 2009), wine yeast strains with the *KNR4* gene deleted released increased amounts of mannoproteins and the resultant strains produced wines showing attenuated responses in protein haze tests. *KNR4* codes for Knr4p, a regulatory protein required for the correct targeting of the Stl2p MAP kinase and it is also part of the main cell integrity pathway participating in the coordination of cell wall synthesis with bud emergence (Basmaji *et al.,* 2006; Gonzales-Ramos *et al*., 2009). Gonzales-Ramos *et al*. (2009) also revealed that deletion of *GPI7* or *FKS1* and *GAS1* in the EC1118 background also results in increased release of mannoproteins. However, the protein haze of wines fermented with these strains was observed to be similar to or higher than for the unmodified strain. Mannoproteins responsible for haze reduction may be specific and only produced by some yeast strains. *GPI7* encodes an enzyme required for the synthesis of the *GPI* anchor, a structure mediating the linkage of some proteins to the plasma membrane or to the cell wall, and most *S. cerevisiae* cell wall mannoproteins are synthesized as GPI-anchored precursors and covalently linked to the cell wall through a GPI anchor remnant (Richard *et al.,* 2002). *GAS1* encodes a glycoprotein of the plasma membrane with a β-1,3-glucanosyltransferase activity and is involved in the elongation of β-1,3-glucan branches (Mouyana *et al.,* 2000). There is therefore further need to manipulate the cell wall regulatory processes affecting the synthesis and the degradation of the yeast cell wall with the aim of producing yeast strains releasing large quantities of haze reducing mannoproteins.

Considering that yeast autolysis resulting in increased release of mannoproteins into wine can take several months under enological conditions (Feuillat *et al.,* 2003). As a result methods or techniques aiming at reducing this time period have been another biotechnological target. In a study by Teparic *et al.* (2004), it was found that the lack of the non-covalently bound wall proteins (non-covalently linked, SDS-soluble wall proteins) Scw4p, Scw10p and Bgl2p increases the mortality of *Saccharomyces cerevisiae* cells grown exponentially under standard laboratory conditions, as assayed by methylene blue staining. However, the autolytic ability of the mutant strains was not assessed. In a study by Zhang *et al.* (1999), a genetically controlled cell lysis

was achieved in *S. cerevisiae* by the repression of *PKC1* gene which regulates the synthesis of cell wall glucan. In the same study, a double mutant, carrying both pMET3-SRB1/PSA1 and pMET3-*PKC1* cassettes in place of *SRB1/PSA1* and *PKC1,* was constructed and found to permit the efficient release of both homologous and heterologous proteins. To shorten time of aging wine on lees and production cost, such strains can be used in wine making conditions to increase the released mannoproteins with the aim of improving wine quality. In a separate study by Cebollero *et al.* (2009), genetic engineering techniques were used to construct an autolytic industrial strain by expressing the csc1-1 allele from the *RDN1* locus. The expression of this mutant allele, that causes a ‘‘constitutive autophagy phenotype,’’ resulted in accelerated autolysis of the recombinant strain. Whether the wine industry will use genetically modified wine yeast strains in future remains highly uncertain. Besides, the use of such autolytic strains may also result in the production of off-flavours in wine due to several reasons such as the oxidation of lipids. Nevertheless, the data suggest that similar results should be achievable through standard breeding and selection strategies.

2.6 Future perspectives

Understanding haze formation in wine is important for the future development of improved methods to reduce this serious problem. Protein haze formation in wines is a dynamic process with several wine protein and non-proteinaceous components being responsible for causing wine haze. Although proteomics has the potential to identify the proteins involved in haze formation and allow significant progress to be made, its use is not yet widespread.

A standard test needs to be established to assess wine for its potential to form haze. Moreover, results must also be validated using a large selection of wines because wine composition is known to influence protein heat-stability and aggregation (Mesquita *et al.,* 2001). Most wines containing the same set of structurally related proteins, differ in the micro-heterogeneities evidenced within a same protein family (Monteiro *et al.,* 2001; Mesquita *et al*., 2001; Sauvage *et al.,* 2010) which is wine-dependent and thus induce conflicting results (Mesquita *et al.,* 2001).

There is also a need to correlate the test results with realistic storage conditions to validate the protein stability of wine.

Despite decades of research being carried out, the mechanism for protein haze formation in wines also remains at least in part to be elucidated. In addition to the identification of the role played by other wine compounds in haze formation, additional information with regards to the sensitivity of different wine proteins to heat induced precipitation and their adsorption by bentonite or other clarifying agents is essential. Besides assessing the impact of clarifying agents on wine quality, it would be useful to determine and characterize the rate of clarifying agent reactions under various wine conditions, especially the influence of pH, temperature, and ethanol content, and the extent of potential clarifying agent-metal interactions if any. Yeast derived mannoproteins may also have immense benefits for the winemaking process, specifically in protein stability and aspects of wine quality. Therefore given the potential use of mannoproteins in wine clarification, an understanding of their nature and of their kinetic of release would be important. Alternative methods of increasing mannoprotein levels need to be entertained and explored such as screening of yeast strains producing large amounts of mannoproteins. It is also imperative to explore the genetic improvement of industrial wine yeasts for mannoprotein production. Finally, it must be kept in mind that many of the studies on wine haze have to be interpreted with care as exploratory work is mostly conducted on a laboratory scale and in model solutions under controlled conditions, which do not necessarily reflect the circumstances encountered during standard industrial winemaking.

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Chapter 3

Research results I

Effect of different yeast strains on protein wine haze formation in model wine and Chardonnay must

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Effect of different yeast strains on protein wine haze formation in model wine and Chardonnay must

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3. Abstract

Protein haze in wine is most commonly the result of slow denaturation of wine proteins and is one of the many challenges faced by winemakers. To stabilize wines, clarifying agents such as bentonite are used, but such treatments have a number of well-known disadvantages, and it remains a challenge to find more suitable techniques to counteract or prevent wine haze. Yeast mannoproteins have been previously shown to reduce wine haze levels, and several reports have focused on the development and selection of yeast strains which produce high levels of mannoproteins during fermentation. However, while a large number of commercial wine yeast strains are marketed internationally, little information about the inherent haze-protective capacities of these strains exist. In this study, we evaluated a number of commercial wine yeast strains, as well as several strains overexpressing mannoprotein-encoding flocculation genes, *FLO1*, *FLO5*, and *FLO11* to determine the effect on wine haze formation in synthetic wine and in chardonnay grape juice. Data showed very different results when assays were carried out in grape must compared to synthetic must, highlighting possible problems with the experimental procedures commonly used for the evaluation of haze protection. For this reason, we also evaluated several parameters which may impact on the accuracy of the haze formation tests as used by other research groups. Chardonnay grape must fermented by *Saccharomyces paradoxus* strains revealed significantly greater haze protective ability than *Saccharomyces cerevisiae* strains. It was further observed from sporulating a *S. cerevisiae* and *S. paradoxus* hybrid strain that the haze protection phenotype depicted by the *S. paradoxus* strains is a polygenic trait. In addition, increased expression of *Flo* genes and associated cell surface properties did not reduce wine haze.

Key words: Yeast strains, protein haze, fermentation media

3.1 Introduction

The specific cause of haze formation in white wine during storage remains unclear (Dufrechau *et al.,* 2010, Esteruelas *et al.,* 2011*,* Marangon *et al.,* 2011a; Marangon *et al.,* 2011b). Pathogenesis-related proteins (PR), and more specifically thaumatin-like proteins and chitinases derived from the grapes have been most commonly implicated in the formation of such haze (Esteruelas *et al.,* 2009; Batista *et al.,* 2009; Falconer *et al.,* 2010), yet the underlying mechanisms remain poorly understood. Factors such as processing of wine, pH (Batista *et al.,* 2009), alcohol concentration (Mesquita *et al.,* 2001), temperature storage of wine (Mesquita *et al.,* 2001) and metals (Besse *et al.,* 2000) may contribute to wine haze. Mesquita *et al.* (2001)

observed that the pattern of protein instability with increasing temperature is typical of each wine and suggested that haze is not only determined by the grape proteins but also by a combination of non-protein factors. Such factors include polyphenols, polysaccharides, and metal ions (Fenchak *et al.,* 2001, Pocock *et al.,* 2007). Bayly and Berg (1967) were unable to relate the total wine protein contents to the degree of protein haze pointing to specific proteins (Esteruelas *et al.,* 2009) or other wine components being involved in haze formation (Pocock *et al.,* 2007). Furthermore, haze does not develop in model wine solutions containing alcohol, grape proteins and tartaric acid, and sulphate ions have been shown to be essential for haze formation in white wines (Pocock *et al.,* 2007, Marangon *et al.,* 2011a, Marangon *et al.,* 2011b). A recently proposed mechanism of wine haze formation is based on the effect of time-temperature variation on protein aggregation (Dufrechau *et al.,* 2010, Marangon *et al.,* 2011a). Current data by Marangon *et al.* (2011b) using a model wine system revealed that wine haze formation is also influenced by ionic strength and ionic content of model wine. Model systems have frequently been used to investigate factors causing haze in wine (Fenchak *et al.,* 2001; Trela *et al.,* 2008; Dupin *et al.,* 2000, Batista *et al.,* 2010). However they fail to completely replicate the complexity of wines.

The most common method used by wine makers to prevent haze involves the use of bentonite clay that adsorbs positively charged proteins and thus stabilises the wine (Sauvage *et al*., 2010). However, the lack of a standardized haze assay results in inconsistent applications of clarifying agents. The appropriate dosage of bentonite is most commonly determined by a heat test (Esteruelas *et al.,* 2009, Pocock and Waters, 2006). A comparison of different protein stability haze assays by Esteruelas *et al.* (2009) led to the recommendation that rapid heating yielded a precipitate most similar in chemical composition to the haze precipitate formed under natural conditions. However, there is considerable variation in the heating temperatures, heating time and haze measurement wavelength used for the heat assay (Fenchack *et al*., 2011, Brown *et al.,* 2007; Batista *et al.,* 2009; Gonzalez-Ramos *et al.,* 2009) thus pointing to the need to standardize the assay further. Furthermore, it has been suggested that addition of bentonite

may have a negative impact on wine quality (Salazar *et al.,* 2006) and further investigations for alternative methods to prevent or reduce wine haze formation are warranted.

During winemaking, yeast mannoproteins are released during alcoholic fermentation by secretion and autolysis (Palmisano *et al*., 2010; Rowe *et al.,* 2010; Quiros *et al.,* 2011) and are believed to reduce the development of haze during wine storage (Dupin *et al.,* 2000, Rowe *et al.,* 2010). Yeast strains normally used in wine making release quantities of mannoproteins that are too low to be of enological significance and subsequently a demand for wine yeast strains able to release more mannoproteins has developed. Aging of wine on lees is an alternative approach that can be used to increase the release of yeast mannoproteins (Feulliat *et al.,* 2003) but not all wines are aged and therefore this method has limited application.

While it is generally thought that current wine yeast strains do not secrete sufficient amounts of mannoproteins to protect wine from haze formation, little data are available about the relative ability of existing strains to contribute to wine stabilization. In this study, the differences in parameters used in the heat protein stability assays were evaluated and an assay method similar to that recommended by Pocock and Waters (2006) was used. The data show that the development of wine haze depended upon the strains of *S. cerevisiae* and *S. paradoxus* used to ferment grape must or synthetic medium either supplemented with bovine serum albumin or thaumatin to generate haze. Surprisingly, haze assays in real grape juice fermentation and in synthetic must yielded opposite results, suggesting that haze assays as carried out in many research projects may misrepresent the haze-protective activity of proteins secreted by different strains. The nature and type of other mannoproteins responsible for wine haze reduction besides the haze protection factor proteins and invertase (Brown *et al.,* 2007) still remains to be further elucidated. As the genetic determinants involved in the secretion of mannoproteins during wine fermentation have only been investigated to a limited extent (Gonzalez-Ramos *et al.,* 2009), yeast strains overexpressing *FLO* genes encoding mannoproteins were also evaluated in this study for their wine haze protecting capacity. However these strains yielded no

reduction in haze when compared with their wild type parental strains in fermented synthetic wine medium and grape must.

3.2. Materials and methods

3.2.1 Preparation of model wine synthetic wine medium and Chardonnay must

A model wine solution containing 4 g/l tartaric acid and 12% (v/v) ethanol dissolved in deionized water was prepared as described by Batista *et al.* (2009). Sodium hydroxide was used to adjust the pH to 3.3. Varying amounts of protein were added as bovine serum albumin (BSA; Sigma-Aldrich, catalogue number, A7906; Dupin *et al.,* 2000) and potassium sulphate (Sigma-Aldrich, catalogue number, P0772). A chemically defined synthetic grape medium (MS300) was prepared (Bely *et al.,* 1990) and consists of a basal medium of (per litre) 100 g glucose, 100 g fructose, 3 g tartaric acid, 0.3 g citric acid, 6 g malic acid, 2 g potassium dihydrogen phosphate, 0.2 g magnesium sulphate, 0.3 g ammonium sulphate, 0.3 g myo-inositol, pH 3.3 and sterilized at 121 ^{o}C for 15 min. To the basal MS300 medium, 65.45 ml of stock solution A, 5 ml stock solution B, 50 ml stock solution C and 5 ml stock solution D were added and made up to 1 litre with sterile distilled water. Stock solution A contained (per litre) 1.4 g tyrosine, 13.7 g tryptophan, 2.5 g isoleucine, 3.4 g aspartic acid, 9.2 g glutamic acid, 28.6 g arginine, 3.7 g leucine, 5.8 g threonine, 1.4 g glycine, 38.6 g glutamine, 11.1 g alanine, 3.4 g valine, 2.4 g methionine, 2.9 g phenylalanine, 6 g serine, 2.5 g histidine, 1.3 g lysine, 1 g cysteine, and 46.8 g proline. Stock solution B contained 4 mg $MnSO_4$. H_20, 4 mg $ZnSO_4$. 7 H_20, 1 mg $CuSO_4$. 5 H_2O, 1 mg potassium iodine, 0.4 mg $CoCl_2$. $6H_2O$, 1 mg $(NH_4)_6$ $Mo_7O_{24.}$ $4H_2O$ and 1 mg boric acid. Stock solution C contained (per litre) 0.03 mg (d-) biotin, 3 mg thiamine, 3 mg pyridoxine. HCl, 20 mg nicotinic acid and 15 mg d-pantothenic acid. A 100 mg/l stock solution of biotin was prepared and 0.3 ml was added to stock solution C. Stock solution D contained 0.05 ml Tween 80, 1.5 mg ergosterol and 0.5 mg sodium oleate to permit anaerobic growth. All stock solutions were filter sterilized. Chardonnay grape berries hand harvested at Stellenbosch (South Africa) in 2010 were used to make grape juice for fermentations. The grape juice had a 23.1 Brix value, 237 g/l sugars, pH of 3.6, total acidity of 3.12 % and alpha amino nitrogen content of 183 mg/l.

3.2.2 Fermentation conditions

The fermentations were carried out in triplicate at 21^{o}C in 120 ml bottles with a 50 ml working volume without agitation and fitted with air caps. For the aging experiments, fermentations were carried out in 500 ml Erlenmeyer flasks with a total volume of 300 ml of either MS300 medium or Chardonnay grape juice. Yeast cell cultures were pre-cultured in YPD broth at 30° C overnight before inoculating 10^6 cells/ ml into MS300 or Chardonnay grape must. Commercial wine strains and strains over-expressing the *FLO* 1, 5 and 11 genes (including the non-flocculent parental strains, BM45 and VIN13) (Table 3.1), were used to ferment chemically defined MS300 medium and grape juice to dryness over 19 days. Residual glucose and fructose concentrations were less than 5 g/l as measured using a D-glucose/fructose kit (Amersham).

Table 3.1: *S. cerevisiae*[a], *S. paradoxus*[b] and hybrid[ab] yeast strains used in this study

Strain	Genotype	Source
BM45[a]	Industrial wine yeast strain	Lallemand Inc. (Montreal, Canada)
BM45-F5A[a]	*FLO5p::SMRA-ADH2p*	Govender *et al.,* 2010
BM45-F11A[a]	*FLO11p::SMRA-ADH2p*	Govender *et al.,* 2010
VIN13[a]	Commercial yeast strain	Anchor Yeast (Cape Town, South Africa)
VIN13-F1A[a]	*FLO1p::SMRA-ADH2p*	Govender *et al.,* 2010
VIN13-F5A[a]	*FLO5p::SMRA-ADH2p*	Govender *et al.,* 2010
VIN13-F11A[a]	*FLO11p::SMRA-ADH2p*	Govender *et al.,* 2010
L2323[a]	Industrial wine yeast strain	Lallemand Inc.
WE372[a]	Industrial wine yeast strain	Anchor Yeast
N96[a]	Commercial yeast strain	Anchor Yeast
NT50[a]	Commercial yeast strain	Anchor Yeast
L2226[a]	Commercial yeast strain	Lallemand Inc.
Fermicru XL[a]	Commercial yeast strain	DSM Food Specialties B.V. (Netherlands)
NT112[a]	Commercial yeast strain	Anchor Yeast
SC22[a]	Commercial yeast strain	Bio Springer (France)
D254[a]	Commercial yeast strain	Lallemand Inc. (Montreal, Canada)
EC1118[a]	Commercial yeast strain	Lallemand Inc. (Montreal, Canada)
Exotic[ab]	Commercial yeast strain VIN13×RO88 hybrid	Mocke, 2005
P 01-167[b]	Industrial wine yeast strain	Phaff Yeast Collection (University of California, Davis, CA, USA)
P 01-208[b]	Industrial wine yeast strain	Phaff Yeast Collection
P 01-146[b]	Industrial wine yeast strain	Phaff Yeast Collection
RO88[b]	Industrial wine yeast strain	Redžepović *et al.,* 2003

In some instances, MS300 medium was supplemented with BSA or thaumatin from *Thaumatococcus daniellii* (a tropical flowering plant; Sigma-Aldrich, Catalogue number T7638) to a final concentration of 0.5 g/l. Fermentations were monitored by measuring weight loss and

briefly agitated before weighing. Fermented medium containing yeast lees was aged at 21^{o}C. Samples were taken bi-weekly for the haze assays. During the aging period, air caps were kept on to exclude oxygen. Samples were only agitated before sampling.

3.2.3 Heat stability test

Before performing the heat stability test, BSA and potassium sulphate were added to fermented MS300 medium (Pocock *et al*., 2007). No BSA or potassium sulphate was added to fermented Chardonnay grape juice before carrying out the haze assay. The heat stability of wine samples was determined as described by Pocock and Waters (2006) with all measurements made in triplicate with the appropriate controls. Briefly, the assay was carried out by centrifuging fermented MS300 medium and Chardonnay grape juice at 5,000 rpm for 5 min to remove cells. After taking absorbance readings at 520 nm, the model wine solution and the resultant supernatant from the fermented Chardonnay juice and MS300 medium were heated at 80°C for 2 h and then cooled to 4°C for 16 h. For subsequent aging experiments 1 g/l each of BSA and potassium sulphate were used whereas 0.5 g/l was used for experiments when haze formation differences were measured after alcoholic fermentation. A_{520} was measured after acclimatization at room temperature for 30 min. Haze was measured by calculating the difference in absorbance before and after heating of the sample (Waters *et al*., 1992). Samples were considered protein unstable when the difference in absorbance between heated and unheated controls is greater than 0.02 absorbance units (Pocock and Waters 2006; Pocock *et al.,* 2007).

3.2.4 Protein concentration determination

Total soluble protein of the fermented MS300 supernatant after centrifugation at 5, 000 rpm for 5 min was determined by the Bradford method (1976) with BSA as a standard. Mannoprotein content of wines at the end of alcoholic fermentation and on wines aged on lees was quantified using glucose and mannose content of wines using the D-mannose, D-fructose and D-glucose assay kit (Megazyme, K-MANGL).

3.2.5 Sporulation

Sporulation and micromanipulation were carried out on the yeast strain Exotic, a hybrid between RO88 and VIN13 using a protocol described by McClary *et al.* (1959) and Bauer (1992). A single colony of yeast was inoculated in 5ml YPD broth, grown overnight. The yeast cells were allowed to sediment and the sediment was discarded gently. The pellet was spotted on sporulation medium consisting of 0.18 % potassium chloride, 0.1 % glucose, 0.82 % sodium acetate trihydrate, 2.5 % yeast extract and 1.5 % agar (McClary *et al.,* 1959). The spores were prepared following a protocol described by Bauer *at al.* (1992). The cells were sporulated at room temperature for 7 days. The spores cell walls were lyzed with zymolyase (Sigma-Aldrich, catalogue # L7651). The cell suspension was centrifuged, and the pellet resuspended in 1 ml sterile water in order to obtain 10^5 to 5 x 10^5 asci/ml. 0.1 ml of diethyl ether was added and the suspension vortexed vigorously for 1 min. The cells were left at room temperature for 20 min, centrifuged and the pellet washed twice in sterile water. The asci were plated on YPD-agar (Biolab, Merck, South Africa). After 2 days, haploid colonies were distinguished from diploid colonies because of their smaller size.

3.2.6 Statistical analysis

The data are expressed as the mean ± standard deviation from three biological and three technical replicates. Statistical comparisons between values were performed with a multi-factorial ANOVA using STATISTICA 10 ($p<0.05$).

3.3 Results

3.3.1 A comparison of methods to estimate wine haze levels in synthetic wine

In order to optimize and standardize our haze formation assays, we tested several parameters previously described as important for haze formation. Such parameters included assessing wine haze formation using different wavelengths and the use of potassium sulphate, BSA and thaumatin to generate haze in MS300 medium fermented using various yeast strains. A significant decrease in haze absorbance values was observed when the wavelength increased

from 490 nm to 650 nm regardless of the strains used. As the haze absorbance was only slightly lower at 520 nm than at 490 nm, an absorbance of 520 nm was selected for further investigations similar to the absorbance used in other studies (Pocock and Waters, 2006). When BSA and potassium sulphate concentrations were increased using different concentration ratios in the model wine medium, a greater increase in haze absorbance was observed with BSA than with potassium sulphate. A similar pattern was also observed in fermented MS300 medium (results not shown).

3.3.2 Comparison of haze development in synthetic and Chardonnay musts

Greater haze absorbance was generally observed when BSA and potassium sulphate were added to generate haze after fermentation of MS300 medium without BSA when compared to haze levels produced by adding only potassium sulphate after fermentation to MS300 containing BSA (Fig. 3.1a, b). These results confirm the importance of sulphate ions in the development of haze under the defined conditions used here.

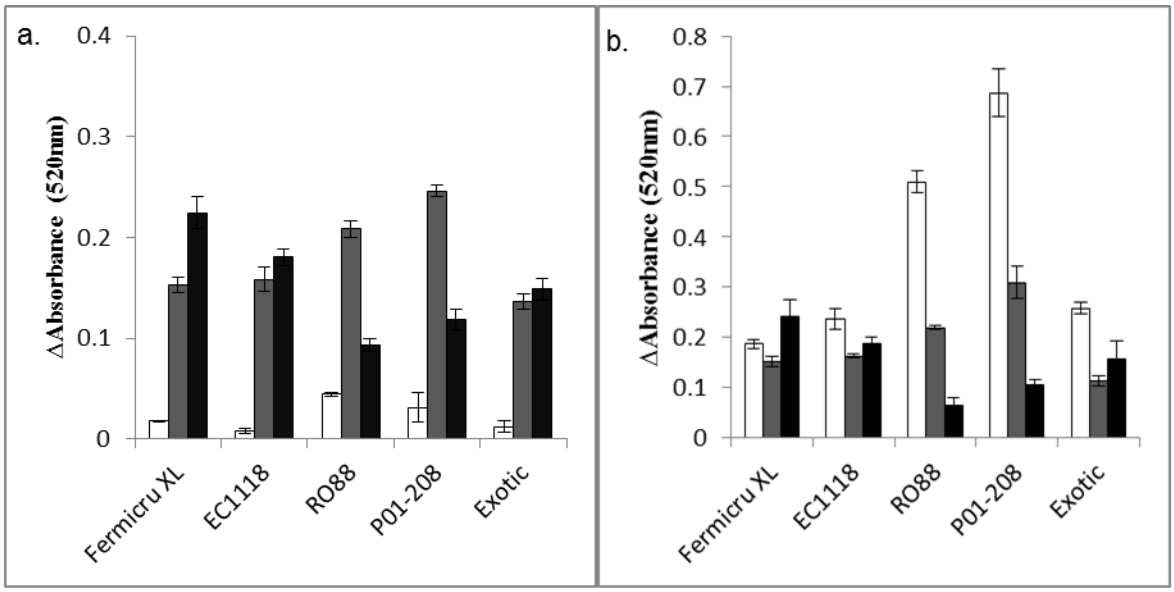

Figure 3.1: Haze levels (mean difference in absorbance before and after heating ± standard deviation of triplicate measurements) in MS300 (□), in MS300 containing 0.5 g/l bovine serum albumin (▩) and in MS300 containing 0.5 g/l thaumatin (■) fermented with *S. cerevisiae* (Fermicru XL, EC1118), *S. paradoxus* (RO88, P01-208) and *S.cerevisiae/S.paradoxus* hybrid (Exotic) strains. a, No BSA or potassium sulphate added during haze assay. b, during haze assay 0.5g/l BSA and 0.5g/l sulphate were added to fermented MS300 (□) to create haze whereas 0.5g/l sulphate only was added to MS300 containing either BSA (▩) or thaumatin (■)after fermentation.

Remarkably high haze levels were observed in MS300 medium fermented with *S. paradoxus* strains P01-208 and RO88 with or without potassium sulphate added to generate haze (Fig 3.1b). However, while significant differences ($p < 0.05$) in total protein concentrations produced by the various strains were found (Fig. 3.2), these differences could not be correlated to the haze levels. A correlation of 0.28 was observed between total protein and haze levels. Moreover no differences were observed for total mannoproteins estimated using mannose and glucose levels (data not shown) between *S. paradoxus* and *S. cerevisiae* strains at the end of alcoholic fermentation and for the wines aged on lees. These findings confirm the involvement of a specific protein(s) influencing the development of wine haze as previously described (Bayly and Berg, 1967; Mesquita *et al*., 2001).

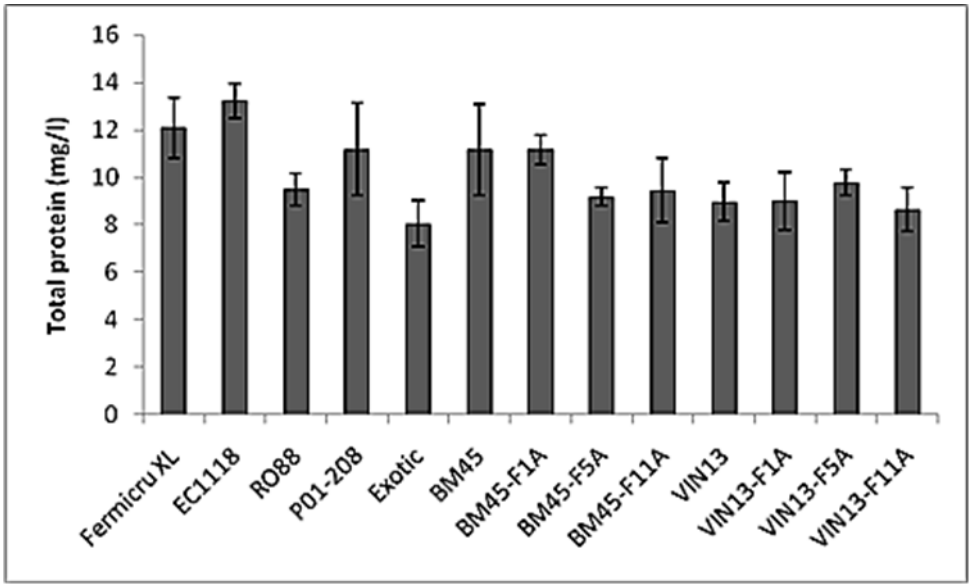

Figure 3.2: Total protein (mean ± standard deviation of triplicate measurements) in MS300 medium without added BSA fermented by various yeast strains

To investigate the effect of thaumatin on wine haze formation, commercially available thaumatin was used as a protein source due to its similarity to grape thaumatin-like protein shown to be one of the proteins responsible for wine haze formation (Pocock and Waters, 2006). Surprisingly, no significant differences were observed between haze levels produced by most wild type strains when potassium sulphate was added to MS300 medium containing thaumatin compared to when no potassium sulphate was added during the haze assay (Fig. 3.1a, b). Lower haze levels were observed in MS300 fermented containing thaumatin and in fermented

Chardonnay must by the *S. paradoxus* strains P01-208 and RO88 when compared to *S. cerevisiae* strains (Fig. 3.1b: Fig. 3.4 respectively). This observation points to the possibility that these strains release greater amounts of compounds that bind to thaumatin/ grape protein thereby reducing haze development. Interestingly Exotic, a hybrid cross between *S. cerevisiae* (VIN13) and *S. paradoxus* strain (RO88) showed intermediate phenotype between the parental strains. In general for all the yeast strains screened in this study, remarkably high haze levels were observed in MS300 medium fermented with *S. paradoxus* strains in comparison to *S. cerevisiae* strains (Fig. 3.3) with BSA and sulphate being used to generate haze during the haze assay.

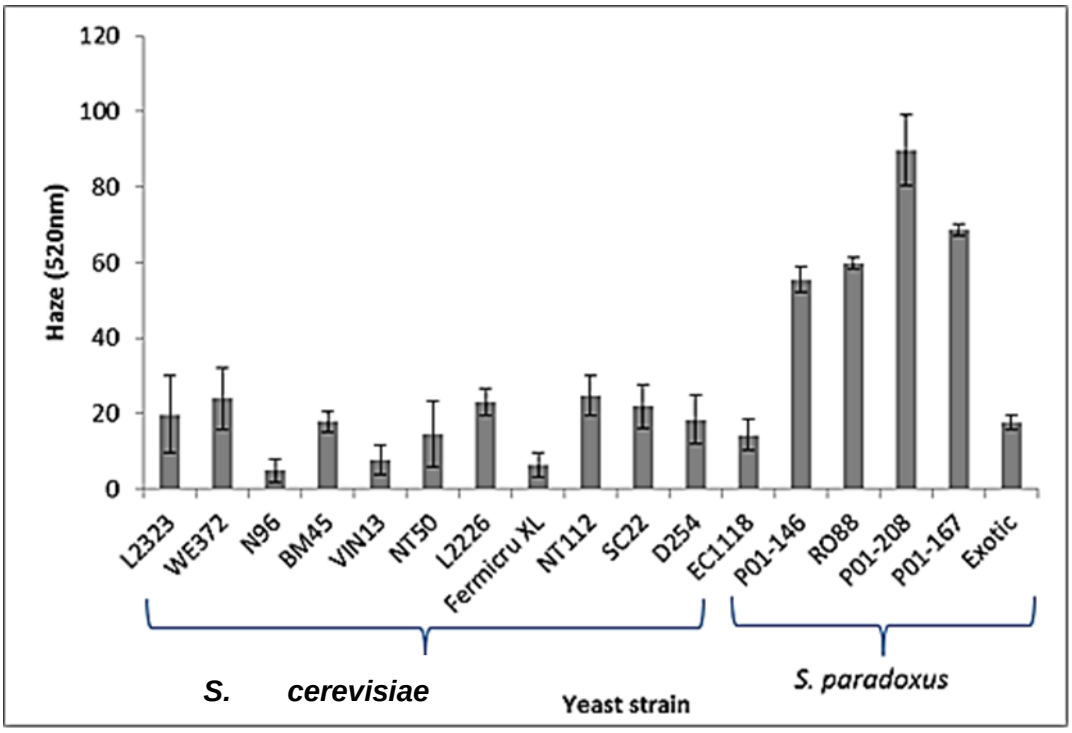

Figure 3.3: Haze levels (mean difference in absorbance before and after heating ± standard deviation of triplicate measurements) in MS300. To create haze during the haze assay, 0.5 g/l each of sulphate and BSA were added to MS300. (Data shown here was normalized).

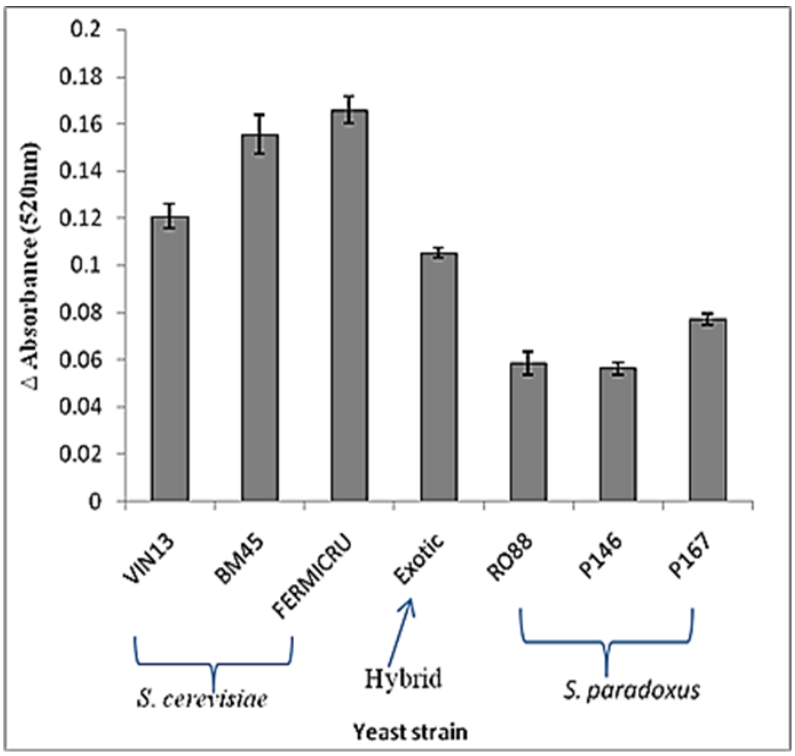

Figure 3.4: Differences in haze levels (mean difference in absorbance before and after heating ± standard deviation of triplicate measurements) between *S. cerevisiae, S. cerevisiae*/*S. paradoxus* hybrid and *S. paradoxus* yeast strains formed in fermented chardonnay grape must juice at the end of fermentation. No potassium sulphate and protein were added during the haze assay

3.3.3 Effect of Flo proteins on wine haze levels

Contradicting results on the effect of mannoproteins have been reported, with some studies reporting a reduction in wine haze (Dupin *et al*., 2000) while others reporting increased wine haze levels in strains releasing high quantities of mannoproteins (Gonzalez-Ramos *et al.,* 2008). Brown *et al.* (2007) and Gonzalez-Ramos *et al.* (2008) demonstrated that genetic modification of the yeast by over-expressing and deleting some cell wall related genes can produce wines showing reduced haze when assayed. To investigate the effect of Flo mannoproteins, strains over-expressing the *FLO* genes were used to ferment MS300 medium. *FLO1, FLO5*, and *FLO11* flocculation genes were expressed under the transcriptional control of the *ADH2* promoter gene in VIN13 and BM45 wine yeast strains (Govender *et al.,* 2008). In general, slight haze was observed in MS300 medium with no added BSA fermented by BM45, VIN13 and the genetically engineered mutants (Fig 3.5a). These levels are below values considered to be reliable (Pocock and Waters, 2006; Pocock *et al.,* 2007). Strains BM45-F5A, VIN13-F5A and VIN13-F11A produced the highest haze in fermented MS300 with thaumatin added (Fig. 3.5a).

A similar haze level pattern was also observed when potassium sulphate was added to generate haze (Fig 3.5a, b).

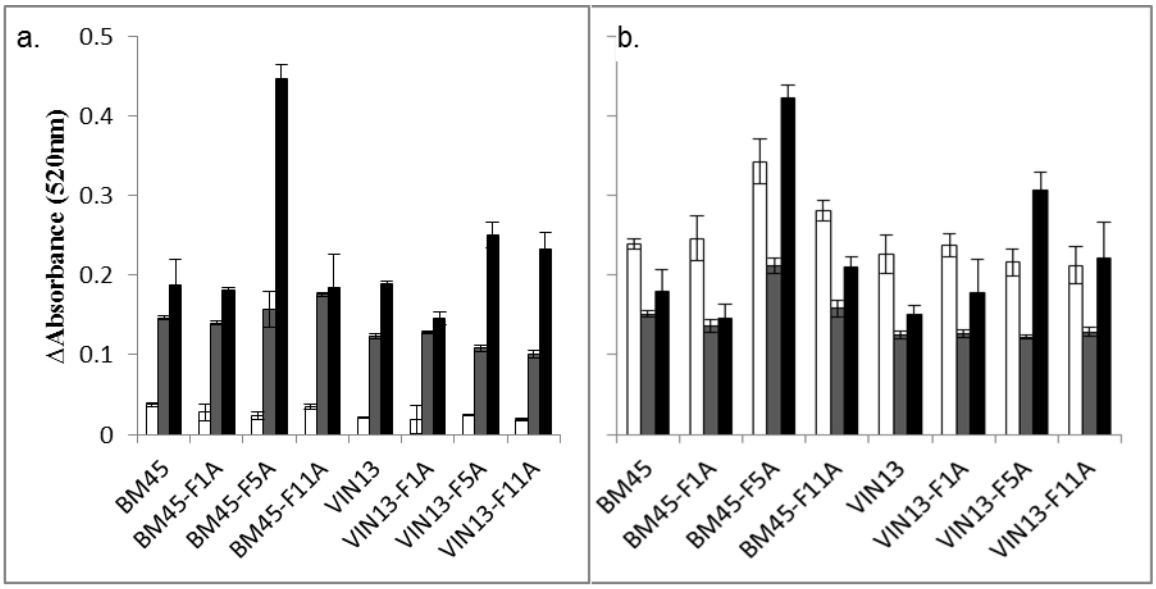

Figure 3.5: Haze levels (mean difference in absorbance before and after heating ± standard deviation of triplicate measurements) in MS300 (□), in MS300 containing either 5 g/l bovine serum albumin (▒) and thaumatin (■) fermented by different *S. cerevisiae* wine strains and strains overexpressing the FLO genes. a, No BSA or potassium sulphate added during haze assay. b, during haze assay 0.5g/l BSA and 0.5g/l potassium sulphate added to fermented MS300 (□) to create haze whereas 0.5g/l potassium sulphate only was added to MS300 containing BSA (▒) and thaumatin (■) after fermentation.

3.3.4 Effect of wine aging on lees on wine haze formation

To investigate the effect of aging on wine haze formation, fermented MS300 medium and Chardonnay grape juice were aged on lees for a period of 12 weeks after fermentation. Lower haze levels were observed in fermented grape must fermented by RO88 yeast strain when compared to the other strains used (Fig. 3.6). There was a general decrease in wine haze levels in fermented grape must juice during the aging period for all the wines fermented with different yeast strains and the spontaneously fermented grape juice. Higher haze levels were observed in *S. paradoxus* strain RO88 as compared to other yeast strains during the aging period (Fig. 3.7) in fermented MS300 medium. These results suggest that the yeast strains possibly release compounds that reduce the haze forming precipitated aggregates in Chardonnay wine whereas in synthetic medium, the yeast compounds actually contribute to the development of haze.

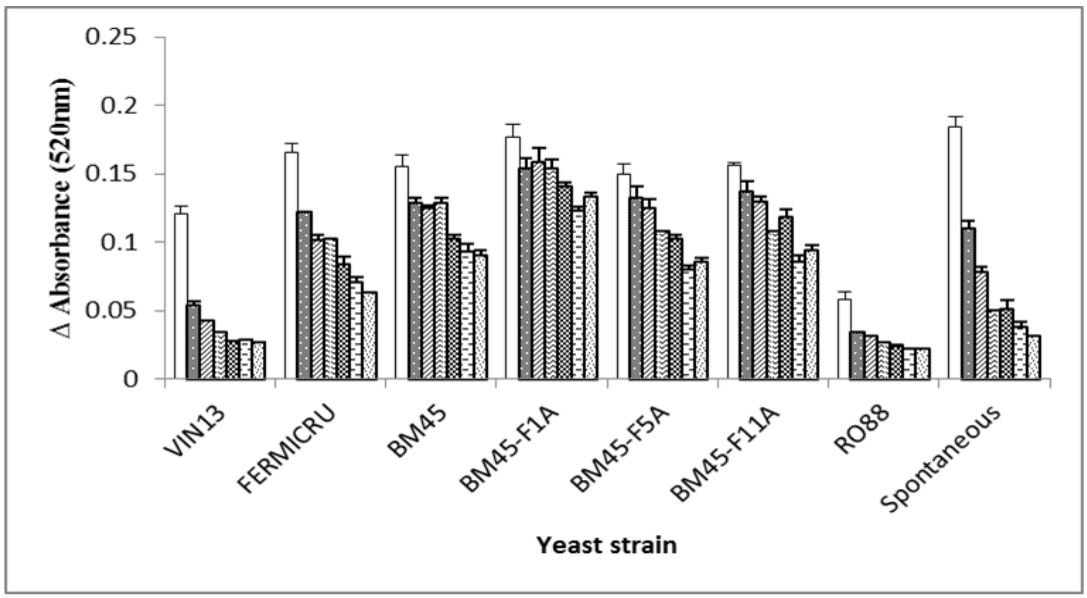

Figure 3.6: Bi-weekly haze levels (mean difference in absorbance before and after heating ± standard deviation of triplicate measurements) formed in fermented Chardonnay grape must during aging.). *S. cerevisiae* (Vin13, FERMICRU, BM45, BM45-F1A, BM45-F5A, BM45-F11A) and *S. paradoxus* (RO88) strains were used to ferment the grape must and also grape must was allowed to ferment spontaneously. Haze measurements were done at the start of the aging process (week 0) ( ) and every two weeks thereafter ((week 2 ( ), week 4 ( ), week 6 ( ), week 8 ( ), week 10 ( ) and week 12 ( ). No BSA or potassium sulphate were added when carrying out the haze assay

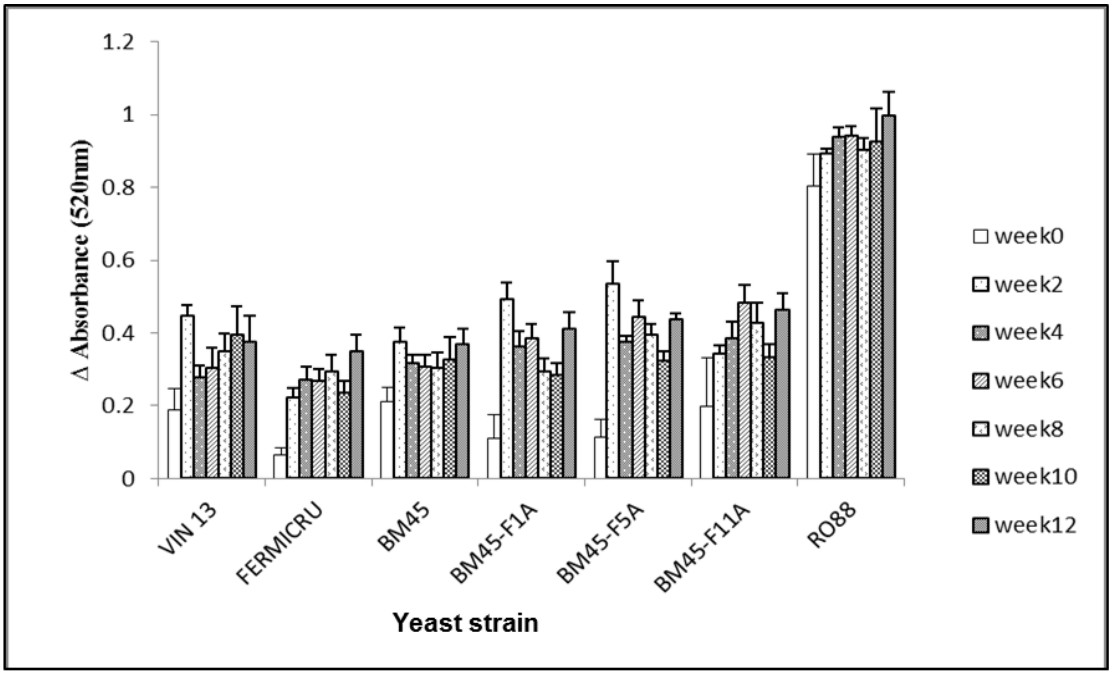

Figure 3.7: Bi-weekly haze levels (mean difference in absorbance before and after heating ± standard deviation of triplicate measurements) formed in fermented MS300 during aging at week 0 ( ), week 2 ( ), week 4 ( ), week 6 ( ), week 8 ( ), week 10 ( ) and week 12 ( ). *S. cerevisiae* (VIN13, FERMICRU, BM45, BM45-F1A, BM45-F5A, BM45-F11A) and *S. paradoxus* (RO88) strains were used to ferment MS300. Week zero haze measurements represents the start of aging. To generate wine haze, bovine serum albumin (1 g/l) and potassium sulphate (1 g/l) were added to the fermented medium before carrying out the haze assay. A gram per litre BSA and potassium sulphate were used instead of 0.5 g/l in order to clearly see the reduction in haze differences if any, during aging between the strains.

3.3.5 Genetic analysis of haze protective activities

In order to investigate whether the haze protective activity of *S. paradoxus* strains was a monogenic or multigenic trait, we made use of a hybrid S. cerevisiae/S. paradoxus strain. The haze protection capabilities of self-diploidised spores of the hybrid strain Exotic were analyzed. The wines fermented using the diploid spores followed neither RO88 nor VIN13 haze levels but lower haze levels than RO88, intermediate haze levels between RO88 and VIN13, and higher haze levels than VIN13 were obtained. The phenotype observed from the first filial spores after fermentation in Chardonnay grape must therefore indicated that the haze protection capacity was not a single gene phenotype (Figure 3.8) but a polygenic trait.

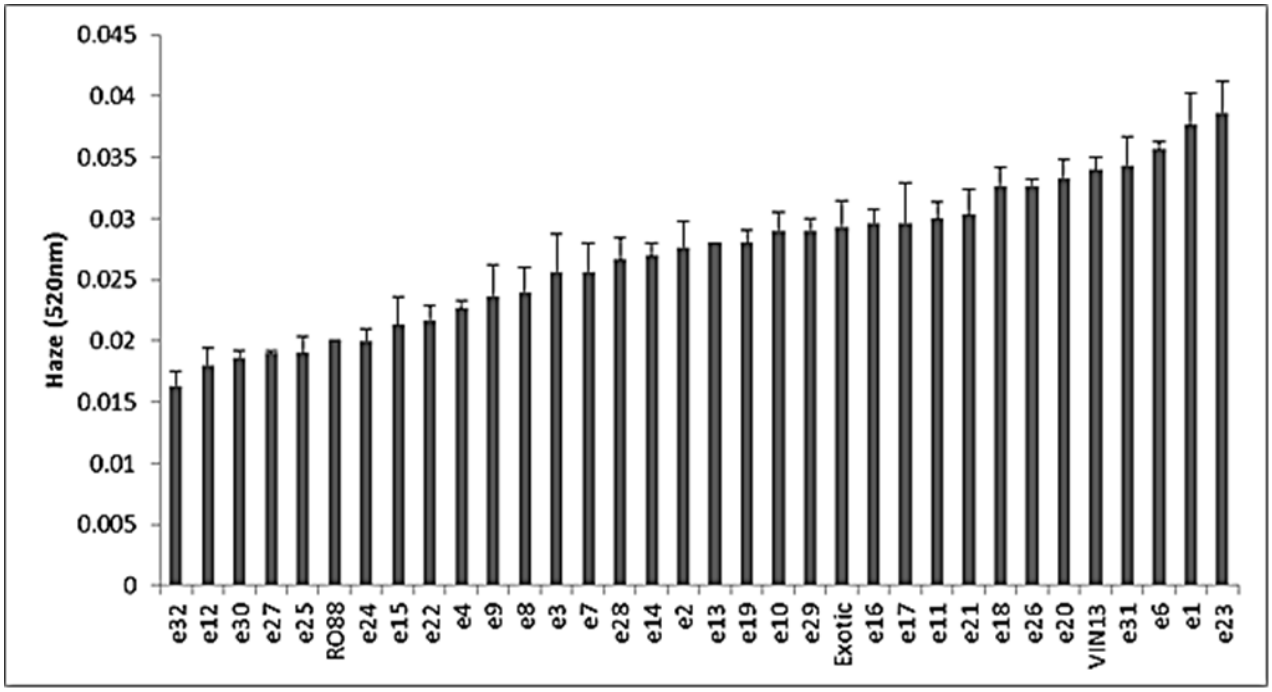

Figure 3.8: Haze formation capacities of F_1 spores obtained from hybrid strain Exotic. Samples were taken at the end of Chardonnay alcoholic fermentation.

3.4 Discussion

Our comparative studies confirm the complex nature of haze development in wine and the resulting problem of establishing accurate and reliable tools for the evaluation of the potential of haze formation. Such evaluations are essential in determining the amount of agent to be added and therefore may directly impact on the nature and quality of the final product. Variations in the measurement of haze formation in model solutions and in fermented synthetic must mostly confirmed previous data regarding the impact of protein concentration and sulphate. Importantly however, our data clearly show that model solutions such as those commonly used in research can at best only provide approximations of real haze formation potential and of haze protective activities of yeast, and at worst may produce results that are contrary to those observed in real grape must. Indeed, haze formation in synthetic and real grape musts yielded opposite results when assessing the inherent haze protective capacity of fermenting yeast strains.

Of equal importance is the information that commercial wine yeast strains differ in their ability to provide haze-protective activity in ways that could be of commercial importance. Most interestingly, the data suggest that strains of *S. paradoxus* or of hybrids between *S. paradoxus* and *S. cerevisiae* display significantly more haze protective activity than any of the evaluated *S. cerevisiae* wine yeast strains. The molecular nature of this haze protective activity of the evaluated S*. paradoxus* strains will require further investigation.

The data generated with strains overexpressing the cell wall- based Flo proteins suggest that the increased production of these mannoproteins appears not to impact on haze formation. More importantly, and considering the data presented by Govender *et al*. (2008; 2010), the FLO-overexpressing strains used here are known to display very significant differences in general cell wall related properties such as adhesion ability and hydrophobicity. The data therefore strongly suggest that such general yeast cell wall properties have no impact on haze formation or prevention.

Higher haze levels were observed when both the concentrations of BSA and potassium sulphate were increased confirming the effect of protein and sulphate on wine haze in model systems (Mesquita *et al.,* 2001; Pocock *et al.,* 2007). It will be interesting to evaluate whether the same effect can be observed in wine. The sulphate content in Chianti red wines was reported by Tamasi *et al.* (2010) to range from 399 to 902 mg (K_2SO_4)/l but the wines with high sulphate concentrations did not show any haze or any precipitate. This indicated that most protein and some sulphate may have co-precipitated with phenols and tannins in red wine before bottling. This is in contrast to white wine where haze is most commonly observed regardless of low phenol and tannin levels in white musts (Esteruelas *et al.,* 2011; Sierbet *et al.,* 2009). Surprisingly, in fermented MS300 containing thaumatin, there was no increase in haze when potassium sulphate was added. These results may demonstrate the importance of potassium sulphate in other model system used to mimic wine haze formation (Pocock *et al.,* 2007) but not in fermented MS300 were thaumatin was added.

Yeast strains appear to release compounds that modulate wine protein composition as demonstrated by the differences observed in haze levels in wine fermented with different yeast strains. Most research on wine haze has been mainly focused on evaluating the effect of wine composition and their interaction on haze levels but not on the variation caused by the actual yeast strain fermenting the wine (Batista *et al.,* 2009; Marangon *et al.,* 2011a;, Mesquita *et al.,* 2001; Pocock *et al*., 2007; Waters *et al.,* 2005). The differences in haze levels observed in wine produced from different cultivars may be due to the different yeast strain used which may have modified the intrinsic parameters of the fermented wine. Contradicting results on the levels of protein produced in wine making have been found, with some authors reporting a decrease in total protein concentration (Dizy and Bisson, 1999; Manteau *et al.,* 2003), while others observing an increase in soluble protein content after alcoholic fermentation (Bayly and Berg, 1967; Fukui and Yokotsuka, 2003) in fermented must. In our study, *S. paradoxus* strains produced much higher haze levels in synthetic wine fermented without BSA when compared to that fermented in the presence of BSA (Fig. 1b). The reduced haze levels may be due to reduced BSA levels at the end of fermentation available to generate haze in the presence of

potassium sulphate. The presence of stabilizing factors released by yeast over time (Vincezi *et al.,* 2011) could also be involved in the removal and decline in haze levels. Matsuka *et al.* (1999) demonstrated that BSA binds optimally to the surface of yeast protoplast at the isoelectric point of BSA. Moreover, *S. cerevisiae* PIR1 strain selected from Pinot noir grapes in the Champagne area was shown to secrete an acid proteolytic activity against BSA (Younes *et al.,* 2011). However, the actual contribution of the yeast cell wall has not been studied in sufficient depth to understand its precise role in wine protein haze formation.

Haze protective effect of *S. paradoxus* strains was observed in fermented Chardonnay wine and in fermented MS300 where thaumatin was added. However, higher haze levels were also observed for the same strains in fermented MS300 where BSA and potassium sulphate was added. These findings may have important implications for haze measurement and therefore questions studies where BSA was used to generate haze (Lagace and Bisson, 1990; Dupin *et al.,* 2000; Fenchack *et al.,* 2001; Mesquita *et al.,* 2001; Trela, 2008). The positive correlation ($p<0.05$) between haze levels in fermented grape must and MS300 where thaumatin has been added to create haze reveals the similarity in behavior of grape proteins and thaumatin protein extracted from the tropical flowering plant *T. daniellii*. High haze observed in wines aged on lees in fermented MS300 could be due to yeast mannoproteins released interacting with BSA added to generate haze thus resulting in high haze being formed.

In this work, effect of Flop over-expression under regulation of the *ADH2* promoter was investigated on wine protein stability. The genetically engineered strains did not reduce the haze relative to their parental strains. Higher haze observed in BM45 Flop overexpressing strains when compared to VIN13-Flop overexpressing strains, indicates the effect of strain background on wine haze formation (Gonzalez-Ramos and Gonzalez, 2006). Siebert *et al.* (2009) suggested that variations in the hydrophobicity level of different yeast protein classes could be involved in the degree of protein hazing of white wines. However, no subtle differences in haze were observed in fermented grape juice in this study when these Flo protein over-expressing strains were used and demonstrate that the FLO genes overexpression does not appear to have an

impact on wine haze reduction. Similarly no haze differences between wild type strains and some strains overexpressing high levels of mannoproteins have been reported (Brown *et al.,* 2007; Gonzalez-Ramos *et al.,* 2009).

This study has demonstrated that most of the current predictive assays used by researchers give different results thus making it difficult for one to compare the different results. Although various factors have been studied individually, the use of model systems is questionable when studying wine haze as it is the compounds originating from the grape and yeast and their interaction that contributes to haze formation. Solving this question is a complex task due to wine variability, incomplete knowledge concerning the precise structures and physicochemical properties of wine proteins and the mechanisms involved in their instability (Batista *et al.,* 2009, Mesquita *et al.,* 2001). Moreover, results must also be validated using a large selection of wines because wine composition is known to influence protein heat-stability and aggregation (Mesquita *et al.,* 2001). Most wines contain the same set of structurally related proteins but differ in properties within a same protein family (Mesquita *et al.,* 2001, Sauvage *et al*., 2010) and thus yield conflicting results (Mesquita *et al.,* 2001). There is also need to correlate the test results with realistic storage conditions to validate the protein stability of wine. We propose that the yeast strain used in fermentation must be considered as a factor when investigating various factors important in wine haze formation.

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Chapter 4

Research results II

Exoproteomic profiling of wine yeast strains differing in wine haze protection capacities

This manuscript will be submitted for publication in **The Proteome Research**

Exoproteomic profiling of wine yeast strains differing in wine haze protection capacities

4. Abstract

While many data sets suggest that some wine proteins have positive impacts on the organoleptic characteristics of wine, proteins have more frequently been associated with the formation of wine protein haze, a serious commercial problem for the global industry. Of the different types of proteins present in wine, yeast mannoproteins in particular have been shown to positively contribute to several enological properties of wine, including wine haze reduction. In the previous chapter we demonstrated that the *Saccharomyces paradoxus* strains have better wine haze protective ability than commonly used *Saccharomyces cerevisiae* wine yeast strains. In this chapter, we further investigate this *S. paradoxus* phenotype. The main aim of the study was to investigate whether the wine haze reduction phenotype of the *S. paradoxus* strain RO88 can be linked to the nature and amount of the secreted proteome when compared to *S. cerevisiae* VIN13.

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) was used to analyze banding profile of several *S. paradoxus* and *S. cerevisiae* protein secretome at the end of alcoholic fermentation. Linear quadrupole ion trap/FT-ICR mass spectrometry **(**LTQ-FT-ICR MS) and isobaric tags for relative and absolute quantitation (iTRAQ) analysis were further used to analyse proteins and the exo-proteome of the two selected yeast strains at the end of alcoholic fermentation. One dimensional gel electrophoretic analysis of total deglycosylated proteins revealed similar banding patterns in all *S. cerevisiae* strains which differed slightly from the *S. paradoxus* strain. Several proteins were identified from the single protein band that most significantly differed between the *S. paradoxus* and *S. cerevisiae* strains, but their nature did not provide specific insights into the differences between the two strains. For this reason, iTRAQ analysis was carried out on the total secretome and demonstrated that the *S. paradoxus* strain (RO88) released higher levels of cell wall related mannoproteins when compared to the *S. cerevisiae* strain (VIN13), which released significantly higher levels of metabolic enzymes. The individual impact of some of the proteins that were highly released by the RO88 yeast strain on wine haze formation was further assessed through the use of the corresponding deletion mutants from the EUROSCARF library and by expressing the corresponding *S. paradoxus* genes in *S. cerevisiae*. The data suggest that some of these proteins may indeed individually contribute to the differences in wine haze protection activity between *S. paradoxus* and *S. cerevisiae* strains.

4.1 Introduction

Considering the cost and the negative impact of clarifying agents employed in the global wine industry, it is imperative to investigate alternative methods of protein stabilization in wine. Over the past decade, several studies have suggested that mannoproteins secreted by yeast could play a major role in controlling haze formation (Dupin *et al*., 2000). There is however a paucity of data on the genetic determinants involved in the secretion and specific impact of mannoproteins during wine making, although a number of studies have been carried out (Waters *et al.,* 1993; Brown *et al.,* 2007; Gonzalez-Ramos *et al.,* 2006; 2008; 2009). In addition, data on the identity of yeast mannoproteins specifically responsible for haze diminution remains limited, even though some have been identified (Brown *et al*., 2007). These data suggest that yeast with better mannoprotein production capacity could make a significant contribution to solving the problem of unwanted haze formation.

The protein composition of the cell wall and of the sectretome is highly dynamic, varying depending on yeast strain and growth conditions such as nutrient availability, temperature, external pH, and oxygen level (Insenser *et al*., 2010). Besides being temporally and spatially controlled (Insenser *et al*., 2010), protein release is also dependent on cellular processes including budding, mating and dimorphic transition which results in dynamic cell wall changes. Yeast protein release has been shown to be also cell cycle dependent (Frykman and Srienc *et al.,* 2001).

Several studies have assessed the secretome of wine yeast strains (Kwon *et al*., 2004; Giovani *et al*., 2010; Palmisano *et al.,* 2010), but most of these studies provide qualitative and not quantitative data (Kwon *et al.,* 2004; Cilindre *et al*., 2008; Palmisano *et al*., 2010). Kwon *et al.,* (2004) identified twenty proteins from a Sauvignon Blanc wine in which 12 were of yeast origin, five proteins derived from the grape, two from bacteria, and one from fungi. In a separate study, Palmisano *et al.* (2010) found several grape glycoproteins including invertase and pathogenesis-related (PR) proteins, and also yeast glycosylated proteins in wine after the vinification process. Most recently Insenser *et al.,* (2010) and Branconi *et al.*, (2011) described

the yeast cell surface proteome (surfome), and classified proteins according to cellular processes including the control of cell wall organization, cell rescue, defence, and virulence, protein fate, protein synthesis and metabolism. Other studies investigating yeast protein secretome quantified mannoproteins using mannose levels in wine (Fusi *et al.,* 2010; Quirós *et al*., 2011; Rowe *et al.,* 2010). The limitation of this method is linked to the fact that glycoproteins have varying mannan chain lengths and not all proteins released by yeast strains are mannoproteins.

In the previous study, yeast strains with significant differences in their ability to protect wine from haze formation were identified. With the aim of establishing whether differences in the quantity and/or nature of the proteins secreted during fermentation were potentially responsible for the observed haze protection differences, we subjected two yeast strains showing significant divergence in terms of haze protective capability to quantitative proteomic profiling of proteins released at the completion of alcoholic fermentation. The strains assessed were the *S. paradoxus* RO88 and *S. cerevisiae* VIN13. *S. paradoxus* is the closest known relative of *S. cerevisiae,* but research thus far has focused on geographic differentiation, population structure, and linkage disequilibrium (Sweeney *et al.,* 2004; Johnson *et al.,* 2004; Fay and Benavides 2005; reviewed by Borneman *et al.,* 2011).

The data show that the strains secreted similar amounts of total proteins, and showed broadly similar protein and mannoprotein profiles in one dimensional SDS-PAGE when comparing proteins released into synthetic must at the end of fermentation.

To characterize the full exo-proteome of the two strains at the end of fermentation, iTRAQ analysis was used. Significant differences between the two strains in the relative quantity of the secreted proteins were observed. In particular, the *S. paradoxus* exo-proteome showed significantly higher levels of cell wall mannoproteins, while the *S. cerevisiae* exo-proteome was characterized by higher levels of many metabolic enzymes. The potential individual impact on wine haze reduction of the proteins that were highly released by RO88 was evaluated by

expressing the *S. paradoxus* allele of these genes in *S. cerevisiae* VIN13. The data show that expression of the *UTR2, PLB3, PRY3, ECM33, HPF1'* cell wall protein encoding genes did not result in any wine haze reduction while wines fermented by strains expressing *PST1, EXG1, CHS3* and *CRH1* showed a reduction in wine haze formation in comparison to the parental strain VIN13, suggesting that these proteins may indeed contribute to the increased haze-protective activity observed for *S. paradoxus* strains.

4.2 Materials and methods

4.2.1 Fermentation media and conditions

VIN13, a *S. cerevisiae* wine yeast strain commercialized by Anchor Yeast (Cape Town, South Africa) and RO88, a *S. paradoxus* yeast strain (Redžepović *et al.*, 2003) were used in this study. Deletion mutants in the BY4742 background obtained from the European *S. cerevisiae* Archive for Functional Analysis (EUROSCARF) library (Brachmann *et al.,* 1998) were also used in this study (Table 1) to determine the haze formation potential of the chardonnay wines fermented using the deletion mutant strains. *S. cerevisiae* and *S. paradoxus* yeast strains were used to ferment to dryness chardonnay grape juice and chemically defined MS300 (Bely *et al*., 1990) containing 200 g/l of glucose and fructose. Fermentations were carried out in triplicate with yeast cell pre-cultures grown in YPD broth (BD Becton, Dickinson and Company**,** cat # 242820). Chardonnay grape and synthetic musts were inoculated to obtain a final concentration of 10^6 cells/ml. All fermentations were carried out in Erlenmeyer flasks/ glass bottles closed with fermentation caps, with a 100 ml working volume without agitation. The fermentations were conducted in a room maintained at 25^{o}C. Fermentations were monitored using weight-loss and briefly agitated before weighing. Residual glucose and fructose concentrations were less than 5 g/l as measured using a D-glucose/fructose kit (Amersham). Wines were recovered for further analysis, by removing yeast cells by centrifugation.

4.2.2 KDS protein recovery

Protein recovery was evaluated following the protocol by Fusi *et al.* (2010) and Rowe *et al.* (2010). Sodium dodecyl sulphate (SDS), 10% (w/v) was added to wine samples to give a 0.2%

(w/v) final concentration. After heating samples in boiling water bath for 5 min, 2 M KCl was then added to reach a final concentration of 400 mM. Samples were mixed, incubated at 4^{o}C for 45 min. KDS–protein pellets (KDS pellets) were recovered by centrifugation at 13,000rpm for 15 min at 4^{o}C.

4.2.3 Glycosylation enzyme digests

Protein deglycosylation mix was used to deglycosylate both the KDS protein pellet. The enzyme mix contained enzymes including PNGase F (N glycosidase F), Endo-α-N-Acetylgalactosaminidase, Neuraminidase, β1-4 Galactosidase and β-N-acetylglucosaminidase (Biolabs, New England, P6039S).

4.2.4 SDS–PAGE

SDS–PAGE analysis was performed according to Laemmli (1970) in Mini Protean III apparatus (Bio-Rad) as described by Fusi *et al.* (2010). The protein pellets were solubilized in 62.5 mM Tris-HCl buffer (pH 6.8), containing 1.3% (w/v) SDS and 5% (w/v) 2-mercaptoethanol. The solubilized protein solution was heated at 100^{o}C for 5 min and then loaded onto 12% polyacrylamide gels. Electrophoresis was run at 250 volts constant current until the bromophenol blue dye reached the bottom of the gel. Deglycosylated protein gels were stained with either Coomassie Brilliant Blue R250 or silver stain. The Pro-Q Emerald 300 glycoprotein staining kit (Invitrogen, catalogue number P21855) was used to stain non-deglycosylated glycoproteins.

4.2.5 LTQ-FT-ICR mass spectrometer: identification of protein from SDS PAGE gel

The protein was digested with trypsin, dried, reconstituted in 0.1% formic acid and analyzed on a hybrid linear quadrupole ion trap/FT-ICR **(**LTQ-FT-ICR mass spectrometer (Thermo Fisher Scientific) or an LTQ-Orbitrap XL, interfaced with an in-house constructed nano-LC system described in (Carlson *et al.,* 2006). The peptides were separated on a reversed phase column, 200 x 0.050 mm packed in-house with 3 µm Reprosil-Pur C18-AQ particles. The flow through the analytical column was reduced by a split to approximately 100 nl/min and the peptides were

eluted with an acetonitrile gradient and 0.1% formic acid and electrosprayed using 1.4 kV. Peptides were analyzed in a data-dependent mode switching between MS full scan and MS/MS using CID (collision induced dissociation) of the five most abundant doubly, triply or quadruply protonated ions in each MS scan. Database search was performed using MASCOT search program (Version 2.1.0 and 2.2.04, Matrix Science) and the Swiss-prot protein data base for yeast. Positive protein identification was considered for matches within 10 ppm and with at least 95% confidence and a minimum of 3 unique peptides.

4.2.6 Protein purification for iTRAQ analysis

Protein purification was carried out following the protocol described by Palmisano *et al.* (2010). Fermented MS300 was centrifuged at 5000rpm for 5 mins, to remove cells and concentrated using Millipore Membrane Centrifugal Filter devices with a molecular-weight cut-off of 10 kDa. Ice-cold ethanol solution containing 15% (w/v) trichloro-acetic acid (TCA) was used to dilute concentrates at 4 °C for 2h. The solution was centrifuged at 13000rpm for 10 min at 4 °C, and the pellet was washed with ice-cold ethanol and centrifuged. The vacuum dried protein pellet was reduced by solubilized in 100 µl of 6 M urea, 2 M thiourea, and 10 mM DTT. The proteins were then alkylated with 50 mM iodoacetamide for 40 min at room temperature in the dark.

4.2.7 iTRAQ (isobaric tags for relative and absolute quantification) analysis: Sample preparation

Total protein concentration was determined using Pierce BCA Protein Assay (Thermo Scientific). After pooling tubes in each group (VIN13 and RO88) there was 85 µg protein in each sample. Each sample was diluted with 0.5M TEAB (triethyl ammonium bicarbonate) and then diluted with milli-Q water to a 4-fold dilution to a pH >8. SDS (sodium dodecyl sulphate) solution to a final concentration of 0.1% and trypsin (dissolved in milli-Q water), ratio 1:10, was added to each sample. Digestion was performed overnight in 37°C.

4.2.8 Label with iTRAQ reagents

iTRAQ reagents 117 (VIN 13) and 119 (RO88) were dissolved in ethanol and added to the respective sample according to the manufacturer's protocol (Applied Biosystems). After labeling, the samples were combined and concentrated. TMT-labeled peptides were separated with Strong Cation Exchange Chromatography (SCX). The concentrated peptides were acidified by 10% formic acid and diluted with SCX solvent A (25 mM ammonium formate, pH 2.8, 20% acetonitrile (ACN)) and injected onto a PolySULFOETHYL A SCX column (2.1 mm i.d. × 10 cm length, 5 µm particle size, 300 Å pore size). SCX chromatography and fractionation was carried out on an ÄKTA purifier system (GE healthcare) at 0.25 mL/min flow rate using the following gradient: 0% B (500 mM ammonium formate, pH 2.8, 20% ACN) for 5 min; 0-40% B for 20 min; 40-100% B for 10 min and 100% B held for 10 min. UV absorbance at 254 and 280 nm was monitored while fractions were collected at 0.5 mL intervals and dried down in a Speed Vac. The peptide containing fractions were desalted on Pep Clean C18 spin columns according to manufacturer's instructions (Thermo Fisher Scientific).

4.2.9 LC-MS/MS Analysis on LTQ-Orbitrap-Volos

The desalted and dried fractions were reconstituted into 0.1 % formic acid and analyzed on a LTQ-Orbitrap-Velos (Thermo Fisher Scientific) interfaced with an in-house constructed nano-LC column. Two-micro liter sample injections were made with an Easy-nLC autosampler (Thermo Fisher Scientific, Inc., Waltham, MA, USA), running at 200 nl/min. The peptides were trapped on a pre-column (45 x 0.075 mm i.d.) and separated on a reversed phase column, 200 x 0.075 mm, packed in-house with 3 µm Reprosil-Pur C18-AQ particles. The gradient was as followed; 0-90 min 5-37% acetonitrile (ACN), 0.1% formic acid, 90-93 min 37-90% ACN, 0.1% formic acid and the last 5 min at 90% ACN, 0.1% formic acid.

LTQ-Orbitrap Velos settings were: spray voltage 1.4 kV, 1 microscan for MS1 scans at 60 000 resolutions (m/z 400), full MS mass range m/z 400-2000. The LTQ-Orbitrap Velos was operated in a data-dependent mode with one MS1 FTMS scan precursor ions followed by CID (collision induced dissociation) and HCD (high energy collision dissociation), MS2 scans of the five most

abundant protonated ions in each FTMS scan. The settings for the MS2 were as follows: 1 microscans for HCD-MS2 at 7500 resolution (at m/z 400), mass range m/z 100-2000 with a collision energy of 50%, 1 microscans for CID-MS2 with a collision energy of 30%.

4.2.10 Database Search and iTRAQ Quantification

MS raw data files from all 13 SCX fractions for the iTRAQ set were merged for relative quantification and identification using Proteome Discoverer version 1.2 (Thermo Fisher Scientific). Database search was performed by Mascot search engine using the following criteria: fungi in Uniref100 protein database, MS peptide tolerance as 5 ppm, MS/MS tolerance as 0.05 Da, trypsin digestion allowing 2 missed cleavages with variable modifications; methionine oxidation, cysteine carbamethylation, tyrosine iTRAQ-label and fixed modifications; N-terminal iTRAQ-label, lysine iTRAQ-label. The detected protein threshold in the software was set to 99% confidence and identified proteins were grouped by sharing the same sequences to minimize redundancy.

For iTRAQ quantification, the ratios of iTRAQ reporter ion intensities in MS/MS spectra (m/z 117.11, 119.11) from raw data sets were used to calculate fold changes between samples. Only peptides unique for a given protein were considered for relative quantitation, excluding those common to other isoforms or proteins of the same family. The resulting ratios were then exported into Excel for manual data interpretation.

4.2.11 Bioinformatics analysis

The identified proteins were assigned a Gene Ontology (http://www.geneontology.org) term according to their molecular function, and they were statistically grouped into functional categories using Xcalibur, Proteome Discoverer Version 1.3; Thermo Fisher Scientific Inc©) to identify overrepresented GO categories.

Table 4.1: Yeast strains used in this study. a: Deletion mutant strains in BY4742 (*S. cerevisiae*) background were obtained from the EUROSCARF library.

Strain name/ gene	Description
VIN13 (*S. cerevisiae*)	Wine yeast strain (Anchor Yeast, Cape Town, South Africa)
RO88 (*S. paradoxus*)	Wine yeast strain (Redžepović *et al.,* 2003)
BY4742	*MATα ; his3Δ 1; leu2Δ 0; lys2Δ 0; ura3Δ 0*
YFR045W[a]	BY4742 Δ *yfr045; yfr045::OFP::KanMX4*
SCW4[a]	BY4742 Δ *scw4; scw4::OFP::KanMX4*
DSE1[a]	BY4742 Δ *dse1; dse1::OFP::KanMX4*
SHC1[a]	BY4742 Δ *shc1; shc1::OFP::KanMX4*
CWP2[a]	BY4742 Δ *cwp2; cwp2::OFP::KanMX4*
YJL160C[a]	BY4742 Δ *yjl160C; jjl160C::OFP::KanMX4*
SUN4[a]	BY4742 Δ *sun4; sun4::OFP::KanMX4*
HPF1[a]	BY4742 Δ *hpf11; hpf11::OFP::KanMX4*
SKT5[a]	BY4742 Δ *skt5; skt5 ::OFP::KanMX4*
TIP1[a]	BY4742 Δ *tip1; tip1::OFP::KanMX4*
YBR016W[a]	BY4742 Δ *ybr016w; ybr016w::OFP::KanMX4*
CHS3[a]	BY4742 Δ *chs3; chs3::OFP::KanMX4*
RCR1[a]	BY4742 Δ *rcr1; rcr1::OFP::KanMX4*
HPF2[a]	BY4742 Δ *hpf2; hpf2::OFP::KanMX4*
SBE2[a]	BY4742 Δ *sbe2; sbe2::OFP::KanMX4*
CTS2[a]	BY4742 Δ *cts2; cts2::OFP::KanMX4*
VPS52[a]	BY4742 Δ *vps52; vps52::OFP::KanMX4*
UTR2[a]	BY4742 Δ *utr2; utr2::OFP::KanMX4*
YEA4[a]	BY4742 Δ *yea4; yea4::OFP::KanMX4*
PCM1[a]	BY4742 Δ *pcm1; pcm1::OFP::KanMX4*
SCW11[a]	BY4742 Δ *scw11; scw11::OFP::KanMX4*
YGL081W[a]	BY4742 Δ *ygl081W; ygl081W::OFP::KanMX4*
PMT6[a]	BY4742 Δ *pmt6; pmt6::OFP::KanMX4*
CRH1[a]	BY4742 Δ *crh1; crh1::OFP::KanMX4*
CHS7[a]	BY4742 Δ *chs7; chs7::OFP::KanMX4*
SUC2[a]	BY4742 Δ *suc2; suc2::OFP::KanMX4*
HOC1[a]	BY4742 Δ *hoc1; hoc1 ::OFP::KanMX4*
CWP1[a]	BY4742 Δ *cwp1; cwp1::OFP::KanMX4*
SAC1[a]	BY4742 Δ *sac1; sac1::OFP::KanMX4*
CDA2[a]	BY4742 Δ *cda2; cda2::OFP::KanMX4*
CDA1[a]	BY4742 Δ *cda1; cda1 ::OFP::KanMX4*
CHS5[a]	BY4742 Δ *chs5; chs5::OFP::KanMX4*
SCW10[a]	BY4742 Δ *scw10; scw10::OFP::KanMX4*
DFG5[a]	BY4742 Δ *dfg5; dfg5::OFP::KanMX4*
RIM21[a]	BY4742 Δ *rim21; rim21::OFP::KanMX4*
YNL190W[a]	BY4742 Δ ynl190w*;* ynl190w*::OFP::KanMX4*
YGP1[a]	BY4742 Δ *ygp11; ygp1::OFP::KanMX4*
CHS1[a]	BY4742 Δ *chs1; chs11::OFP::KanMX4*
BN14[a]	BY4742 Δ *bn14; bn14::OFP::KanMX4*
AGA1[a]	BY4742 Δ *aga1; aga1::OFP::KanMX4*

Table 4.1 (cont.)

Strain name/ gene	Description
Genes overexpressed in VIN13 with RO88 ORF	
VIN13-UTR2	VIN13[*pDMP- PGK1$_P$-UTR2- PGK1$_T$]*
VIN13-PLB3	VIN13-[*pDMP - PGK1$_P$ -PLB3- PGK1$_T$]*
VIN13-PST1	VIN13-[*pDMP - PGK1$_P$ -PST1- PGK1$_T$]*
VIN13-EXG1	VIN13-[*pDMP - PGK1$_P$ -EXG1- PGK1$_T$]*
VIN13-ECM33	VIN13-[*pDMP - PGK1$_P$ -ECM33- PGK1$_T$]*
VIN13-PRY3	VIN13-[*pDMP - PGK1$_P$ -PRY3- PGK1$_T$]*
VIN13-HPF1'	VIN13-[*pDMP - PGK1$_P$ -HPF1'- PGK1$_T$]*
VIN13-CHS3	VIN13-[*pDMP - PGK1$_P$ -CHS3- PGK1$_T$]*
VIN13- CRH1	VIN13-[*pDMP - PGK1$_P$ -CRH1- PGK1$_T$]*
VIN13-pDMPL	VIN13-[*pDMP - PGK1$_P$ -PGK1-PGK1$_T$]*

4.2.12 Molecular biology techniques

General molecular biology techniques were used to construct the overexpression cassettes (Sambrook *et al.,* 1989) to generate strains listed in Table 4.1. High-fidelity DNA polymerase Phusion® (Finnzymes, catalogue # F530L) was used for all the PCR reactions. Finnzymes Phusion® High-Fidelity DNA Polymerase manual was followed for the PCR program and an annealing Tm of 60^{o}C was used for all the primers. For the overexpression of cell wall genes, open reading frame of genes were PCR amplified from RO88 and cloned first in pJet1.2™ vector (Fermentas, cat # K1232) following the CloneJet™ PCR cloning kit instruction manual for further sequencing. The cassettes were then released from the pJet1.2/blunt™ Cloning vector using appropriate restriction enzymes and cloned into pDMPL vector (2 µm *bla LEU2 PGK1$_P$-mmcs-PGK1$_T$ loxP-kanMX-loxp*) (Malherbe, 2009). The genes overexpressed in this study were *UTR2, PLB3, PST1, EXG1, ECM33, PRY3, HPF1', CHS3* and *CRH1*. Yeast transformation of overexpression vector pDMPL with gene of interest was carried out using the electroporation method described by Volschenk *et al.* (2004). After transformation, cells were diluted in 1 ml YPD broth and incubated for 16 h at 30°C and 200 rpm to allow expression of the resistance allele before selective pressure was applied. Transformants were selected on YPD plates containing 250 mg/l G418 after 2 to 3 days of incubation at 30°C (Gonzalez-Ramos *et al.,* 2008).

The kanamycin resistance phenotypes of transformants were confirmed by streaking out colonies on fresh selection medium. Positive strains were grown in YPD broth at 30°C and 200 rpm. A protocol described by Ausubel *et al.* (2003) was used for genomic DNA extraction. Quantitative real-time PCR was used to determine overexpressed gene expression levels. Total RNA was isolated as previously described by Schmitt *et al.* (1990) and DNase I (Roche diagnostics) treatment was used to eliminate DNA contamination. One µg of the extracted RNA was used as template for cDNA synthesis using the ImProm-II™ reverse transcription system according to the manufacturer instructions (Promega). cDNA samples were diluted to 100 ng/µl with nuclease free water before real-time PCR analysis. Primers designed using Primer Express software ver. 3 (Applied Biosystems, CA, USA) listed in Table 4.2, were used for qRT-PCR analysis. KAPA SYBR® FAST Universal 2X qPCR Master Mix (5 mL) (Kapa Biosystems Cape Town, South Africa, catalogue # KK4601) was used for real-time PCR. The 7500 cycler (Applied Biosystems) was used for qRT-PCR runs and cycling conditions during qRT-PCR were as follows: 95˚C for 5 minutes, 95˚C for 1 minute, 40 cycles of 95˚C for 15 seconds followed by 60˚C for 1 minute. A dissociation curve analysis was included to verify amplicon authenticity and collection of spectral data were performed with SYBR Green for the detection of genes overexpressed. QRT-PCR reaction runs were performed in triplicate. Relative expression data was normalized to the relative expression value of the housekeeping gene *PDA1* in each respective sample thus giving normalized relative expression for a target gene as $2^{-Ct\ (target)}/2^{-Ct\ (PDA1)}$.

4.2.13 Gene sequencing and Phylogenetic trees

The open-reading frames of *UTR2, PLB3, PST1, EXG1, ECM33, PRY3, HPF1', CHS3* and *CRH1* genes were PCR-amplified from both VIN13 and RO88 yeast strains using High-fidelity DNA polymerase Phusion® (Finnzymes, catalogue # F530L). The ORFs were cloned into pJet1.2/blunt™ cloning vector. All sequencing reactions were performed by the Central Analytical Facility (Stellenbosch University, South Africa).

DNA sequencing was performed on both strands using universal pJET1.2/blunt™ Cloning vector sequencing primers supplied with the CloneJet1.2 kit (pJet1.2 Forward Sequencing Primer 5'-CGACTCACTATAGGGAGAGCGGC-3' and pJet1.2 Reverse Sequencing Primer 5'-AAGAACATCGATTTTCCATGGCAG-3'). Primer walking was done on ORF genes longer that 1300bp. Nucleotide sequence data were assembled and Expasy tools (ExPASy, University of Geneva, Switzerland) was used to translate the nucleotide sequences to protein sequences. BioEdit (Bioedit program; Ibis Therapeutics, Carlsbad, CA, USA) was used for multiple sequence alignment. The basic local alignment search tool (BLASTp and fungal genome blast) of the National Center of Biotechnology Information (NCBI website: http://blast.ncbi.nlm.nih.gov/Blast.cgi)) were used for searching for other available protein sequences from other yeast strains to compare with VIN13 and RO88 sequences obtained in this study.

Table 4.2: List of primers used in the study (restriction sites are underlined)

Primer	Primer sequence (5' to 3')
Primers used for amplifying ORF from RO88 and VIN13 (5' to 3')	
CHS3-F *Sal*I	GATCGTCGACAGAATGACCGGCTTGAATG
CHS3-R *Spe*I	GATCACTAGTTCTATGCAACGAAGGAGTCAC
CRH1-F *Nhe*I	GATCGCTAGCACCATGAAAGTGCTTGACC
CRH1-R *Sac*I	GATCCCGCGGGTCTCAAATGTTACTTAGTGATCTACG
UTR2-F *Sal*I	GATCGTCGACATGGCAATCGTTAATAGTTGG
UTR2-R *Nde*I	GATCCATATGGTTCTTAGATGCAGATCCTCCTA
PST1-F *Nde*I	GATCCATATGGAGAAGCAAAAAAAAAAAGCTCGC
PST1-R *Spe*I	GATCACTAGTCGTGAATGACAGGTACTAGTTATCA
PLB3-F *Nhe*I	GATCGCTAGCCGCCGTTTTAAGAGGACTAA
PBL3-R *Sal*I	GATCGTCGACTTATACTGCTCCGGTAAACATC
ECM33-F *Nde*I	GATCCATATGCAATGCAATTCAAGAACGC
ECM33-R *Spe*I	GATCACTAGTCAGTGATGAACCAACCGTC
PRY3-F *Sac*II	GATCCCGCGGAACGCTTATGCTGGAGTTTC
PRY3-R *Sal*I	GATCGTCGACGAGGAGTCTAGAAGGCGAAC
HPF1'-F *Sal*I	GATCGTCGACTGGTAAGATCTCGCCTAATTG
HPF1'- R *Nde*I	GATCCATATGGGAAATGGCAGTAGTCAATG
EXG1-F *Nde*I	GATCCATATGTACCAACTAAAATGCTTTCGC
EXG1-R *Spe*I	GATCACTAGTGGACTGAGGGCGACTTAGT
Real-time PCR primers (5' to 3')	
CHS3-F-rt	TCACCTGGATGTTTTACCATCAAG
CHS3-R-rt	CCACTCCGACGAGTTGCAT
CRH1-F-rt	CGCGGCTGCCGAAAG
CRH1-R-rt	GCAGTGCTAGAAGCTGCAGTTG
UTR2-F-rt	AGGGCTGTTTGGTATGGTAAGG
UTR2-R-rt	GACCACACCAGCAAGATGTGA
PST1-F-rt	TTTGGCTGCTACTTCCTCTTCTTC
PST1-R-rt	CGTGGCATGTGAGCTTATGG
PLB3-F-rt	GCTAACAGGTGCGGGTGTTC

Table 4.2 (cont.)

Primer	Primer sequence (5' to 3')
PLB3-R-rt	CCATGCTCATAAGCACCTTCAG
ECM33-F-rt	TCCAAGGCCGCTTTCAGTAA
ECM33-R-rt	TCAATGACCTTCAATTGTGTGTTG
HPF1'-F-rt	TTTGCTATTGGTACCGGATTCTC
HPF1'-R-rt	GCACCTGCAAAGATTCCTTCA
EXG1-F-rt	CCATCGGTTGGGCTAGAAAC
EXG1-R-rt	CGGCACCATGCAAATCAA
PRY3-F-rt	GCAGAACTACGCCGACCAAT
PRY3-R-rt	TGGGCCATCGGAATGC
PST1-F-rt	AGGCAATGCCGCTATCATG
PST1-R-rt	CCGTCAAAAGACCAATCAAAGTG

For phylogenetic studies, multiple sequence alignments of protein sequences were created using MEGA v5.1 software (Kumar *et al.,* 2008) by applying default parameters. MEGA v5.1 software package was used to construct phylogenetic trees using the neighbour-joining algorithm with Poisson correction model (Zuckerkandl and Pauling, 1965).

4.2.14 Wine haze formation potential of genetically modified yeast strains

Heat test assays were performed to determine the protein haze formation potential of wines fermented by the genetically modified wine strains and parental wild type strains listed in Table 4.1 as described by Pocock and Waters (2006). Absorbance at 520 nm of 1 ml of wine sample was measured before heating at 80 ^{o}C for 2 h. The samples were then incubated at 4 ^{o}C for 16 h and cooled to room temperature before taking the final absorbance readings at 520 nm.

4.3 Results

4.3.1 Quantification of total secreted proteins by different wine yeast strains

Several wine yeast strains, representing the species *S. cerevisiae* and *S. paradoxus,* were selected for significant differences in haze reduction capability as described in Chapter 3, and were used to characterize the secreted proteins under wine fermentative conditions. Analysis of total protein content using the Bradford assay indicated no significant differences in the total amount of proteins in fermented synthetic must or in Chardonnay grape must (Figure 4.1). As expected, the total protein concentration in Chardonnay was significantly higher, reflecting that the majority of proteins in wine are grape rather than yeast derived.

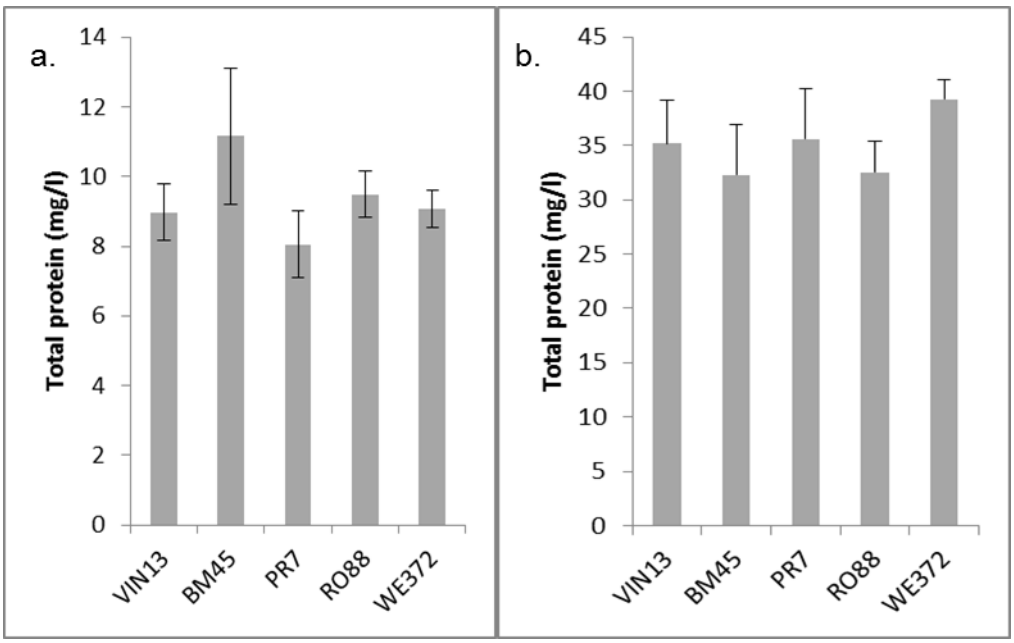

Figure 4.1: Total protein content in (a) synthetic grape must and (b) chardonnay wine measured using Bradford reagent. Samples were collected at the end of alcoholic fermentation after removing the cells from the fermented wine.

4.3.2 Protein and glycoprotein qualitative analysis

In order to broadly compare the sizes and quantities of secreted proteins and mannoproteins by the haze protecting *S. paradoxus* strain RO88 and the non-haze protecting strain *S. cerevisiae*, proteins and glycoproteins released into fermented Chardonnay grape must and synthetic must were separated using SDS PAGE after KDS precipitation. As expected, the chardonnay-extracted proteins showed very similar protein banding patterns after staining proteins and glycoproteins (Figure 4.2a). A thick band with an electrophoretic mobility corresponding to ~22 kDa may correspond to thaumatin-like protein (Fusi *et al*., 2010) and was observed in all samples, while the 30 kDa mass proteins may correspond to grape chitinases (Ferreira *et al.,* 2002; Vincezi and Curioni, 2005; Fusi *et al.,* 2010). It is interesting to further note that most of these grape proteins were stained with Pro-Q Emerald 300 glycoprotein gel stain kit with no protein stain added indicating that they are glycosylated.

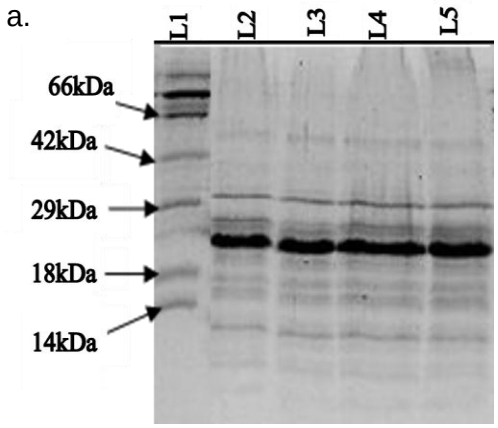

Figure 4.2a: SDS−PAGE separation of proteins extracted from fermented Chardonnay grape must. The proteins were resolved in 12% (w/v) acrylamide and stained with Pro-Q Emerald 300 glycoprotein gel stain kit, followed by SYPRO Ruby protein gel stain. Candy Cane Glycoprotein molecular weight standards were run on lane 1 and the molecular weight in kDa of each protein marker is shown in the left lane. Fermented chardonnay grape juice KDS precipitated wine proteins were also run. Lane 2: RO88 3: WE372 4: VIN13 5: BM45

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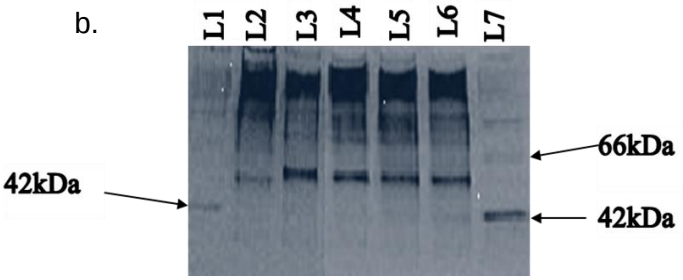

Figure 4.2b: SDS−PAGE separation of proteins extracted from fermented synthetic must. The glycoproteins were resolved in 12% (w/v) acrylamide and stained with Pro-Q Emerald 300 glycoprotein gel stain kit. Candy Cane Glycoprotein molecular weight standards (C21852) containing a mixture of glycosylated and non-glycosylated proteins were run on lane 1 and 7. The molecular weight of each protein marker was shown in the left lane. Proteins isolated by KDS precipitation from fermented MS300 were also run. Lane 2: RO88 3: L2226 4: WE372 5: BM45 and 6: VIN13.

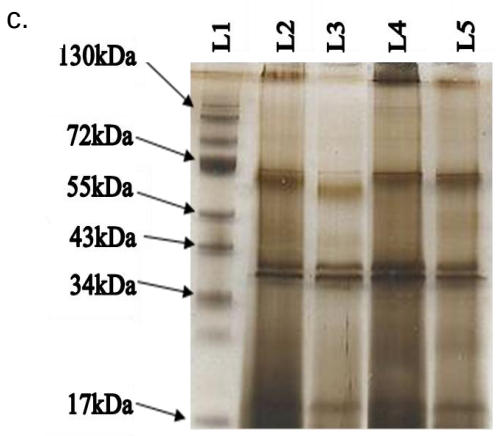

Figure 4.2c: SDS−PAGE separation of deglycosylated proteins extracted from fermented synthetic must. The proteins were resolved in 12% (w/v) acrylamide and stained with silver stain. Protein Page Ruler ladder (#SM0671) molecular weight marker was run on lane 1 and the molecular weight of each protein marker is shown in the left lane. Fermented synthetic must KDS precipitated proteins were first deglycosylated using the Deglycosylation mix before running on SDS PAGE gel. Lane 2: WE372 3: RO88 4: BM45 5: VIN13.

When glycoproteins from the synthetic must were compared (Figure 4.2b), the overall pattern was also very similar between all strains. However, several glycoproteins did not properly enter the gel, resulting in a diffuse band at a high molecular weight.

Consequently the secreted proteome was deglycosylated in order to be in a position to clearly view the different electrophoretic mobilities of the proteins and glycoproteins (Figure 4.2c). The overall pattern was again very similar between strains, but the gels suggested some differences in relative concentrations of individual proteins. A prominent but diffuse RO88 protein band between 60 and 70 KDa was visually the most obvious difference between this strain and the *S. cerevisiae* strains. The band was manually cut from SDS PAGE gel and the corresponding proteins were identified using MALDI TOF MS/MS. Eight different proteins, including Plb1p, Scw10p, Scw4p, Ecm33p, Pst1p (Hpf2p), Gas1p and Inv1p were identified from this single band, but no quantification was possible, thus making it difficult to draw any conclusion. For this reason, a global analysis of secreted proteome was undertaken using iTRAQ.

4.3.3 Global protein identification and quantification using iTRAQ analysis

A total of about 90 proteins and glycoproteins from both VIN13 and RO88 strains were identified from fermented synthetic must supernatant using iTRAQ analysis. Different classes of proteins were identified, and several of the proteins such as proteins showing anti-oxidant activity have not previously been identified from wine. However most of the glycoproteins identified by Palmisano *et al.* (2010), Rowe *et al.* (2010) and D'Amato *et al.* (2011) were also identified in this study. All RO88 proteins identified from the band cut from SDS-PAGE gel in section 4.3.2 were also identified using iTRAQ.

Tables 4.3 and 4.4 show the concentration ratios of quantified proteins and glycoproteins that were released at a higher level in VIN13 (Table 3) or by RO88 (Table 4.4). Interestingly, several proteins that have been linked to haze protection were expressed at significantly higher levels by the *S. paradoxus* strain. Hpf1´p is indeed the protein with the strongest concentration difference between the two strains, with 11 times higher concentration in the must fermented by

S. paradoxus, while Hpf2p/Pst1p is found in 2 times higher levels. Glucanases and glycosidases that are involved in cell wall remodeling, and which include Exg1p and 2p, Suc2p and Egt2p, are also secreted at higher levels in the *S. paradoxus* strain (Dupin *et al.,* 2000). Other highly released proteins are the protein and lipid binding proteins such as Trx1p and 2p, glycolipid anchored surface protein and Tal1p.

Table 4.3: Proteins released in higher (25%) concentrations in *S. cerevisiae* strain (VIN 13) relative to *S. paradoxus* strain (RO88)

Protein name and gene name	Fold increase
Homoisocitrate dehydrogenase, LYS12	7.399
Pab1p-binding protein, PBP1	7.387
NAPDH dehydrogenase (Old yellow enzyme), OYE2	4.393
Aconitate hydratase, ACO1/GLU1	2.607
Aconitate hydratase, mitochondrial, ACO1	2.388
ATP synthase subunit alpha, ATP1	2.129
Glyceraldehyde-3-phosphate dehydrogenase, TDH1	2.035
60S ribosomal protein L5, RPL5	2.006
5-methyltetrahydropteroyl triglutamate homocysteine methyltransferase, MET6	1.981
40S ribosomal protein S9-B, RPS9B	1.856
Glycoprotein, YGP1	1.834
Lys1p	1.794
NADP-specific glutamate dehydrogenase 1, GDH1	1.657
Phosphoglucomutase, PGM2	1.647
Pyruvate kinase 1, CDC19	1.616
Cell wall protein, SCRG_00832	1.606
Phosphoglycerate kinase, PGK1	1.514

Table 4.4: Proteins released in higher (25%) concentrations in *S. paradoxus* (RO88) relative to *S. cerevisiae* strain (VIN 13)

Protein name and gene name	Fold change	Protein name and gene name	Fold change
Daughter-specific expression-related protein, *DSE4*	1.515	Glycolipid-anchored surface protein 3	1.965
Phosphoglycerate mutase 1, *GPM1*	1.538	YLR179C-LIKE PROTEIN	2.012
carboxyPEPtidase Y-deficient, *PEP4*	1.587	Similar to uniprot|P04838 *Saccharomyces cerevisiae* YLL039c UBI4 ubiquitin	2.160
Heat shock protein, *HSP26*	1.637	3-isopropylmalate dehydrogenase	2.208
Glycosylphosphatidylinositol (GPI)-anchored cell wall endoglucanase, *EGT2*	1.650	Invertase-Sucrose fermentation, SUC2	2.681
Glycophospholipid-anchored surface protein, *GAS1*	1.675	EXo-1,3-beta-Glucanase, EXG1	3.247
Target of Sbf, *TOS1*	1.706	ExtraCellular Mutant, ECM33	3.322
Phospholipase B, *PLB3*	1.715	ThioRedoXin, TRX1	3.344
Glyceraldehyde-3-phosphate dehydrogenase 3, *TDH3*	1.736	Pathogen Related in Yeast, PRY3	3.584
Exo-1,3-beta-glucanase, *EXG2*	1.776	Transaldolase, TAL1	4.608
Cell wall mannoprotein-Protoplasts-Secreted *PST1/HPF2*	1.818	ThioRedoXin, TRX2	4.739
60S ribosomal protein L17-B, *RPL17B*	1.835	Mitochondrial Matrix Factor, MMF1	4.878
Glutaredoxin-2, mitochondrial, *GRX2*	1.845	YIL169C, HPF1'	11.111

The Gene Ontology (GO) analysis of all proteins and glycoproteins was performed using the *S. cerevisiae* Swiss-Prot data set as reference for comparative analysis (Supplementary Figure 4.1a; b; c; d). For all the GO analysis, proteins differentially released by 25 % were compared between *S. cerevisiae* and *S. paradoxus*. The overrepresented biological processes in VIN 13 were proteins involved in metabolic processes with a total of 61.45 % in comparison to 42.27 % observed in RO88. Moreover the identified proteins in VIN13 had 32.71 % catalytic activity and 7.48 % protein binding whereas RO88 had 39.39 % catalytic activity and 10.10 % protein binding (Supplementary Figure 4.1c; d). About 2 % of proteins highly released in RO88 were also involved in enzyme regulation while these were absent in proteins highly released by VIN13. No annotation was available for 18.18 % of the identified RO88 proteins when compared to 11.21 % in VIN13 for the molecular function analysis thus indicating that annotation of the

yeast proteins is still in its preliminary base despite the availability of the whole yeast genome sequence.

4.3.4 Assessment of individual proteins on haze protection

To further verify whether the increased levels of certain mannoproteins released by the *S. paradoxus* strain might be directly contributing to the haze protective properties of the strain, two strategies were followed. In a first approach, a number of deletion strains from the BY4742 EUROSCARF haploid strain collection were used to assess haze protective properties. The deletion strains included mutants of genes identified in the exoproteome analysis and also some strains mutated for genes that had previously been shown or suggested to contribute to haze protection. While this genetic background may not present a good model to analyze haze protective properties as further discussed in the discussion section, the collection may nevertheless allow the identification of interesting genetic targets. In a second approach, the genes encoding proteins that were highly released in *S. paradoxus* strain were cloned from this strain and overexpressed in *S. cerevisiae* to directly assess individual impacts.

Most deletion mutants in the BY4742 background did not differ in haze protective activity when compared to the parental strain BY4742, except for the Δ*ygp1* and Δ*yjl160c* mutant strains which showed reduced haze formation (Figure 4.3). Yjl160cp is a putative protein of unknown function and a member of the PIR (proteins with internal repeats) family of cell wall proteins. Ygp1 protein is a cell wall-related secretory glycoprotein which plays a role in stress adaptation (Destruelle *et al.,* 1994). The meaning of this finding is not clear at this stage, and we are currently following up on these two proteins with the gene deletions in commercial wine yeast strains for further assessment of the impact these genes have on wine haze formation.

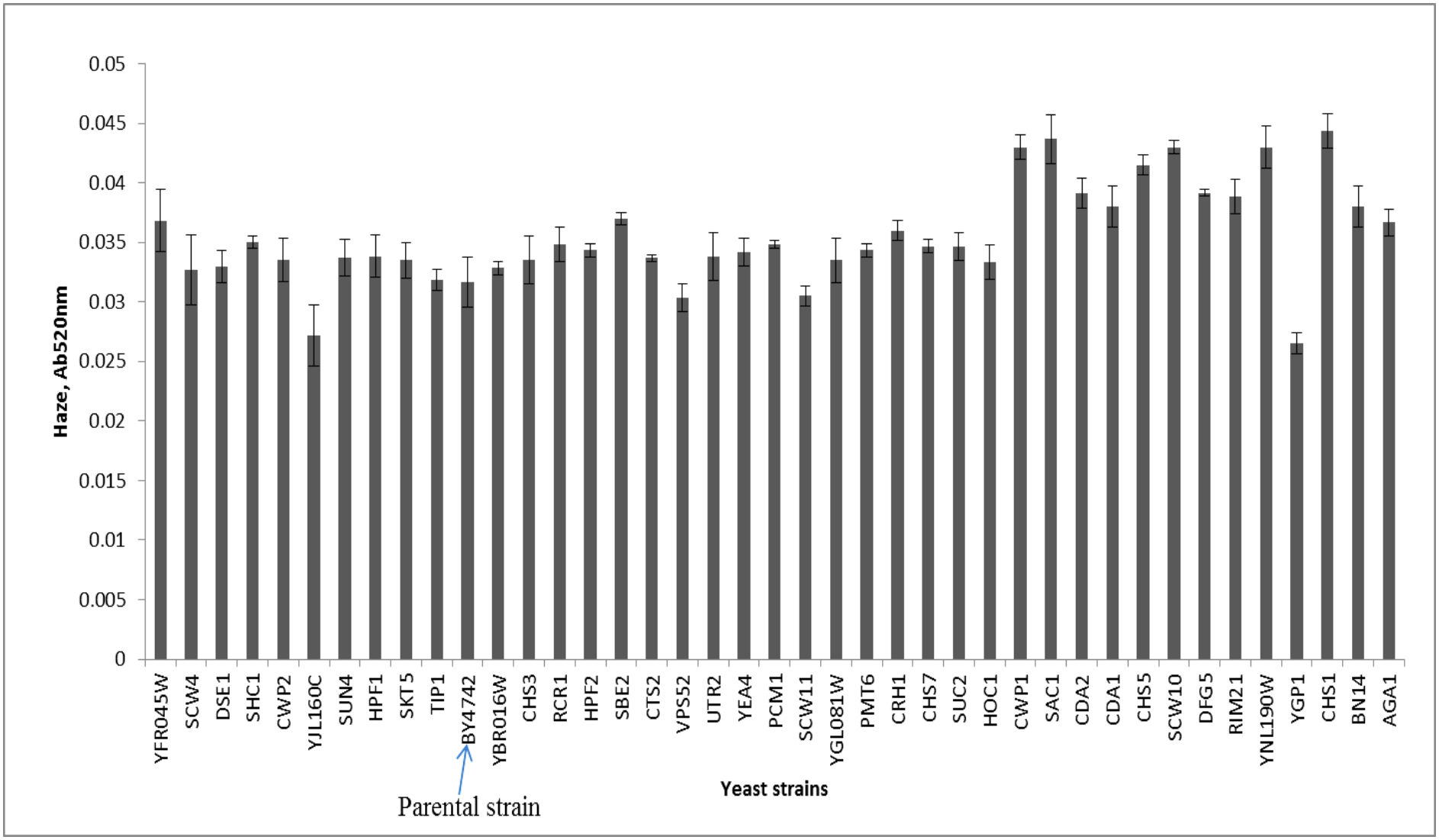

Figure 4.3: Haze formation potential in Chardonnay wines fermented using the deletion mutants on BY4742 background from the EUROSCARF library. Wine also fermented by BY4742 parental strain was assayed for protein haze formation.

4.3.5 Cloning of *S. paradoxus* genes and phylogenetic analysis

UTR2, PLB3, PST1, EXG1, ECM33, PRY3, HPF1', CHS3 and *CRH1* genes (described in Table 4.5) were cloned and sequenced from both VIN13 and RO88 and the translated amino acid sequences were compared with those found in the genome databases. Alignment of the protein sequences is shown in Supplementary Figure 4.2 using ClustalW and ClustalX version 2 (Larkin *et al.,* 2007; Goujon *et al.,* 2010).

Table 4.5: Description of proteins used in this study for both overexpression and phylogenetic analysis

Protein name and gene name	Description
Chitin transglycosylase, ***UTR2***	Chitin transglycosylase that functions in the transfer of chitin to β(1-6) and β(1-3) glucans in the cell wall;
Phospholipase B, ***PLB3***	A lysophospholipase hydrolyzes phosphatidylinositol and phosphatidylserine and displays transacylase activity in vitro
Cell wall mannoprotein-Protoplasts-Secreted, ***PST1/HPF2***	GPI-attached cell wall protein secreted by regenerating protoplasts; up-regulated by activation of the cell integrity pathway (Redundant function to ECM33)
Exo-1,3-beta-Glucanase ***EXG1***	Major exo-1,3-β-glucanase of the cell wall, involved in cell wall β-glucan assembly
Extracellular Mutant, ***ECM33***	GPI-anchored protein of unknown function, has a possible role in apical bud growth
Pathogen Related in Yeast, ***PRY3***	Cell wall protein with a role in mating efficiency and expression of full-length transcript is daughter cell-specific
Haze protection factor 1', ***HPF1'***	Putative protein of unknown function
Chitin synthase III, ***CHS3***	Chitin synthase III catalyzes the transfer of N-acetylglucosamine (GlcNAc) to chitin; required for synthesis of the majority of cell wall chitin, the chitin ring during bud emergence, and spore wall chitosan
Chitin transglycosylase, ***CRH1***	Chitin transglycosylase functions in the transfer of chitin to β(1-6) and β(1-3) glucans in the cell wall and its expression induced by cell wall stress

The phylogenetic relationship between *S. cerevisiae* strains and *S. paradoxus* strain RO88 were studied by constructing neighbour-joining trees based on amino acid sequences of the cell wall genes. As shown in Supplementary Figure 4.3a-i, most wine yeast strains clustered together although differences were observed for certain proteins. Amino acid differences in protein sequence ranged from 1.5 % for Plb3p to 25.5 % for the Pry3p when comparing VIN13 and RO88 sequences (Supplementary Figure 4.2*).*

These results however have to be interpreted with caution as the output tree of a phylogenetic analysis does not necessarily accurately represent the species evolutionary history as the analysis can be confounded by horizontal/lateral gene transfer, duplication and deletion, hybridization between species, convergent evolution, and conserved sequences. Furthermore, some positions in the protein coding genes are more variable than others and some genes may evolve faster than others.

4.3.6 Expression levels of the overexpressed genes

Quantitative RT-PCR was carried out on the transformed VIN13 where nine cell wall genes amplified from *S. paradoxus* strain RO88 were cloned in pDMPL vector and overexpressed under the constitutive *PGK1* promoter in *S. cerevisiae* VIN13 strain and control strain to assess the increase in expression levels in conditions of wine fermentation. All the genes ORF were sequenced to confirm that the correct gene was overexpressed. RT-PCR data of gene expression comparing transformed and wild type VIN13 show that all genes were indeed expressed at higher levels in the transformed strains, with particularly significant increase in the case of *PST1, ECM33, HPF1, CHS3* and *CRH1* (Figure 4.5). Each gene exists and is natively expressed in the *S. cerevisiae* strain VIN13, but the figure shows the expression ratio of transformant / wild type. It is important to highlight that the fold increases do not directly relate to protein expression, since some genes are bound to be naturally very low in the wild type while others may already be highly expressed. In this case the expression of a cloned gene might be significantly higher when compared to wild type, but in absolute amounts (protein concentration) the corresponding protein might still be at lower levels than a protein with a lower fold increase.

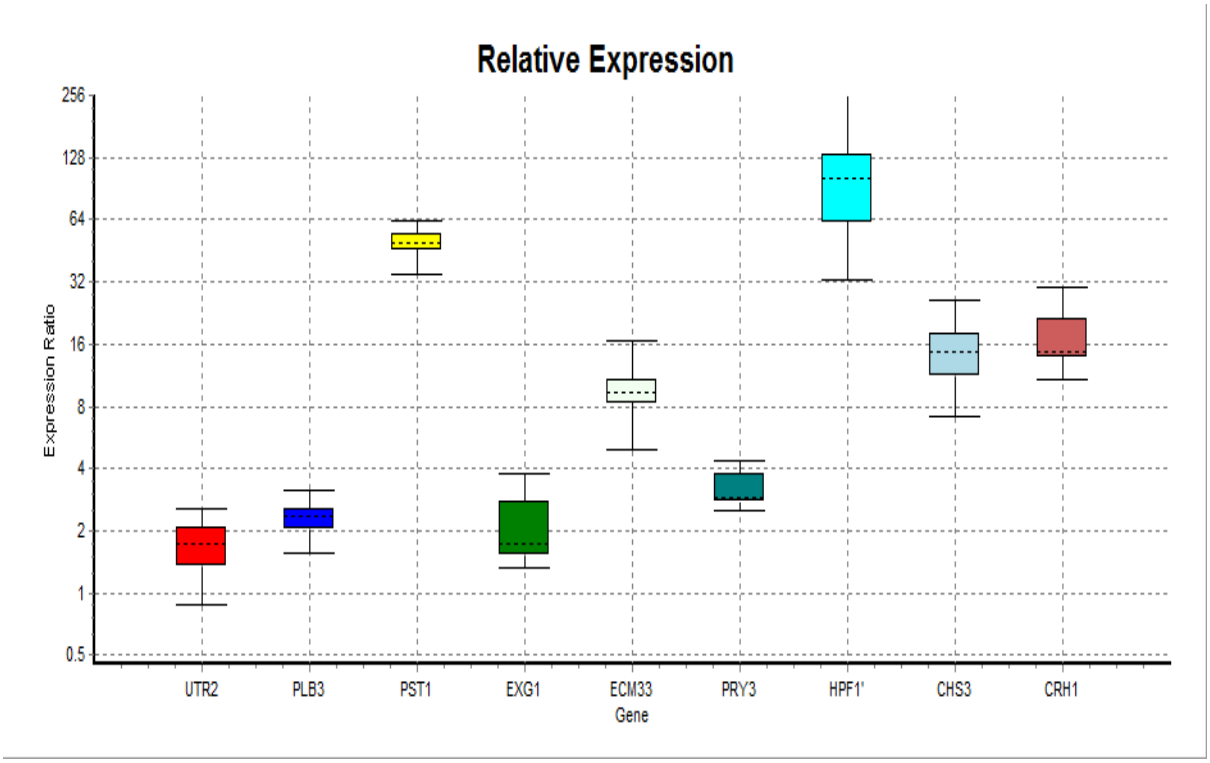

Figure 4.5: qRT-PCR relative expression levels of genes overexpressed in VIN13. Samples were taken at the end of alcoholic fermentation. Relative expression data was normalized to the relative expression value of the housekeeping gene *PDA1* in each respective sample thus giving normalized relative expression for a target gene as $2^{-Ct\ (target)}/2^{-Ct\ (PDA1)}$. Wild type VIN13 was used as a reference strain. Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

4.3.7 Wine haze assays for the genetically modified strains

Heat test assays were carried out to determine the potential haze formation capacity using the heat test. Overexpression of cell wall genes *UTR2, PLB3, PRY3, ECM33, HPF1'* resulted in no wine haze reduction whereas wines fermented by mutants overexpressing *PST1, EXG1, CHS3* and *CRH1* revealed a significant reduction in wine haze formation potential (Figure 4.6) when compared to the parental strain VIN13-pDML and to VIN13 transformed with an empty pDMPL empty vector.

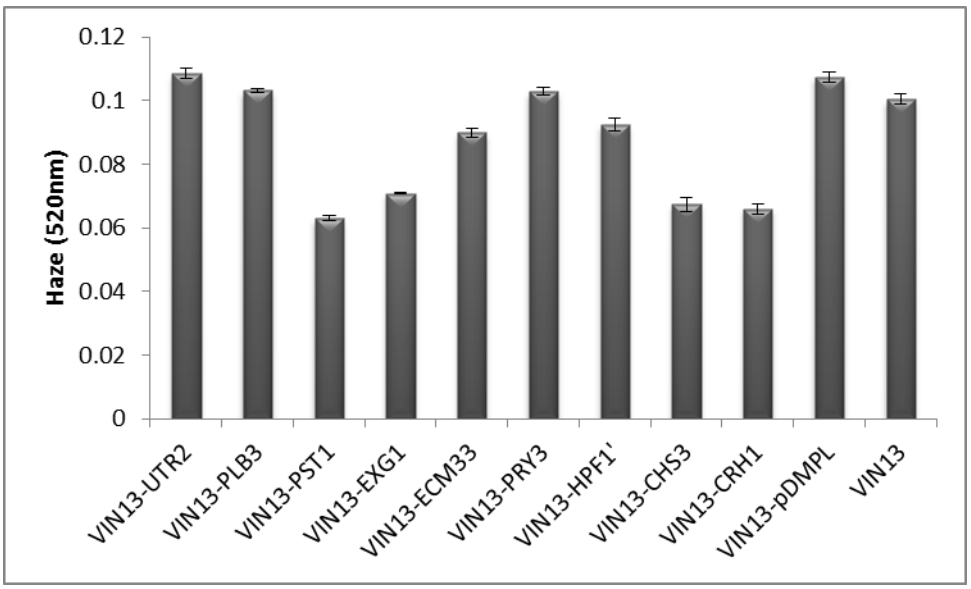

Figure 4.6: Haze levels of Chardonnay wines fermented by VIN13 parental strain and VIN13 mutants overexpressing genes in pDMPL vector. VIN13-pDMPL is the strain VIN13 transformed with pDMPL vector overexpressing no gene.

4.4 Discussion

To enhance our understanding of the yeast proteins released in wine, SDS PAGE was used to visualize the different proteomic banding pattern between strains. iTRAQ analysis was also used to identify and quantify the total proteins and glycoproteins released into wine media. Global proteomic analysis was carried out to compare *S. paradoxus* (RO88) and *S. cerevisiae* strains (VIN13) showing different haze protection capacities. No differences were observed in total protein content measured in both fermented synthetic must and chardonnay must between *S. cerevisiae* and *S. paradoxus* strains. *Saccharomyces cerevisiae* and its close congener *S. paradoxus* are typically indistinguishable by the phenotypic criteria of classical yeast taxonomy, except for their evolutionarily differences revealed by hybrid spore inviability and genomic sequence divergence (Sweeney *et al*., 2004; Fay and Benavides, 2005). However, no studies to date have investigated the differences between *S. cerevisiae* and *S. paradoxus* with reference to exoproteomic differences. Considering the absence of differences in total protein quantities between *S. cerevisiae* strains and *S. paradoxus* strains, it can be hypothesized that the nature of the protein identity is important regardless of the total protein released by a yeast strain.

In most wine proteomic studies, protein profiling has been done in fermented grape juice but not in model wine solution (Weiss *et al.,* 1998, Palmisano *et al.,* 2010). Analysing the proteins in wine limits the identification of yeast proteins due to the presence of grape and bacterial proteins (Kwon *et al.,* 2004), demonstrated by the multiple bands observed in protein gel extracted from Chardonnay wine in this study. These other proteins may be present in higher concentrations thus masking yeast proteins present in low concentrations in wine (Feulliat *et al.,* 2003). In wine most of the identified proteins in previous studies are grape proteins (Cilindre *et al.,* 2008; Fusi *et al.,* 2010; Palmisano *et al.,* 2010 D'Amato *et al.,* 2011). Moreover, *Botrytis cinerea* if present from infected grapes, have been implicated in degrading both must and wine proteins as it exhibits proteolytic activity (Marchal *et al.,* 2006; Cilindre *et al.,* 2008). This results in modified wine proteomic profiles which will not be a true qualitative and quantitative representation of proteins normally present in wines. In this study, it was further observed that most grape proteins are glycosylated as they stained with glycoprotein staining kit which is specific for glycosylated proteins. The Pro-Q® Emerald 300 glycoprotein stain reacts with periodate-oxidized carbohydrate groups thus creating a bright green-fluorescent signal on glycoproteins (Serrano *et al.,* 2005). This observation may perhaps explain the stability of these grape proteins under wine making conditions (Waters *et al.,* 1998) as glycosylation is thought to confer additional protein stability (Helenius and Aebi, 2004).

Minor differences in electrophoretic gel mobilities of proteins and glycoproteins were observed in synthetic must between *S. paradoxus* RO88 and all the *S. cerevisiae* strains evaluated, including VIN13, BM45 and WE372. These results suggested some differences in the nature of the proteins released during fermentation by the different yeast species. Vincezi *et al*. (2011) also observed strain differences in yeast polysaccharides released into wine. Diffuse band observed in glycoprotein gel stained with Pro-Q Emerald 300 glycoprotein gel stain kit can be explained by the glycoproteins being most likely characterized by higher molecular mass greater than the average gel pore diameter or, by a scarce affinity for SDS, leading to low charge/mass ratio (Fusi *et al.,* 2010). Several proteins were identified in a single band manually cut from an SDS PAGE gel. This could be due to 'spot over-lapping' which occurs when proteins have

similar electrophoretic behaviour as already observed by Cilindre *et al.,* 2008 and Westbrook *et al*. (2001).

A total of 90 glycosylated and non-glycosylated yeast proteins were identified using the iTRAQ analysis in this study. Most of the proteins highly released in VIN13 relative to RO88 where metabolic proteins whereas RO88 released more cell wall proteins. Most of the proteins have not been reported as present on the cell surface from previous studies, revealing that the yeast cell surface is composed not only of typical but also by atypical cell wall proteins (Nombela *et al*., 2006; Inseser *et al*., 2010). Proteins with enzyme regulator function and catalytic activity highly released by the *S. paradoxus* strains further confirms the known fact that *S. paradoxus* strains releases more glycolytic proteins such as pectinases in comparison to *S. cerevisiae* strains. However more *S. paradoxus* proteins were not annotated compared *S. cerevisiae* strains indicating that yeast annotation is still at its preliminary phase. The proteins identified in this study were similar to those identified by Kwon *et al.* (2004), Inseser *et al.* (2010) and Branconi *et al.* (2011). High levels of Hpf1´p and Hpf2p released by RO88 strain could be the most likely protein candidates responsible for haze protective capabilities of the strain.

To investigate the effect of some proteins highly released by the *S. paradoxus* strain and those which have been shown previously to be have an impact on wine haze reduction, strains from the EUROSCARF deletion mutant library were used to assess their haze protective capacity. Lower wine haze formation was obtained for wines fermented by BY4742Δ*yjl160c,* a putative protein of unknown function and a member of the PIR and BY4742Δ*ygp1* a cell wall-related secretory glycoprotein (Destruelle *et al.,* 1994). Deletion of these two genes in BY4742 background may have led to cell wall modifications such as release of high levels of haze protecting material which may explain the observed reduced wine haze. Protein haze development in wine is thought to be antagonized by specific yeast glycoproteins (Waters *et al.,* 1998; Gonzalez-Ramos *et al.,* 2008; Vincezi *et al.,* 2011). Other cell wall deletion mutants did not impact on wine haze formation including the deletion of haze protection factor genes,

possibly due to the BY4742 yeast genetic background. Moreover, Gonzalez-Ramos *et al.* (2006) observed the effect of yeast genetic background on the release of mannoproteins and polysaccharides. BY4742 genetic background is also not a perfect model for studying wine haze formation potential as it does not ferment well at high sugar concentrations applicable to wine making conditions (Gonzalez-Ramos *et al.,* 2006) and has poor secretion properties in comparison to commercial wine yeast strains. In addition BY4742 differs in cell wall properties when compared to wine yeast strains as for it has example been shown to carry a *FLO 8* gene deletion (Liu *et al.,* 1996).

Overexpression of cell wall genes in *S. cerevisiae* VIN13 with RO88 open reading frames was carried out in this study to determine the impact of the proteins or genetically engineered strain on wine haze reduction. Overexpression of *PST1, EXG1, CHS3* and *CRH1* resulted in slight wine haze reduction whereas the other genes did not differ from the parental strain. Reduced levels of wine haze confirm the results obtained by Brown *et al.* (2007) on the importance of *HPF2/PST1* on wine haze reduction. Overexpression of *CHS3*, a chitin synthase and *CRH1*, a chitin transglycosylase that functions in the transfer of chitin to β-1,6- and β-1,3-glucans in the cell wall may possibly indicate the importance of cell wall chitin on wine haze reduction (Vincezi *et al*., 2005). Overexpression of *EXG1* by Jiang *et al.* (1995) resulted in a slight reduction in cell wall β-1,6-glucan, which may explain the reduction in wine haze in our study due to possible high release of cell wall polysaccharides as a result of reduced cell wall β-1,6-glucan layer responsible for inter-linking mannoproteins and chitin to the yeast cell wall.

In this study, the cell wall protein sequences overexpressed in VIN13 were obtained from sequencing VIN13 and RO88 corresponding genes. The de novo translated protein sequences were used for phylogenetic analysis to compare to other proteins from other *S. cerevisiae* strains found in the NCBI database. In all the genes analysed, differences in protein sequence clustering were observed between RO88 and VIN13. However, there is a need to determine whether these differences in sequences have any impact on the enzyme activity, protein folding,

and overall cell walls' charge among other factors which may impact on the protein's phenotype such as the ability to reduce wine haze. Kellis *et al.* (2003) when comparing de novo protein coding sequences for *S. cerevisiae, S. paradoxus, Saccharomyces. mikatae* and *Saccharomyces bayanus* observed that most of the nucleotide changes in protein-coding regions are silent and a small number of events closely spaced compensatory indels that affect the translation of small contiguous amino-acid stretches further suggest additional mechanisms of rapid protein change. In addition these events include the loss and gain of stop codons (by a nucleotide substitution or a frame-shifting indel), which may result in the rapid change of protein segments or the translation of previously non-coding regions. On the other hand our results may be consisted with observations made by Borneman *et al.* (2010) who observed genetic variation comprising both single nucleotide polymorphisms and large-scale insertions and deletions in ORF between wine and brewing yeast strains.

4.5 Conclusion

There were differences in proteomic and glycoproteomic electrophoretic profiles between *S. cerevisiae* and *S. paradoxus* strains as demonstrated by the glycoproteins and proteins' electrophoretic abilities in SDS PAGE gel. Based on the iTRAQ results, cell wall glycoproteins were highly expressed in *S. paradoxus* strain in comparison to *S. cerevisiae* strain which possibly could account for the observed haze formation phenotypic differences between the two strains. Other cell wall proteins besides Hpf1´p and Hpf2p highly released by RO88 yeast strain may indeed contribute to the observed difference between *S. paradoxus* and *S. cerevisiae* as demonstrated by the overexpression study. In conclusion, the differences in the nature and quantities of the proteins released by different yeast strains maybe at least in part responsible for the wine haze protection.

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iTRAQ MS analysis was provided by the Proteomics Core Facility at Sahlgrenska Academy, University of Gothenburg, Sweden

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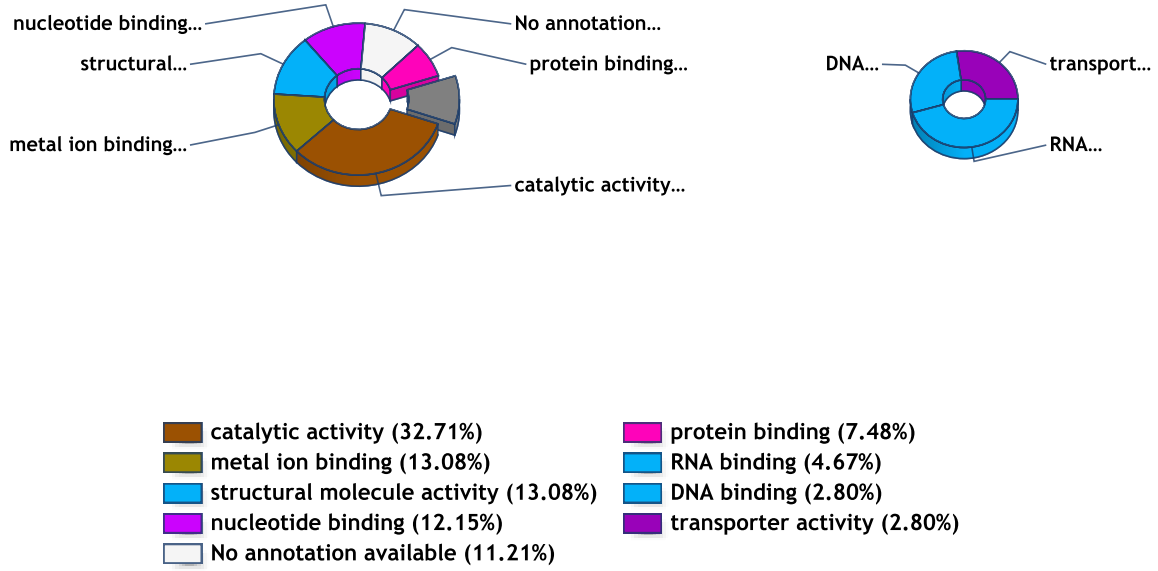
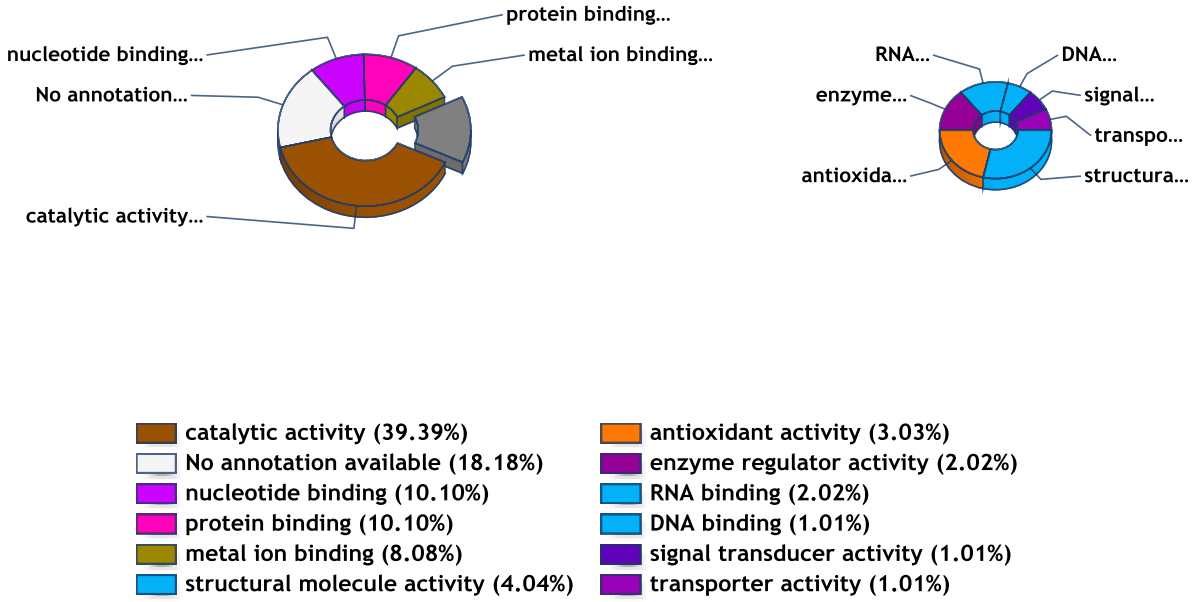
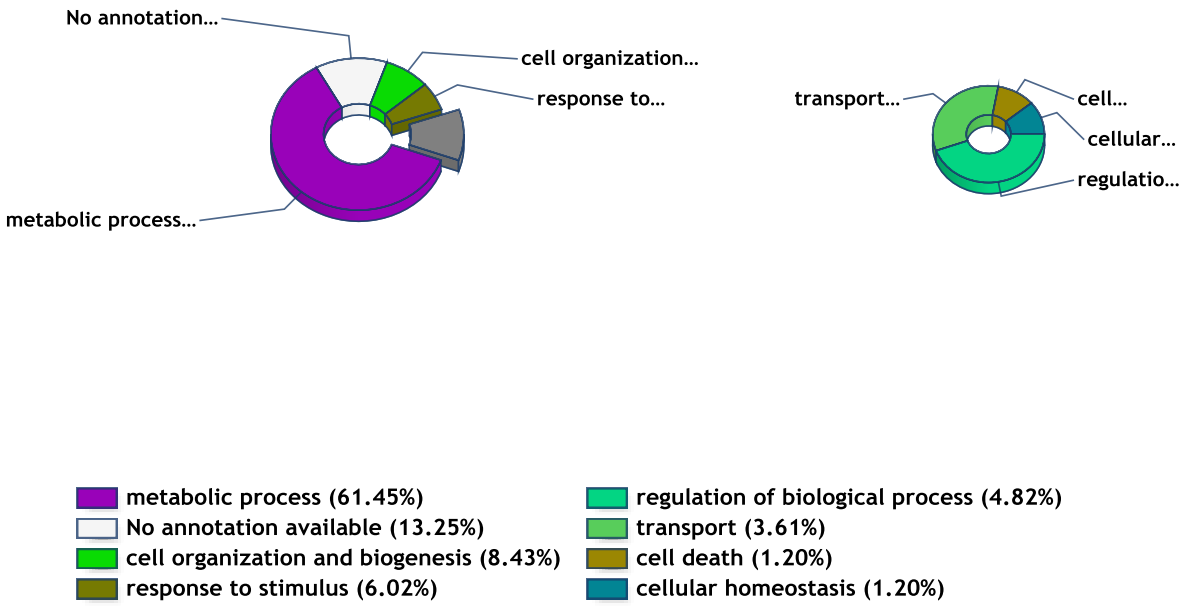
4.8 Supplementary figures



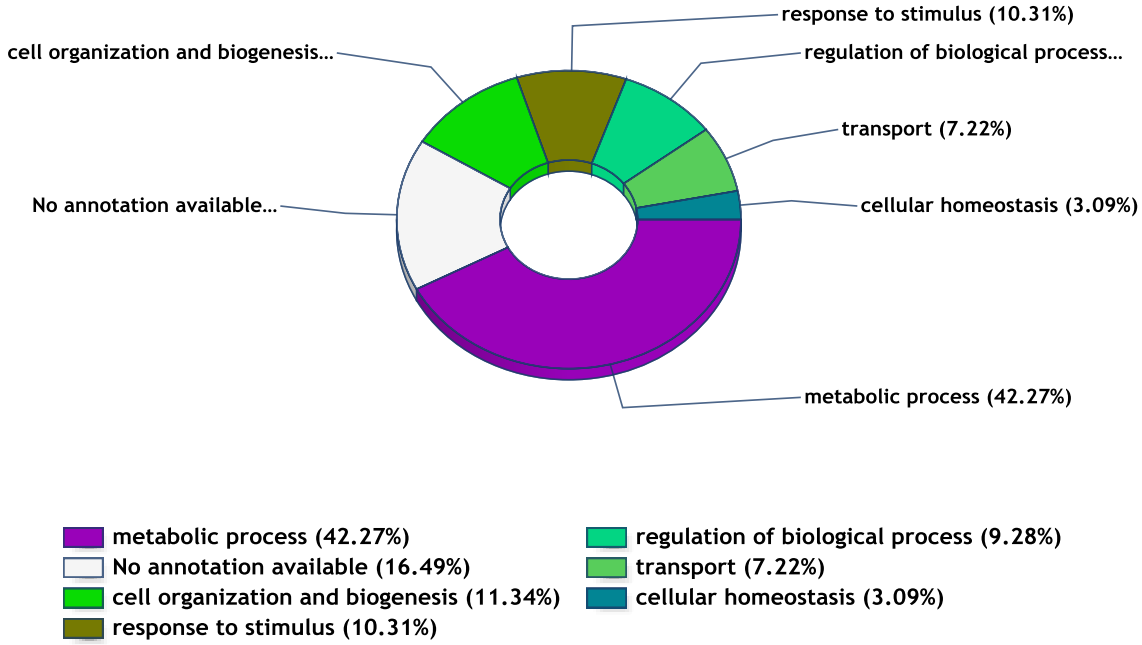
Supplementary Figure 4.1a: Gene Ontology analysis of molecular function of proteins highly released by 25% in VIN13.



Supplementary Figure 4.1b: Gene Ontology analysis of molecular function of proteins highly released by 25% in RO88.



Supplementary Figure 4.1c: Gene Ontology analysis of biological processes of proteins highly released by 25% in VIN13. Values are in percentages.



Supplementary Figure 4.1d: Gene Ontology analysis of biological processes of proteins highly released by 25% in RO88. Values are in percentages.

Utr2p

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RO88p-Utr2p          MAIVNSWLICLVSIFSFVVRVEAATFCNATQACPEDKPCCSQYGECGTGQYCLNNCDVRY
EC1118-Utr2p         MAIVNSWLICLVSIFSFVVRVEAATFCNATQACPEDKPCCSQYGECGTGQYCLNNCDVRY
AWRI1631-Utr2p       MAIVNSWLICLVSIFSFVVRVEAATFCNATQACPEDKPCCSQYGECGTGQYCLNNCDVRY
VIN13-Utr2p          MAIVNSWLICLVSIFSFVVRVEAATFCNATQACPEDKPCCSQYGECGTGQYCLNNCDVRY
VL3-Utr2p            ------------------------------------------------------------
YJM789-Utr2p         MAIVNSWLICLVSVFSFVVRVEAATFCNATQACPEDKPCCSQYGECGTGQYCLNNCDVRY
Kyokai7              MAIVNSWLICLVSVFSFVVRVEAATFCNATQACPKDKPCCSQYGECGTGQYCLNNCDVRY


S288C-Utr2p          SFSHDSCMPVPICKSSSTKFKDYSSKLGNANTFLGNVSEADWLYTGDVLDYDDEESLILA
RO88p-Utr2p          SFSHDSCMPVPICKSSSTKFKDYSSKLGNANTFLGNVSEADWLYTGDVLDYDDEESLILA
EC1118-Utr2p         SFSHDSCMPVPICKSSSTKFKDYSSKLGNANTFLGNVSEADWLYTGDVLDYDDEESLILA
AWRI1631-Utr2p       SFSHDSCMPVPICKSSSTKFKDYSSKLGNANTFLGNVSEADWLYTGDVLDYDDEESLILA
VIN13-Utr2p          SFSHDSCMPVPICKSSSTKFKDYSSKLGNANTFLGNVSEADWLYTGDVLDYDDEESLILA
VL3-Utr2p            -------MPVPICKSSSTKFKDYSSKLGNANTFLGNVSEADWLYTGDVLDYDDEESLILA
YJM789-Utr2p         SFSHDSCMPVPICKSSSTKFKDYSSKLGNANTFLGNVSEADWLYTGDVLDYDDEESLILA
Kyokai7              SFSHDSCMPVPICKSSSTKFKDYSSKLGNANTFLGNVSEADWLYTGDVLDYDDEESLILT
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S288C-Utr2p          MPKNSGGTVLSSTRAVWYGKVSARIKTSHLAGVVTGFILYSGAGDELDYEFVGADLETAQ
RO88p-Utr2p          MPKNSGGTVLSSTRAVWYGKVSARIKTSHLAGVVTGFILYSGAGDELDYEFVGADLETAQ
EC1118-Utr2p         MPKNSGGTVLSSTRAVWYGKVSARIKTSHLAGVVTGFILYSGAGDELDYEFVGADLETAQ
AWRI1631-Utr2p       MPKNSGGTVLSSTRAVWYGKVSARIKTSHLAGVVTGFILYSGAGDELDYEFVGADLETAQ
VIN13-Utr2p          MPKNSGGTVLSSTRAVWYGKVSARIKTSHLAGVVTGFILYSGAGDELDYEFVGADLETAQ
VL3-Utr2p            MPKNSGGTVLSSTRAVWYGKVSARIKTSHLAGVVTGFILYSGAGDELDYEFVGADLETAQ
YJM789-Utr2p         MPKNSGGTVLSSTRAVWYGKVSARIKTSHLGGVVTGFILYSGAGDELDYEFVGADLETAQ
Kyokai7              MPKNSGGTVLSSTRAVWYGKVSARIKTSHLAGVVTGFILYSGAGDELDYEFVGADLETAQ
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S288C-Utr2p          TNFYWESVLNYTNSANISTTDTFENYHTYELDWHEDYVTWSIDGVVGRTLYKNETYNATT
RO88p-Utr2p          TNFYWESVLNYTNSANISTTDTFENYHTYELDWHEDYVTWSIDGVVGRTLYKNETYNATT
EC1118-Utr2p         TNFYWESVLNYTNSANISTTDTFENYHTYELDWHEDYVTWSIDGVVGRTLYKNETYNATT
AWRI1631-Utr2p       TNFYWESVLNYTNSANISTTDTFENYHTYELDWHEDYVTWSIDGVVGRTLYKNETYNATT
VIN13-Utr2p          TNFYWESVLNYTNSANISTTDTFENYHTYELDWHEDYVTWSIDGVVGRTLYKNETYNATT
VL3-Utr2p            TNFYWESVLNYTNSANISTTDTFENYHTYELDWHEDYVTWSIDGVVGRTLYKNETYNATT
YJM789-Utr2p         TNFYWESVLNYTNSANISTTDTFENYHTYELDWHEDYVTWSIDGVVGRTLYKNETYNATT
Kyokai7              TNFYWESVLNYTNSANISTTDTFENYHTYELDWHEDYVTWSIDGVVGRTLYKNETYNATT
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S288C-Utr2p          QKYQYPQTPSKVDISIWPGGNSTNAPGTIAWSGGEINWDASDISNPGYYYAIVNEVNITC
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EC1118-Utr2p         QKYQYPQTPSKVDISIWPGGNSTNAPGTIAWSGGEINWDAADISNPGYYYAIVNEVNITC
AWRI1631-Utr2p       QKYQYPQTPSKVDISIWPGGNSTNAPGTIAWSGGEINWDAADISNPGYYYAIVNEVNITC
VIN13-Utr2p          QKYQYPQTPSKVDISIWPGGNSTNAPGTIAWSGGEINWDAADISNPGYYYAIVNEVNITC
VL3-Utr2p            QKYQYPQTPSKVDISIWPGGNSTNAPGTIAWSGGEINWDAADISNPGYYYAIVNEVNITC
YJM789-Utr2p         QKYQYPQTPSKVDISIWPGGNSTNAPGTIAWSGGEINWDASDISNPGYYYAIVNEVNITC
Kyokai7              QKYQYPQTPSKVDISIWPGGNSTNAPGTIAWSGGEINWDASDISNPGYYYAIVNEVNITC
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EC1118-Utr2p         YDPPSDTKKNGTSAYVYTSSSEFLAKDIAITDDEVTMDSDEGSGLDPHKGATTSSTQKSS
AWRI1631-Utr2p       YDPPSDTKKNGTSAYVYTSSSEFLAKDIAITDDEVTMDSDEGSGLDPHKGATTSSTQKSS
VIN13-Utr2p          YDPPSDTKKNGTSAYVYTSSSEFLAKDIAITDDEVTMDSDEGSGLDPHKGATTSSTQKSS
VL3-Utr2p            YDPPSDTKKNGTSAYVYTSSSEFLAKDIAITDDEVTMDSDEGSGLDPHKGATTSSTQKSS
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Kyokai7              YDPPSDTKKNGTSAYVYTSSSEFLAKDIAITDDEVMMDSDEGSGLDPHKGATTSSTQKSS
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RO88p-Utr2p          SSTATSSSKTSSDHSSSTKKSSKTSSTASSSSSSSSSSSSSSSTATKNGDKVVSSVSSSV
EC1118-Utr2p         SSTATSSSKTSSDHSSSTKKSSKTSSTASSSSSSSSSS-SSS-TATKNGDKVVSSVSSSV
AWRI1631-Utr2p       SSTATSSSKTSSDHSSSTKKSSKTSSTASSSSSSSSSS-SSSSTATKNGDKVVSSVSSSV
VIN13-Utr2p          SSTATSSSKTSSDHSSSTKKSSKTSSTASSSSSSSSSS-SSSSTATKNGDKVVSSVSSSV
VL3-Utr2p            SSTATSSSKTSSDHSSSTKKSSKTSSTASSSSSSSSSS-SSSSTATKNGDKVVSSVSSSV
YJM789-Utr2p         SSTATSSSKTSSDHSSSTKKSSKTSSTASSSSSSSSSSSSSS-TATKNGDKVVSSVSSSV
Kyokai7              SSTATSSSKTSSDHSSSTKKSSKTSSTASSSSSSSSSSSSSSSTATKNGDKVVSSVSSSV
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RO88p-Utr2p          TSQTQTTSSVSGSASSSTSSMSGNNAGANVAANWRLTVLCVILGYVL
EC1118-Utr2p         TSQTQTTSSVSGSASSSTSSMSGNNAGANVAANWHLTVLCVILGYVL
AWRI1631-Utr2p       TSQTQTTSSVSGSASSSTSSMSGNNAGANVAANWHLTVLCVILGYVL
VIN13-Utr2p          TSQTQTTSSVSGSASSSTSSMSGNNAGANVAANWHLTVLCVILGYVL
VL3-Utr2p            TSQTQTTSSVSGSASSSTSSMSGNNAGANVAANWHLTVLCVILGYVL
YJM789-Utr2p         TSQTQTTSSVSGSASSSTSSMSGNNAGANVAANWRLTVLCVILGYVL
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Kyokai7             TSQTQTTSSVSGSASSSTSSMSGNNAGANVAANWRLTVLCVILGYVL
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Plb3p

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FostersB            MIRPLCSKIIISYIFAISQFLLAANAWSPTDSYVPGTVSCPDDINLVREATSISQNESAW
S288C-PLB3p         MIRPLCSKIIISYIFAISQFLLAANAWSPTDSYVPGTVSCPDDINLVREATSISQNESAW
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VL3                 MIRPLCSKIIISYIFAISQFLLAANAWSPADSYVPGTVSCPDDINLVREATSISQNESAW
YJM789              MIRPLCSKIIISYIFAISQFLLAANAWSPADSYVPGTVSCPDDINLVREATSISQNESAW
RO88p-              MIRPLCSKIIISYIFAISQFLLAANAWSPTDSYVPGTVSCPDDINLVREATSISQNESAW
VIN13-              MIRPLCSKIIISYIFAISQFLLAANAWSPTDSYVPGTVSCPDDINLVREATSISQNESAW
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FostersB            LEKRNKVTSVALKDFLTRATANFSDSSEVLSKLFNDGNSENLPKIAVAVSGGGYRSMLTG
S288C-PLB3p         LEKRNKVTSVALKDFLTRATANFSDSSEVLSKLFNDGNSENLPKIAVAVSGGGYRSMLTG
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VL3                 LEKRNKVTSVALKDFLTRATANFSDSSEVLSKLFNDGNSENLPKIAVAVSGGGYRSMLTG
YJM789              LEKRNKVTSVALKDFLTRATANFSDSSEVLSKLFNDGNSENLPKIAVAVSGGGYRSMLTG
RO88p-              LEKRNKVTSVALKDFLTRATANFSDSSEVLSKLFNDGNSENLPKIAVAVSGGGYRSMLTG
VIN13-              LEKRNKVTSVALKDFLTRATANFSDSSEVLSKLFNDGNSENLPKIAVAVSGGGYRSMLTG
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VL3                 AGVLAAMDNRTEGAYEHGLGGLLQSTTYLSGASGGNWLVGTLALNNWTSVQDILNNMQND
YJM789              AGVLAAMDNRTEGAYEHGLGGLLQSTTYLSGASGGNWLVGTLALNNWTSVQDILNNMQND
RO88p-              AGVLAAMDNRTEGAYEHGLGGLLQSTTYLSGASGGNWLVGTLALNNWTSVQDILNNMQND
VIN13-              AGVLAAMDNRTEGAYEHGLGGLLQSTTYLSGASGGNWLVGTLALNNWTSVQDILNNMQND
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FostersB            DSIWDLSDSIVTPGGINIFKTAKRWDHISNAVESKQNADYNTSLADIWGRALAYNFFPSL
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EC1118-             DSIWDLSDSIVTPGGINIFKTAKRWDHISNAVESKQNADYNTSLADIWGRALAYNFFPSL
VL3                 DSIWDLSDSIVTPGGINIFKTAKRWDHISNAVESKQNADYNTSLADIWGRALAYNFFPSL
YJM789              DSIWDLSDSIVTPGGINIFKTAKRWDHISNAVESKQNADYNTSLADIWGRALAYNFFPSL
RO88p-              DSIWDLSDSIVTPGGINIFKTAKRWDHISNAVESKQNADYNTSLADIWGRALAYNFFPSL
VIN13-              DSIWDLSDSIVTPGGINIFKTAKRWDHISNAVESKQNADYNTSLADIWGRALAYNFFPSL
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FostersB            NRGGIGLTWSSIRDFPVFQNAEMPFPISVADGRYPGTKVINLNATVFEFNPFEMGSWDPS
S288C-PLB3p         NRGGIGLTWSSIRDFPVFQNAEMPFPISVADGRYPGTKVINLNATVFEFNPFEMGSWDPS
EC1118-             NRGGIGLTWSSIRDFPVFQNAEMPFPISVADGRYPGTKVINLNATVFEFNPFEMGSWDPS
VL3                 NRGGIGLTWSSIRDFPVFQNAEMPFPISVADGRYPGTKVINLNATVFEFNPFEMGSWDPS
YJM789              NRGGIGLTWSSIRDFPVFQNAEMPFPISVADGRYPGTKVINLNATVFEFNPFEMGSWDPS
RO88p-              NRGGIGLTWSSIRDFPVFQNAEMPFPISVADGRYPGTKVINLNATVFEFNPFGMGSWDPS
VIN13-              NRGGIGLTWSSIRDFPVFQNAEMPFPISVADGRYPGTKVINLNATVFEFKSFEMGSWDPS
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FostersB            LNSFANVKYLGTNVSNGVPLERGKCTAGFDNAGFIMGTSSTLFNQFLLRINSTHLPSFIT
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YJM789              LNSFANVKYLGTNVSNGVPLERGKCTAGFDNAGFIMGTSSTLFNQFLLRINSTHLPSFIT
RO88p-              LNSLANVKYLGANVSNGVPLERGKCTAGFDNAGFIMGTSFTLFNQFLLRINSTHLPSFIT
VIN13-              LNSFANVKYLGTNVSNGVPLERGKCTAGLDNAGFIMGT**C**SNLFNQLLLRINSTHLPSFIT
                    ***:*******:****************:*********. .****:**************

Kyokai              RLARHFLKDLSQDFNDIAVYSPNPFKDTKFLDSDYTTSIVDSDSLFLVDGGEDDENVPVL
FostersB            RLARHFLKDLSQDFNDIAVYSPNPFKDTKFLDSDYTTSIVDSDSLFLVDGGEDDENVPVL
S288C-PLB3p         RLARHFLKDLSQDFNDIAVYSPNPFKDTKFLDSDYTTSIVDSDSLFLVDGGEDDENVPVL
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VL3                 RLARHFLKDLSQDFNDIAVYSPNPFKDTKFLDSDYTTSIVDSDSLFLVDGGEDDENVPVL
YJM789              RLARHFLKDLSQDFNDIAVYSPNPFKDTKFLDSDYTTSIVDSDSLFLVDGGEDDENVPVL
RO88p-              RLARHFLKDLSQDFNDIAVYSPNPFKDTKFLDSDYTTSIVDSDSLFLVDGGEDDENVPVL
VIN13-              RLARHFLKDLSQDFNDIAVYSPNPFKDTKFLDSDYTTSIVDSDSLFLVDGGEDDENVPVL
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Kyokai              PLIQKERDVDIIFAVDNSADMRLAWPDGSSLVHTYERQFVKQGQGMSFPYVPDTNTFVNL
FostersB            PLIQKERDVDIIFAVDNSADMRLAWPDGSSLVHTYERQFVKQGQGMSFPYVPDTNTFVNL
S288C-PLB3p         PLIQKERDVDIIFAVDNSADMRLAWPDGSSLVHTYERQFVKQGQGMSFPYVPDTNTFVNL
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EC1118-          PLIQKERDVDIIFAVDNSADMRLAWPDGSSLVHTYERQFVKQGQGMSFPYVPDTNTFVNL
VL3              PLIQKERDVDIIFAVDNSADMRLAWPDGSSLVHTYERQFVKQGQGMSFPYVPDTNTFVNL
YJM789           PLIQKERDVDIIFAVDNSADMRLAWPDGSSLVHTYERQFVKQGQGMSFPYVPDTNTFVNL
RO88p-           PLIQKERDVDIIFAVDNSADMRLAWPDGSSLVHTYERQFVKQGQGMSFPYVPDTNTFVNL
VIN13-           PLIQKERDVDIIFAVDNSADMRLAWPDGSSLVHTYERQFVKQGQGMSFPYVPDTNTFVNL
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Kyokai           GLNKKPTFFGCDANNLTDLQYIPPLVVYLPNAEYSFNSNQSAFKLSYSESQRRSMIQNGF
FostersB         GLNKKPTFFGCDANNLTDLQYIPPLVVYLPNAEYSFNSNQSAFKLSYSESQRRSMIQNGF
S288C-PLB3p      GLNKKPTFFGCDANNLTDLQYIPPLVVYLPNAEYSFNSNQSAFKLSYSESQRRSMIQNGF
EC1118-          GLNKKPTFFGCDANNLTDLQYIPPLVVYLPNAEYSFNSNQSAFKLSYSESQRRSMIQNGF
VL3              GLNKKPTFFGCDANNLTDLQYIPPLVVYLPNAEYSFNSNQSAFKLSYSESQRRSMIQNGF
YJM789           GLNKKPTFFGCDANNLTDLQYIPPLVVYLPNAEYSFNSNQSAFKLSYSESQRRSMIQNGF
RO88p-           GLNKKPTFFGCDANNLTDLQYIPPLVVYLPNAEYSFNSNQSAFKLSYSESQRRSMIQNGF
VIN13-           GLNKKPTFFGCDANNLTDLQYIPPLVVYLPNAEYSFNSNQSAFKLSYSESQRRSMIQNGF
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Kyokai           EIATRNNFTDDPEFMGCVGCAIIRRKQQALNITLPPECETCFKNYCWNGTLDTTPLPDVE
FostersB         EIATRNNFTDDPEFMGCVGCAIIRRKQQALNITLPPECETCFKNYCWNGTLDTTPLPDVE
S288C-PLB3p      EIATRNNFTDDPEFMGCVGCAIIRRKQQALNITLPPECETCFKNYCWNGTLDTTPLPDVE
EC1118-          EIATRNNFTDDPEFMGCVGCAIIRRKQQALNITLPPECETCFKNYCWNGTLDTTPLPDVE
VL3              EIATRNNFTDDPEFMGCVGCAIIRRKQQALNITLPPECETCFKNYCWNGTLDTTPLPDVE
YJM789           EIATRNNFTDDPEFMGCVGCAIIRRKQQALNITLPPECETCFKNYCWNGTLDTTPLPDVE
RO88p-           EIATRNNFTDDPEFMGCVGCAIIRRKQQALNITLPPECETCFKNYCWNGTLDTTPLPDVE
VIN13-           EIATRNNFTDDPEFMGCVGCAIIRRKQQALNITLPPECETCFKNYCWNGTLDTTPLPDVE
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Kyokai           KDVHHSFINVNSFNSSIGQEESLYAGSSASQSSSSSSSS----EIPSATATLEKKAATNS
FostersB         KDVHHSFINVNSFNSSIGQEESLYAGSSASQSSSSSSSS----EIPSATATLEKKAATNS
S288C-PLB3p      KDVHHSFINVNSFNSSIGQEESLYAGSSASQSSSSSSSSSSSSSEIPSATATLEKKAATNS
EC1118-          KDVHHSFINVNSFNSSIGQEESLYAGSSASQSSSSSSSSSSSSSEIPSATATLEKKAATNS
VL3              KDVHHSFINVNSFNSSIGQEESLYAGSSASQSSSSSSSSSSSSSEIPSATATLEKKAATNS
YJM789           KDVHHSFINVNSFNSSIGQEESLYAGSSASQSSSSSSSSSSSSSEIPSATATLEKKAATNS
RO88p-           KDVHHSFINVNSFNSSIGQEESLYAGSSASQSSSSSSSSSSSSSEIPSATATLEKKAATNS
VIN13-           KDVHHSFINVNSFNSSIGQEESLYAGSSASQSSSSSSSSSSSSSEIPSATATLEKKAATNS
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Kyokai           GSHLSGISVKFSAMIMLTLLMFTGAV-
FostersB         GSHLSGISVKFSAMIMLTLLMFTGAV-
S288C-PLB3p      GSHLSGISVKFSAMIMLTLLMFTGAV-
EC1118-          GSHLSGINVKFSAMIMLTLLMFTGAV-
VL3              GSHLSGIXVKFSAMIMLTLLMFTGAV-
YJM789           GSHLSGISVKFSAMIMLTLLMFTGAV-
RO88p-           GSHLSGISVKFSAMIMLTLLMFTGAV-
VIN13-           GSHLSGISVKFSAMIMLTLLMFTGAV-

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Pst1

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Lalvin           MQLHSLIASTALLITSALAATSSSSSIPSSCTISSHATATAQSDLDKYSRCDTLVGNLTI
EC1118           MQLHSLIASTALLITSALAATSSSSSIPSSCTISSHATATAQSDLDKYSRCDTLVGNLTI
S288CPST1        MQLHSLIASTALLITSALAATSSSSSIPSSCTISSHATATAQSDLDKYSRCDTLVGNLTI
VIN13PST1        MQLHSLIASTALLITSALAATSSSSSIPSSCTISSHATATAQSDLDKYSRCDTLVGNLTI
YJM789           MQLHSLIASTALLITSALAATSSSSSIPSSCTISSHATATAQSDLDKYSRCDTLVGNLTI
Kyokai7          MQLHSLIASTALLITSALAATSSSSSIPSSCTISSHATATAQSDLDKYSRCDTLVGNLTI
FostersO         MQLHSLIASTALLITSALAATSSSSSIPSSCTISSHATATAQSDLDKYSRCDTLVGNLTI
AWRI796          MQLHSLIASTALLITSALAATSSSSSIPSSCTISSHATATAQSDLDKYSRCDTLVGNLTI
RO88PST1         ----------------------------GEAKKKSSHATATAQSDLDKYSRCDTLVGNLTV
                                             .: . **************************:

Lalvin           GGGLKTGALANVKEINGSLTIFNATNLTSFAADSLESITDSLNLQSLTILTSASFGSLQS
EC1118           GGGLKTGALANVKEINGSLTIFNATNLTSFAADSLESITDSLNLQSLTILTSASFGSLQS
S288CPST1        GGGLKTGALANVKEINGSLTIFNATNLTSFAADSLESITDSLNLQSLTILTSASFGSLQS
VIN13PST1        GGGLKTGALANVKEINGSLTIFNATNLTSFAADSLESITDSLNLQSLTILTSASFGSLQS
YJM789           GGGLKTGALANVKEINGSLTIFNATNLTSFAADSLESITDSLNLQSLTILTSASFGSLQS
Kyokai7          GGGLKTGALANVKEINGSLTIFNATNLTSFAADSLESITDSLNLQSLTILTSASFGSLQS
FostersO         GGGLKTGALANVKEINGSLTIFNATNLTSFAADSLESITDSLNLQSLTILTSASFGSLQS
AWRI796          GGGLKTGALANVKEINGSLTIFNATNLTSFAADSLESITDSLNLQSLTILTSASFGSLQS
RO88PST1         GGGLKTGALANVKEIKGSLTIFNATNLTSFAADSLESITDSLNLQSLTILTSASFGSLQN
                 ***************:******************************************.

Lalvin           VDSIKLITLPAISSFTSNIKSANNIYISDTSLQSVDGFSALKKVNVFNVNNNKKLTSIKS
EC1118           VDSIKLITLPAISSFTSNIKSANNIYISDTSLQSVDGFSALKKVNVFNVNNNKKLTSIKS
S288CPST1        VDSIKLITLPAISSFTSNIKSANNIYISDTSLQSVDGFSALKKVNVFNVNNNKKLTSIKS
VIN13PST1        VDSIKLITLPAISSFTSNIKSANNIYISDTSLQSVDGFSALKKVNVFNVNNNKKLTSIKS
YJM789           VDSIKLITLPAISSFTSNIKSANNIYISDTSLQSVDGFSALKKVNVFNVNNNKKLTSIKS
Kyokai7          VDSIKLITLPAISSFTSNIKSANNIYISDTSLQSVDGFSALKKVNVFNVNNNKKLTSIKS
FostersO         VDSIKLITLPAISSFTSNIKSANNIYISDTSLQSVDGFSALKKVNVFNVNNNKKLTSIKS
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AWRI796         VDSIKLITLPAISSFTSNIKSANNIYISDTSLQSVDGFSALKKVNVFNVNNNKKLTSIKS
RO88PST1        VNSINLITLPAISAFTSNIKSANNIYISDTSLQSVDGFSALKKVNVFNVNNNKKLTSIKS
                *:**:********:**************************************************

Lalvin          PVETVSDSLQFSFNGNQTKITFDDLVWANNISLTDVHSVSFANLQKINSSLGFINNSISS
EC1118          PVETVSDSLQFSFNGNQTKITFDDLVWANNISLTDVHSVSFANLQKINSSLGFINNSISS
S288CPST1       PVETVSDSLQFSFNGNQTKITFDDLVWANNISLTDVHSVSFANLQKINSSLGFINNSISS
VIN13PST1       PVETVSDSLQFSFNGNQTKITFDDLVWANNISLTDVHSVSFANLQKINSSLGFINNSISS
YJM789          PVETVSDSLQFSFNGNQTKITFDDLVWANNISLTDVHSVSFANLQKINSSLGFINNSISS
Kyokai7         PVETVSDSLQFSFNGNQTKITFDDLVWANNISLTDVHSVSFANLQKINSSLGFINNSISS
FostersO        PVETVSDSLQFSFNGNQTKITFDDLVWANNISLTDVHSVSFANLQKINSSLGFINNSISS
AWRI796         PVETVSDSLQFSFNGNQTKITFDDLVWANNISLTDVHSVSFANLQKINSSLGFINNSISS
RO88PST1        PLETVSDSLQFSFNGNQTKITFDVLVWANNISLTDVHSVSFASLQKINSSLGFINNSISS
                *:********************* ******************.*****************

Lalvin          LNFTKLNTIGQTFSIVSNDYLKNLSFSNLSTIGGALVVANNTGLQKIGGLDNLTTIGGTL
EC1118          LNFTKLNTIGQTFSIVSNDYLKNLSFSNLSTIGGALVVANNTGLQKIGGLDNLTTIGGTL
S288CPST1       LNFTKLNTIGQTFSIVSNDYLKNLSFSNLSTIGGALVVANNTGLQKIGGLDNLTTIGGTL
VIN13PST1       LNFTKLNTIGQTFSIVSNDYLKNLSFSNLSTIGGALVVANNTGLQKIGGLDNLTTIGGTL
YJM789          LNFTKLNTIGQTFSIVSNDYLKNLSFSNLSTIGGALVVANNTGLQKIGGLDNLTTIGGTL
Kyokai7         LNFTKLNTIGQTFSIVSNDYLKNLSFSNLSTIGGALVVANNTGLQKIGGLDNLTTIGGTL
FostersO        LNFTKLNTIGQTFSIVSNDYLKNLSFSNLSTIGGALVVANNTGLQKIGGLDNLTTIGGTL
AWRI796         LNFTKLNTIGQTFSIVSNDYLKNLSFSNLSTIGGALVVANNTGLQKIGGLDNLTTIGGTL
RO88PST1        LNFTKLSTIGQTFSIVSNDYLKNLSFSNLSTIGGALVVANNTGLQKIGGLDNLTTIGGTL
                ******.**************************************************

Lalvin          XVVGNFTSLNLDSLKSVKGGADVESKSSNFSCNALKALQKKGGIKGESFVCKNGASSTSV
EC1118          KVVGNFTSLNLDSLKSVKGGADVESKSSNFSCNALKALQKKGGIKGESFVCKNGASSTSV
S288CPST1       EVVGNFTSLNLDSLKSVKGGADVESKSSNFSCNALKALQKKGGIKGESFVCKNGASSTSV
VIN13PST1       EVVGNFTSLNLDSLKSVKGGADVESKSSNFSCNALKALQKKGGIKGESFVCKNGASSTSV
YJM789          EVVGNFTSLNLDSLKSVKGGADVESKSSNFSCNALKALQKKGGIKGESFVCKNGASSTSV
Kyokai7         EVVGNFTSLNLDSLKSVKGGADVESKSSNFSCNALKALQKKGGIKGESFVCKNGASSTSV
FostersO        EVVGNFTSLNLDSLKSVKGGADVESKSSNFSCNALKALQKKGGIKGESFVCKNGASSTSV
AWRI796         EVVGNFTSLNLDSLKSVKGGADVESKSSNFSCNALKALQKKGGIKGESFVCKNGASSTSV
RO88PST1        EVVGNFTVLNLNSLKSVKGGADVESKSNNFSCNALKALQKKGGIKGESFVCKNGATSTSV
                 ****** ***:***************.****************************:****

Lalvin          KLSSTSKSQSSQTTAKVSKSSSKAEEKKFTSGDIKAAASASSVSSSSASSS-----SSKS
EC1118          KLSSTSKSQSSQTTAKVSKSSSKAEEKKFTSGDIKAAASASSVSSSSASSS-----SSKS
S288CPST1       KLSSTSKSQSSQTTAKVSKSSSKAEEKKFTSGDIKAAASASSVSSSGASSS-----SSKS
VIN13PST1       KLSSTSKSQSSQTTAKVSKSSSKAEEKKFTSGDIKAAASASSVSSSSASSS-----SSKS
YJM789          KLSSTSKSQSSQTTAKVSKSSSKAEEKKFTSGDIKAAASASSVSSSSASSS-----SSKS
Kyokai7         KLSSTSKSQSSQTTAKVSKSSSKAEEKKFTSGDIKAAASASSVSSSSASSS-----SSKS
FostersO        KLSSTSKSQSSQTTAKVSKSSSKAEEKKFTSGDIKAAASASSVSSSSASSS-----SSKS
AWRI796         KLSSTSKSQSSQTTAKVSKSSSKAEEKKFTSGDIKAAASASSVSSSSASSS-----SSKS
RO88PST1        KLSSTSKPQSSKTTSKASKSSSKAEEKKLTSGNIKAAASASSVSSSSASSSSASSSSSKS
                *******.***:**:*.***********:***:*************.****     ****

Lalvin          SKGNAAIMAPIGQTTPLVGLLTAIIMSIM
EC1118          SKGNAAIMAPIGQTTPLVGLLTAIIMSIM
S288CPST1       SKGNAAIMAPIGQTTPLVGLLTAIIMSIM
VIN13PST1       SKGNAAIMAPIGQTTPLVGLLTAIIMSIM
YJM789          SKGNAAIMAPIGQTTPLVGLLTAIIMSIM
Kyokai7         SKGNAAIMAPIGQTTPLVGLLTAIIMSIM
FostersO        SKGNAAIMAPIGQTTPLVGLLTAIIMSIM
AWRI796         SKGNAAIMAPIGQTTPLVGLLTAIIMSIM
RO88PST1        SKGNAAIMAPVGQTTTLIGLLTAMIMSLM
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Exg1p

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S288CEXG1       MLSLKTLLCTLLTVSSVLATPVPARDPSSIQFVHEENKKRYYDYDHGSLGEPIRGVNIGG
VIN13EXG1       MLSLKTLLCTLLTVSSVLATPVPARDPSSIQFVHEENKKRYYDYDHGSLGEPIRGVNIGG
RO88EXG1        MLSLKTLLCTLLTVSSVLATPVPARDPSSIQFVHEENKKRYYDYDHGSLGEPIRGVNIGG
                ************************************************************

S288CEXG1       WLLLEPYITPSLFEAFRTNDDNDEGIPVDEYHFCQYLGKDLAKSRLQSHWSTFYQEQDFA
VIN13EXG1       WLLLEPYITPSLFEAFRTNDDNDEGIPVDEYHFCQYLGKDLAKSRLQSHWSTFYQEQDFA
RO88EXG1        WLVLEPYITPSLFEAFRTNGDNDEGIPVDEYHYCQYLGKDLAKSRLQSHWSTFYQEQDFA
                **:****************.************:**************************

S288CEXG1       NIASQGFNLVRIPIGYWAFQTLDDDPYVSGLQESYLDQAIGWARNNSLKVWVDLHGAAGS
VIN13EXG1       NIASQGFNLVRIPIGYWAFQTLDDDPYVSGLQESYLDQAIGWARNNSLKVWVDLHGAAGS
RO88EXG1        NIASQGFNLVRIPIGYWAFQTLDDDPYVSGLQESYLDQAIGWARNNSLKVWVDLHGAAGS
                ************************************************************

S288CEXG1       QNGFDNSGLRDSYKFLEDSNLAVTTNVLNYILKKYSAEEYLDTVIGIELINEPLGPVLDM
VIN13EXG1       QNGFDNSGLRDSYKFLEDSNLAVTTNVLNYILKKYSAEEYLDTVIGIELINEPLGPVLDM
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RO88EXG1        QNGFDNSGLRDSYKFLEDSNLAVTTNVLNYILKKYSAEEYLDTVIGIELINEPLGPVLDM
                ************************************************************

S288CEXG1       DKMKNDYLAPAYEYLRNNIKSDQVIIIHDAFQPYNYWDDFMTENDGYWGVTIDHHHYQVF
VIN13EXG1       DKMKNDYLAPAYEYLRNNIKSDQVIIIHDAFQPYNYWDDFMTENDGYWGVTIDHHHYQVF
RO88EXG1        DKMKNDYLAPAYEYLRNNIKSDQVIIIHDAFEPYNYWDDFMTENDGYWGVTIDHHHYQVF
                ******************************:*****************************

S288CEXG1       ASDQLERSIDEHIKVACEWGTGVLNESHWTVCGEFAAALTDCTKWLNSVGFGARYDGSWV
VIN13EXG1       ASDQLERSIDEHIKVACEWGTGVLNESHWTVCGEFAAALTDCTKWLNSVGFGARYDGSWV
RO88EXG1        ASDQLQRTMDERIKVACEWGTGVLNESHWTVCGEFAAAMTDCTKWVNSVGFGARYDGSWV
                *****:*::**:*************************:******:**************

S288CEXG1       NGDQTSSYIGSCANNDDIAYWSDERKENTRRYVEAQLDAFEMRGGWIIWCYKTESSLEWD
VIN13EXG1       NGDQTSSYIGSCANNDDIAYWSDERKENTRRYVEAQLDAFEMRGGWIIWCYKTESSLEWD
RO88EXG1        NGDQTSSYIGSCANNDDIAYWSDERKENTRRFVEAQLDAFEMRGGWIIWCYKTESSLEWD
                ******************************:*****************************

S288CEXG1       AQRLMFNGLFPQPLTDRKYPNQCGTISN-
VIN13EXG1       AQRLMFNGLFPQPLTDRKYPNQCGTISN-
RO88EXG1        AQRLMYNGLFPQPLTDRKYPNQCSTISN-

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Ecm33p

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S288CECM33      MQFKNALTATAILSASALAANSTTSIPSSCSIGTSATATAQADLDKISGCSTIVGNLTIT
ESM33           MQFKNALTATAILSASALAANSTTSIPSSCSIGTSATATAQADLDKISGCSTIVGNLTIT
VIN13ECM33      MQFKNALTATAILSASALAANSSTSIPSSCSIGTSATATAQADLDKISGCSTIVGNLTIT
RO88ECM33       MQFKNALTATAILSASALAANSSTSIPSSCSIGTSATATAQADLDKISGCSTIVGNLTIT
                ***********************:************************************

S288CECM33      GDLGSAALASIQEIDGSLTIFNSSSLSSFSADSIKKITGDLNMQELIILTSASFGSLQEV
ESM33           GDLGSAALASIQEIDGSLTIFNSSSLSSFSADSIKKITGDLNMQELIILTSASFGSLQEV
VIN13ECM33      GDLGSAALASIQEIDGSLTIFNSSSLSSFSADSIKKITGDLNMQELIILTSASFGSLQEV
RO88ECM33       GDLGSAALASIQEIDGSLTIFNSSSLSSFSADSIKKITGDLNMQELIILTSASFGSLQEV
                ************************************************************

S288CECM33      DSINMVTLPAISTFSTDLQNANNIIVSDTTLESVEGFSTLKKVNVFNINNNRYLNSFQSS
ESM33           DSINMVTLPAISTFSTDLQNANNIIVSDTTLESVEGFSTLKKVNVFNINNNRYLNSFQSS
VIN13ECM33      DSINMVTLPAISTFSTDLQNANNIIVSDTTLESVEGFSTLKKVNVFNINNNRYLNSFQSS
RO88ECM33       DSINMVTLPAISTFSTDLQNANNIIVSDTTLESVERFSTLKKVNVFNINNNRYLNSFQSS
                ********************************** ************************

S288CECM33      LESVSDSLQFSSNGDNTTLAFDNLVWANNITLRDVNSISFGSLQTVNASLGFINNTLPSL
ESM33           LESVSDSLQFSSNGDNTTLAFDNLVWANNITLRDVNSISFGSLQTVNASLGFINNTLPSL
VIN13ECM33      L-KVSDSLQFSSNGDNTTLAFDNLVWANNITLRDVNSISFGSLQTVNASLGFINNTLPSL
RO88ECM33       LESVSDSLQFSSNGDNTTLAFDNLVWANNITLRDVNSIPFGSLQTVNASLGFINNTLPSL
                * .**********************************.**********************

S288CECM33      NLTQLSKVGQSLSIVSNDELSKAAFSNLTTVGGGFIIANNTQLKVIDGFNKVQTVGGAIE
ESM33           NLTQLSKVGQSLSIVSNDELSKAAFSNLTTVGGGFIIANNTQLKVIDGFNKVQTVGGAIE
VIN13ECM33      NLTQLSKVGQSLSIVSNDELSKAAFSNLTTVGGGFIIANNTQLKVIDGFNKVQTVGGAIE
RO88ECM33       NLTQLSKVGQSLSIVSNDELSKAAFSNLTTVGGGFIIANNTQLKVIDGFNKVQTVGGAIE
                ************************************************************

S288CECM33      VTGNFSTLDLSSLKSVRGGANFDSSSSNFSCNALKKLQSNGAIQGDSFVCKNGATSTSVK
ESM33           VTGNFSTLDLSSLKSVRGGANFDSSSSNFSCNALKKLQSNGAIQGDSFVCKNGATSTSVK
VIN13ECM33      VTGNFSTLDLSSLKSVRGGAKFDSSSSNFSCNALKKLQSNGAIQGDSFVCKNGATSTSVK
RO88ECM33       VTGNFSTLDLSSLKSVRGGAKFDSSSSNFSCNALKKLQSNGAIQGDSFVCKNGATSTSVK
                ********************:****************************************

S288CECM33      LSSTSTESSKSSATSSASSSGDASNAQANVSASASSSSSSSKKSKGAAPELVPATSFMGV
ESM33           LSSTSTESSKSSATSSASSSGDASNAQANVSASASSSSSSSKKSKGAAPELVPATSFMGV
VIN13ECM33      LSSTSTESSKSSATSSASSSGDASNAQANVSASASSSSSSSKKSKGAAPELVPATSFMGV
RO88ECM33       LSSTSTESSKSSATSSASSSGDASNAQANVSASASSSSSSSKKSKGAAPELVPATSFMGV
                ************************************************************

S288CECM33      VAAVGVALL-
ESM33           VAAVAVALL-
VIN13ECM33      VAAVAVALL-
RO88ECM33       VAAVGVALL-
                ****.****
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Pry3p

```
VIN13PRY3       MLEFPISVLLGCLVAVKAQTTFPNFESDVLNEHNKFRALHVDTAPLTWSDTLATYAQNYA
Lalvin          MLEFPISVLLGCLVAVKAQTTFPNFESDVLNEHNKFRALHVDTAPLTWSDTLATYAQNYA
AWRI796         MLEFPISVLLGCLVAVKAQTTFPNFESDVLNEHNKFRALHVDTAPLTWSDTLATYAQNYA
YJM789          MLEFPISVLLGCLVAVKAQTTFPNFESDVLNEHNKFRALHVDTAPLTWSDTLATYAQNYA
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Kyokai7         MLEFPISVLLGCLVAVKAQTTFPNFESDVLNEHNKFRALHVDTAPLTWSDTLATYAQNYA
FostersB        MLEFPISVLLGCLVAVKAQTTFPNFESDVLNEHNKFRALHVDTAPLTWSDTLATYAQNYA
S288C           MLEFPISVLLGCLVAVKAQTTFPNFESDVLNEHNKFRALHVDTAPLTWSDTLATYAQNYA
RO88PRY3        MLEFPISVLLGCLVAARAQTTFPNFESDVLNEHNKFRALHVDTAPLTWSDTLATYAQDYA
                ***************.:****************************************:**

VIN13PRY3       DQYDCSGVLTHSDGPYGENLALGYTDTGAVDAWYGEISKYNYSNPGFSESTGHFTQVVWK
Lalvin          DQYDCSGVLTHSDGPYGENLALGYTDTGAVDAWYGEISKYNYSNPGFSESTGHFTQVVWK
AWRI796         DQYDCSGVLTHSDGPYGENLALGYTDTGAVDAWYGEISKYNYSNPGFSESTGHFTQVVWK
YJM789          DQYDCSGVLTHSDGPYGENLALGYTDTGAVDAWYGEISKYNYSNPGFSESTGHFTQVVWK
Kyokai7         DQYDCSGVLTHSDGPYGENLALGYTDTGAVDAWYGEISKYNYSNPGFSESTGHFTQVVWK
FostersB        DQYDCSGVLTHSDGPYGENLALGYTDTGAVDAWYGEISKYNYSNPGFSESTGHFTQVVWK
S288C           DQYDCSGVLTHSDGPYGENLALGYTDTGAVDAWYGEISKYNYSNPGFSESTGHFTQVVWK
RO88PRY3        DQYDCSGVLTHSDGPYGENLALGYTDTGAVDAWYGEISKYNYSNPGFSESTGHFTQVVWK
                ************************************************************

VIN13PRY3       STAEIGCGYKYCGTTWNNYIVCSYNPPGNYLGEFAEEVEPLISTVS---SSSSSSSS-TS
Lalvin          STAEIGCGYKYCGTTWNNYIVCSYNPPGNYLGEFAEEVEPLISTVS---SSSSSSSS-TS
AWRI796         STAEIGCGYKYCGTTWNNYIVCSYNPPGNYLGEFAEEVEPLISTVS---SSSSSSSS-TS
YJM789          STAEIGCGYKYCGTTWNNYIVCSYNPPGNYLGEFAEEVEPLISTVS---SSSSSSSSSTS
Kyokai7         STAEIGCGYKYCGTTWNNYIVCSYNPPGNYLGEFAEEVEPLISTVS---SSSSSSSSSTS
FostersB        STAEIGCGYKYCGTTWNNYIVCSYNPPGNYLGEFAEEVEPLISTVS---SSSSSSSSSTS
S288C           STAEIGCGYKYCGTTWNNYIVCSYNPPGNYLGEFAEEVEPLISTVS---SSSSSSSS-TS
RO88PRY3        STAEIGCGYKYCGTTWNNYIVCSYNPPGNYLGEFAEEVEPLISAVSSYSSSSSSSSSSTS
                ******************************************:**   ******** **

VIN13PRY3       TTSDTVSTISSSIMPAVAQGYTTTVSSAASSSSLKSTTINPAKTATLTASSSTVITSSTE
Lalvin          TTSDTVSTIXSSIMPAVAQGYTTTVSSAASSSSLKSTTINPAKTATLTASSSTVITSSTE
AWRI796         TTSDTVSTISSSIMPAVAQGYTTTVSSAASSSSLKSTTINPAKTATLTASSSTVITSSTE
YJM789          TTSETVSTISSSIMPAVAQGYTTTVSSAASSSSLKSTTINPAKTATLTASSSTVITSSTE
Kyokai7         TTSDTVSTISSSIMPAVAQGYTTTVSSAVSSSSLKSTTINPAKTATLTASSSTVITSSTE
FostersB        TTSDTVSTISSSIMPAVAQGYTTTVSSAASSSSLKSTTINPAKTATLTASSSTVITSSTE
S288C           TTSDTVSTISSSIMPAVAQGYTTTVSSAASSSSLKSTTINPAKTATLTASSSTVITSSTE
RO88PRY3        STSHTVSTVSSSIMPAVAQGYTTTISSGTTSSSTS-TTINPAKTAGHTASSVVSSASTSS
                :**.****: ***************:**..:*** . *********  **** .  :*::.

VIN13PRY3       SVGSSTVSSASSSSVTTSYATSSSTVVSSDATSSTTT--------TSSVATSSSTTSSDP
Lalvin          SVGSSTVSSASSSSVTTSYATSSSTVVSSDATSSTTT--------TSSVATSSSTTSSDP
AWRI796         SVGSSTVSSASSSSVTTSYATSSSTVVSSDATSSTTT--------TSSVATSSSTTSSDP
YJM789          SVGSSTVSSASSSSVTT----SSSTVVSSDATSSTTT--------TSSVATSSSTTSSDP
Kyokai7         SVGSSTVSSASSSSVTTSYATSSSTVVSSDATSSTTT--------TSSVATSSSTTSSDP
FostersB        SVGSSTVSSASSSSVTTSYATSSSTVVSXDATSSTTT--------TSSVATSSSTTSSDP
S288C           SVGSSTVSSASSSSVTTSYATSSSTVVSSDATSSTTT--------TSSVATSSSTTSSDP
RO88PRY3        SRSVTTLGATSSSTTTSSNPTSSSATSSNPTSSSATSSNPTSSSATSSNPTSSSATSSNP
                * . :*:.::***:.*:    ***:. *  ::**:*:        *** .****:***:*

VIN13PRY3       ASSTTTASSSTTSSDPTSSTAAASS-----------------------------------
Lalvin          ASSTTTASSSTTSSDPTSSTAAASS-----------------------------------
AWRI796         ASSTTTASSSTTSSDPTSSTAAASS-----------------------------------
YJM789          GSSTTTASSSTTSSDLTSSTTSSSSSTTSSDPTSSTAAS----------------------
Kyokai7         ASSTTTASSSTTSSDLTSSTTSSSSSTTSSDPTSSTAAS----------------------
FostersB        ASSTTTASSSTTSSDXTSSTTSSSSSTTSSDPTSSXAAS----------------------
S288C           TSSTAAASS----------------------------------------------------
RO88PRY3        TSSSATSSNPTSSSATSSNPTSSSATSSNPTSSSATSSNPTSSSATSSNPTSSSATPSEA
                 **::::*.

VIN13PRY3       ------------SDPASSSAAASSSASTENAASSSSAISSSSSMVSAPLSSTLTTSTASS
Lalvin          ------------SDPASSSAAASSSASTENAASSSSAISSSSSMVSAPLSSTLTTSTASS
AWRI796         ------------SDPASSSAAASSSASTENAASSSSAISSSSSMVSAPLSSTLTTSTASS
YJM789          ------SSSTTSSDPVSSSAAASSSASTENAASSSSAISSSSSMVSAPLSSTLTTSAASS
Kyokai7         ------SSSTTSSDPVSSSAAASSSASTENAASSSSAISSSSSMVSAPLSSTLTTSAASS
FostersB        ------SSSTTSSDPXSSSAAASSSASTENAASSSXAISSSSSMVSAPLSSTLTTSXASS
S288C           ------------SDPASSSAAASSSASTENAASSSSAISSSSSMVSAPLSSTLTTSTASS
RO88PRY3        ASSSSTSSSSTSSDTPSSISTSSDTTSSIASASSSSATSSTTSIVAGSLSGTSSTPAKSS
                            **. ** :::*.::*:  :**** * **::*:*:..**.* :*.  **

VIN13PRY3       RSVTSSSVNSVKFANTTVFSAQTTSSVSASLSSSVAADDIQGSTSKEATSSVSEHTSIVT
Lalvin          RSVTSSSVNSVKFANTTVFSAQTTSSVSASLSSSVAADDIQGSTSKEATSSVSEHTSIVT
AWRI796         RSVTSSSVNSVKFANTTVFSAQTTSSVSASLSSSVAADDIQGSTSKEATSSVSEHTSIVT
YJM789          RSVTSSSVNSVKFANTTVFSAQTTSSVSASLSSSVAADDIQGSTSKEATSSVSEHTSIVT
Kyokai7         RSVTSSSVNSVKFANTTVFSAQTTSSVSASLSSSVAADDIQGSTSKEATSSVSEHTSIVT
FostersB        RSVTSSSVNSVKFANTTVFXAQTTSSVSASLSSSVAADDIQGSTSKEATSSVSEHTSIVT
S288C           RSVTSNSVNSVKFANTTVFSAQTTSSVSASLSSSVAADDIQGSTSKEATSSVSEHTSIVT
RO88PRY3        GKVASSSANSVRFANTTMYSAETTSSVDASLSSSVSADAILMSTSKKATSSASKSTSIVT
                 .*:*.*.***:*****:: *:*****.*******:** *  ****:****.*: *****

VIN13PRY3       SATNAAQYATRLGSSSRSSSGAVSSS-AVSQSVLNSVIAVNTDVSVTSVSSAAHTTKDTA
Lalvin          SATNAAQYATRLGSSSRSSSGAVSSS-AVSQSVLNSVIAVNTDVSVTSVSSAAHTTKDTA
AWRI796         SAINAAQYATRLGSSSRSSSGAVSSS-AVSQSVLNSVIAVNTDVSVTSVSSAAHTTKDTA
YJM789          SATNAAQYATRLGSSSRSSSGAVSSS-AVSQSALNSVIAVNTDVSVTSVSSAAHTTKDTA
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Kyokai7         SATNAAQYATRLGSSSRSSSGAVSSS-AVSQSALNSVIAVNTDVSVTSVSSAAHTTKDTA
FostersB        SATNAAQYATRLGSSSRSSSGAVSSS-AVSQSXLNSVIAVNTDVSVTSVSSAAHTTKDTA
S288C           SATNAAQYATRLGSSSRSSSGAVSSS-AVSQSVLNSVIAVNTDVSVTSVSSTAHTTKDTA
RO88PRY3        SATNAAQYATRSGSSSGTSSQAITSSPGVSHTSAKTTIAVNAAESVTSVSSLAQTIKYTT
                ** ******** **** :** *::** .**::  ::.****:  ******* *:* * *:

VIN13PRY3       TTSVTASESITSETAQASSSTEKNISNSAATSSSIYSNSASVSGHGVTYAAEYAITS---
Lalvin          TTSVTASESITSETAQASSSTEKNISNSAATSSSIYSNSASVSGHGVTYAAEYAITS---
AWRI796         TTSVTASESITSETAQASSSTEKNISNSAATSSSIYSNSASVSGHGVTYAAEYAITSEQS
YJM789          TTSVTASESITSDTAQVSSSTEKKISNSAATSSSIYSNSASVSGHGVTYAAEYAITSEQS
Kyokai7         TTSVTASESITSDTAQVSSSTEKKISNSAATSSSIYSNSASVSGHGVTYAAEYAITSEQS
FostersB        TTSVTASESITSXTAQVSSSTEKKISNSAATSSSIYSNSASVSGHGVTYAAEYAITSEQS
S288C           TTSVTASESITSETAQASSSTEKNISNSAATSSSIYSNSASVSGHGVTYAAEYAITSEQS
RO88PRY3        TTSMASSEAITSETVQASSSAKNDNSINDATSSRTNSNSASVLASSVTYAAEDAIKSESS
                ***:::**:*** *.*.***:::. * . ****   ****** . .****** **.*

VIN13PRY3       SALATS---VPATNCSSIVKTTTLENSSTTTITAITKSTTTLATTANNSTRAATAVTIDP
Lalvin          SALATS---VPATNCSSIVKTTTLENSSTTTITAITKSXTTLATTANNSTRAATAVTIDP
AWRI796         SALATS---VPATNCSSIVKTTTLENSSTTTITAITKSTTTLATTANNSTRAATAVTIDP
YJM789          SALATS---VPATNRSSIVKTTTLENSSTATITAITESTTTLATTANNYTRAATAVTIDP
Kyokai7         SALATS---VPATNRSSIVKTTTLENSSTATITTITKSATTLATTANNSTRAATAVTIDP
FostersB        SALATS---VPATNXSSIVKTTTLENSSTXTITXITKSXTTLATTANNSTRAATAVTIDP
S288C           SALATS---VPATNCSSIVKTTTLENSSTTTITAITKSTTTLATTANNSTRAATAVTIDP
RO88PRY3        SASTASSISVSATNSSSISETASLETSSAATSTAITRSTTTVATSAYNSTSSTATVTTDH
                ** ::*   *.*** *** :*::**.**: * * **.* **:**:* * * ::::** *

VIN13PRY3       TLDPTDNSASPTDNAKHTSTYGSSSTGASLDSLRTTTSISVSSNTTQLVSTCTSESNYSD
Lalvin          TLDPTDNSASPTDNAKHTSTYGSSSTGASLDSLRTTTSISVSSNTTQLVSTCTSESNYSD
AWRI796         TLDPTDNSASPTDNAKHTSTYGSSSTGASLDSLRTTTSISVSSNTTQLVSTCTSESNYSD
YJM789          TLDPTDNSASPTDNAKHTSTYGSSSTGASLDSLRTTTSISASSNTTQLVSTCTSESDYSD
Kyokai7         TLDPTDNSASPTDNAKHTSTYGSSSTGASLDSLRTTTSISASSNTTQLVSTCTSESDYSD
FostersB        TLDPTDNSASPTDNAKHTSTYGSSSTGASLDSLRTTTSISXSSNTTQLVSTCTSESDYSD
S288C           TLDPTDNSASPTDNAKHTSTYGSSSTGASLDSLRTTTSISVSSNTTQLVSTCTSESDYSD
RO88PRY3        TLDPTQNSVSSADTAKHP-----------------TTSVSTPRNTTLIVSTSTSESDYSD
                *****:**.*.:*.***.                 ***:* . *** :***.****:***

VIN13PRY3       SPSFAISTATTTESNLITNTITASCSTDSNFPISAASSTDETAFTRTISTSCSTLNGAST
Lalvin          SPSFAISTATTTESNLITNTITASCSTDSNFPISAASSTDETAFTRTISTSCSTLNGAST
AWRI796         SPSFAISTATTTESNLITNTITASCSTDSNFPISAASSTDETAFTRTISTSCSTLNGAST
YJM789          SPSFAISTATTTESNLITNTITASCSTDSNFPISAASSTDETAFTRTISTSCSTLNGAST
Kyokai7         SPSFAISTATTTESNLITNTITASCSTDSNLPISAASSTDETAFTRTISTSCSTLNGAST
FostersB        SPSFAISTATTTESNLITNTITASCSTDSNXPISAASSTDETAFTRTISTSCSTLNGAST
S288C           SPSFAISTATTTESNLITNTITASCSTDSNFPTSAASSTDETAFTRTISTSCSTLNGAST
RO88PRY3        KTHLTVSTITTTESNLITNTITASCSTDSKITISTASTADEMVITSTITAPCSTLNGATT
                .. :::** ********************: . *:**::** .:* **::.*******:*

VIN13PRY3       QTSELTTSPMKTNTVVPASSFPSTTTTCLENDDTAFSSIYTEVNAATIINPGETSSLASD
Lalvin          QTSELTTSPMKTNTVVPASSFPSTTTTCLENDDTAFSSIYTEVNAATIINPGETSSLASD
AWRI796         QTSELTTSPMKTNTVVPASSFPSTTTTCLENDDTAFSSIYTEVNAATIINPGETSSLASD
YJM789          QTSELSTSPMKTNTVVPASSFPSTTTTCLENDDTAFSSIYTEVNAATIINPGETSSLASD
Kyokai7         QTSELSTSPMKTNTVVPASSFPSTTTTCLENDNTAFSSIYTEVNAATIINPGETSSLASD
FostersB        QTSELXTSPMKTNTVVPASSSPSTTTTCLENDDTAFSSIYTEVNAATIINPGETSSLASD
S288C           QTSELTTSPMKTNTVVPASSFPSTTTTCLENDDTAFSSIYTEVNAATIINPGETSSLASD
RO88PRY3        QTNEVTTSPSKTNTAVQT------TTNYLENDDTAFSSIYTDVNAATTINPGETSSPANG
                **.*: *** ****.* :      **. ****:********:***** ******** *..

VIN13PRY3       FATSEKPNEPTSVKSTSNEGTSSTTTTYQQTVATLYAKPSSTSLGARTTTGSNGRSTTSQ
Lalvin          FATSEKPNEPTSVKSTSNEGTSSTTTTYQQTVATLYAKPSSTSLGARTTTGSNGRSTTSQ
AWRI796         FATSEKPNEPTSVKSTSNEGTSSTTTTYQQTVATLYAKPSSTSLGARTTTGSNGRSTTSQ
YJM789          FATSEKPNEPTSVKSTSNEGTSSTTTTYQQTVATLYAKPSSTSLGARTTTGSNGRSTTSQ
Kyokai7         FATSEKPNEPTSVKSTSNEGTSSTTTTYQQTVATLYAKPSSTSLGARTTTGSNGRSTTSQ
FostersB        FATSEKPNEPTSVKSTSNEGTSSTTTTYQQTVATLYAKPSSTSLGARTTTGSNGRSTTSQ
S288C           FATSEKPNEPTSVKSTSNEGTSSTTTTYQQTVATLYAKPSSTSLGARTTTGSNGRSTTSQ
RO88PRY3        LTTSEKPNEPASVKFTANEDGASASTTYQQTPVTSYAKPSSKSLGTRTTTSSNGRSTTGQ
                ::********:*** *:**. :*::****** .* ******.***:****.*******.*

VIN13PRY3       QDGSAMHQPTSSIYTQLKEGTSTTAKLSAYEGAATPLSIFQCNSLAGTIAAFVVAVLFAF
Lalvin          QDGSAMHQPTSSIYTQLKEGTSTTAKLSAYEGAATPLSIFQCNSLAGTIAAFVVAVLFAF
AWRI796         QDGSAMHQPTSSIYTQLKEGTSTTAKLSAYEGAATPLSIFQCNSLAGTIAAFVVAVLFAF
YJM789          QDGSAMHQPTSSIYTQLKEGTSTTAKLSAYEGAATPLSIFQCNSLAGTIAAFVVAVLFAF
Kyokai7         QDGSAMHQPTSSIYTQLKEGTSTTAKLSAYEGAATPLSIFQCNSLAGTIAAFVVAVLFAF
FostersB        QDGSAMHQPTSSIYTQLKEGTSTTAKLSAYEGAATPLSIFQCNSLAGTIAAFVVAVLFAF
S288C           QDGSAMHQPTSSIYTQLKEGTSTTAKLSAYEGAATPLSIFQCNSLAGTIAAFVVAVLFAF
RO88PRY3        QKGSATHQPTSSIYTQLKQGTSTTAQLSAYEGAAAPPSIFQCNTLPGTIAAFVVAVLFAF
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VIN13PRY3       -
Lalvin          -
AWRI796         -
YJM789          -
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Kyokai7         -
FostersB        -
S288C           -
RO88PRY3        -
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Chs3p

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VL3-CHS3        --MTGLNGDDPDDYYLNLNQDEESLLRSRHSVGSGAPHRQGSLVRPERSRLNNPDNPHFY
YJM789-CHS3     --MTGLNGDDPDDYYLNLNQDEESLLRSRHSVGSGAPHRQGSLVRPERSRLNNPDNPHFY
S288C-CHS3      --MTGLNGDDPDDYYLNLNQDEESLLRSRHSVGSGAPHRQGSLVRPERSRLNNPDNPHFY
RO88-CHS3       -XMTGLNGDDPDDYYLNLNQDEESLLRSRHSVGSGAPHRQGSLVRPERSRLNNPDNPHFY
VIN13-CHS3      XXMTGLNGDDPDDYYLNLNQDEESLLRSRHSVGSGAPHRQGSLVRPERSRLNNPDNPHFY
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VL3-CHS3        YAQKTQEQMNHLDVLPSSTGVNPNATRRSGSLRSKGSVRSKFSGRETDSYLLQDMNTTDK
YJM789-CHS3     YAQKTQEQMNHLDVLPSSTGVNPNATRRSGSLRSKGSVRSKFSGRETDSYLLQDMNTTDK
S288C-CHS3      YAQKTQEQMNHLDVLPSSTGVNPNATRRSGSLRSKGSVRSKFSGRETDSYLLQDMNTTDK
RO88-CHS3       YAQKTQEQMNHLDVLPSSTGVNPNATRRSGSLRSKGSVRSKFSGRETDSYLLQDMNTTDK
VIN13-CHS3      YAQKTQEQMNHLDVLPSSTGVNPNATRRSGSLRSKGSVRSKFSGRETDSYLLQDMNTTDK
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VL3-CHS3        KASVKISDEGVAEDEFDKDGDVDNFEESSMQPINKSIKPLRKETNDTLSFWQMYCYFITF
YJM789-CHS3     KASVKISDEGVAEDEFDKDGDVDNFEESSMQPINKSIKPLRKETNDTLSFWQMYCYFITF
S288C-CHS3      KASVKISDEGVAEDEFDKDGDVDNFEESSTQPINKSIKPLRKETNDTLSFWQMYCYFITF
RO88-CHS3       KASVKISDEGVAEDEFDKDGDVDNFEESSMQPINKSIKPLRKETNDTLSFWQMYCYFITF
VIN13-CHS3      KASVKISDEGVAEDEFDKDGDVDNFEESSTQPINKSIKPLRKETNDTLSFWQMYCYFITF
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VL3-CHS3        WAPAPILAFCGMPKKERQMAWREKVALISVILYIGAIVAFLTFGFTKTVCSSSKLRLKNN
YJM789-CHS3     WAPAPILAFCGMPKKERQMAWREKVALISVILYIGAIVAFLTFGFTKTVCSSSKLRLKNN
S288C-CHS3      WAPAPILAFCGMPKKERQMAWREKVALISVILYIGAIVAFLTFGFTKTVCSSSKLRLKNN
RO88-CHS3       WAPAPILAFCGMPKKERQMAWREKVALISVILYIGAIVAFLTFGFTKTVCSSSKLRLKNN
VIN13-CHS3      WAPAPILAFCGMPKKERQMAWREKVALISVILYIGAIVAFLTFGFTKTVCSSSKLRLKNN
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VL3-CHS3        EVSTEFVVXNGKAYELDTSSRSGIQDVEVDSDTLYGPWSDAGKDASFLFQNVNGNCHNLI
YJM789-CHS3     EVSTEFVVINGKAYELDTSSRSGIQDVEVDSDTLYGPWSDAGKDASFLFQNVNGNCHNLI
S288C-CHS3      EVSTEFVVINGKAYELDTSSRSGIQDVEVDSDTLYGPWSDAGKDASFLFQNVNGNCHNLI
RO88-CHS3       EVSTEFVVINGKAYELDTSSRSGIQDVEVDSDTLYGPWSDAGKDASFLFQNVNGNCHNLI
VIN13-CHS3      EVSTEFVVINGKAYELDTSSRSGIQDVEVDSDTLYGPWSDAGKDASFLFQNVNGNCHNLI
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VL3-CHS3        TPKSNSSIPHDDDNNLAWYFPCKLKNQDGSSKPNFTVENYAGWNCHTSKEDRDAFYGLKS
YJM789-CHS3     TPKSNSSIPHDDDNNLAWYFPCKLKNQDGSSKPNFTVENYAGWNCHTSKEDRDAFYGLKS
S288C-CHS3      TPKSNSSIPHDDDNNLAWYFPCKLKNQDGSSKPNFTVENYAGWNCHTSKEDRDAFYGLKS
RO88-CHS3       TPKSNSSIPHDDDNNLAWYFPCKLKNQDGSSKPNFTVENYAGWNCHTSKEDRDAFYGLKS
VIN13-CHS3      TPKSNSSIPHDDDNNLAWYFPCKLKKQDGSSKPNFTVENYAGWNCHTSKEDRDAFYGLKS
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VL3-CHS3        KADVYFTWDGIKNSSRNLIVYNGDVLDLDLLDWLEKDDVDYPVVFDDLKTSNLQGYDLSL
YJM789-CHS3     KADVYFTWDGIKNSSRNLIVYNGDVLDLDLLDWLEKDDVDYPVVFDDLKTSNLQGYDLSL
S288C-CHS3      KADVYFTWDGIKNSSRNLIVYNGDVLDLDLLDWLEKDDVDYPVVFDDLKTSNLQGYDLSL
RO88-CHS3       KADVYFTWDGIKNSSRNLIVYNGDVLDLDLLDWLEKDEVDYPVVFDDLKTSNLQGYDLSL
VIN13-CHS3      KADVYFTWDGIKNSSRNLIVYNGDVLDLDLLDWLEKDEVDYPVVFDDLKTSNLQGYDLSL
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VL3-CHS3        VLSNGHERKIARCLSEIIKVGEVDSKTVGCIASDVVLYVSLVFILSVVIIKFIIACYFRW
YJM789-CHS3     VLSNGHERKIARCLSEIIKVGEVDSKTVGCIASDVVLYVSLVFILSVVIIKFIIACYFRW
S288C-CHS3      VLSNGHERKIARCLSEIIKVGEVDSKTVGCIASDVVLYVSLVFILSVVIIKFIIACYFRW
RO88-CHS3       VLSNGHERKIARCLSEIIKVGEVDSKTVGCIASDVVLYVSLVFILSVVIIKFIIACYFRW
VIN13-CHS3      VLSNGHERKIARCLSEIIKVGEVDSKTVGCIASDVVLYVSLVFILSVVIIKFIIACYFRW
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VL3-CHS3        TVARKQGAYIVDNKTMDKHTNDIEDWSNNIQTKAPLKEVDPHLRPKKYSEKSLGHKRAST
YJM789-CHS3     TVARKQGAYIVDNKTMDKHTNDIEDWSNNIQTKAPLKEVDPHLRPKKYSKKSLGHKRAST
S288C-CHS3      TVARKQGAYIVDNKTMDKHTNDIEDWSNNIQTKAPLKEVDPHLRPKKYSKKSLGHKRAST
RO88-CHS3       TVARKQGAYIVDNKTMDKHTNDIEDWSNNIQTKAPLKEVDPHLRPKKYSKKSLGHKRAST
VIN13-CHS3      TVARKQGAYIVDNKTMDKHTNDIEDWSNNIQTKAPLKEVDPHLRPKKYSKKSLGHKRAST
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VL3-CHS3        FDLLKKHSSKMFQFNESVIDLDTSMSSSLQSSGSYRGMTTMTTQNAWKLSNENKAVHSRN
YJM789-CHS3     FDLLKKHSSKMFQFNESVIDLDTSMSSSLQSSGSYRGMTTMTTQNAWKLSNENKAVHSRN
S288C-CHS3      FDLLKKHSSKMFQFNESVIDLDTSMSSSLQSSGSYRGMTTMTTQNAWKLSNENKAVHSRN
RO88-CHS3       FDLLKKHSSKMFQFNESVIDLDTSMSSSLQSSGSYRGMTTMTTQNAWKLSNENKAVHSRN
VIN13-CHS3      FDLLKKHSSKMFQFNESVIDLDTSMSSSLQSSGSYRGMTTMTTQNAWKLSNENKAVHSRN
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VL3-CHS3        PSTLLPTSSMFWNKATSSPVPGSSLIQSLDSTIIHPDIVQQPPLDFMPYGFPLIHTICFV
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YJM789-CHS3       PSTLLPTSSMFWNKATSSPVPGSSLIQSLDSTIIHPDIVQQPPLDFMPYGFPLIHTICFV
S288C-CHS3        PSTLLPTSSMFWNKATSSPVPGSSLIQSLDSTIIHPDIVQQPPLDFMPYGFPLIHTICFV
RO88-CHS3         PSTLLPTSSMFWNKATSSPVPGSSLIQSLDSTIIHPDIVQQPPLDFMPYGFPLIHTICFV
VIN13-CHS3        PSTLLPTSSMFWNKATSSPVPGSSLIQSLDSTIIHPDIVQQPPLDFMPYGFPLIHTICFV
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VL3-CHS3          TCYSEDEEGLRTTLDSLSTTDYPNSHKLLMVVCDGLIKGSGNDKTTPEIALGMMDDFVTP
YJM789-CHS3       TCYSEDEEGLRTTLDSLSTTDYPNSHKLLMVVCDGLIKGSGNDKTTPEIALGMMDDFVTP
S288C-CHS3        TCYSEDEEGLRTTLDSLSTTDYPNSHKLLMVVCDGLIKGSGNDKTTPEIALGMMDDFVTP
RO88-CHS3         TCYSEDEEGLRTTLDSLSTTDYPNSHKLLMVVCDGLIKGSGNDKTTPEIALGMMDDFVTP
VIN13-CHS3        TCYSEDEEGLRTTLDSLSTTDYPNSHKLLMVVCDGLIKGSGNDKTTPEIALGMMDDFVTP
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VL3-CHS3          PDEVKPYSYVAVASGSKRHNMAKIYAGFYKYDDSTIPPENQQRVPIITIVKCGTPAEQGA
YJM789-CHS3       PDEVKPYSYVAVASGSKRHNMAKIYAGFYKYDDSTVPPENQQRVPIITIVKCGTPAEQGA
S288C-CHS3        PDEVKPYSYVAVASGSKRHNMAKIYAGFYKYDDSTIPPENQQRVPIITIVKCGTPAEQGA
RO88-CHS3         PDEVKPYSYVAVASGSKRHNMAKIYAGFYKYDDSTIPPENQQRVPIITIVKCGTPAEQGA
VIN13-CHS3        PDEVKPYSYVAVASGSKRHNMAKIYAGFYKYDDSTIPPENQQRVPIITIVKCGTPAEQGA
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VL3-CHS3          AKPGNRGKRDSQIILMSFLEKITFDERMTQLEFQLLKNIWQITGLMADFYETVLMVDADT
YJM789-CHS3       AKPGNRGKRDSQIILMSFLEKITFDERMTQLEFQLLKNIWQITGLMADFYETVLMVDADT
S288C-CHS3        AKPGNRGKRDSQIILMSFLEKITFDERMTQLEFQLLKNIWQITGLMADFYETVLMVDADT
RO88-CHS3         AKPGNRGKRDSQIILMSFLEKITFDERMTQLEFQLLKNIWQITGLMADFYETVLMVDADT
VIN13-CHS3        AKPGNRGKRDSQIILMSFLEKITFDERMTQLEFQLLKNIWQITGLMADFYETVLMVDADT
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VL3-CHS3          KVFPDALTHMVAEMVKDPLIMGLCGETKIANKAQSWVTAIQVFEYYISHHQAKAFESVFG
YJM789-CHS3       KVFPDALTHMVAEMVKDPLIMGLCGETKIANKAQSWVTAIQVFEYYISHHQAKAFESVFG
S288C-CHS3        KVFPDALTHMVAEMVKDPLIMGLCGETKIANKAQSWVTAIQVFEYYISHHQAKAFESVFG
RO88-CHS3         KVFPDALTHMVAEMVKDPLIMGLCGETKIANKAQSWVTAIQVFEYYISHHQAKAFESVFG
VIN13-CHS3        KVFPDALTHMVAEMVKDPLIMGLCGETKIANKAQSWVTAIQVFEYYISHHQAKAFESVFG
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VL3-CHS3          SVTCLPGCFSMYRIKSPKGSDGYWVPVLANPDIVERYSDNVTNTLHKKNLLLLGEDRFLS
YJM789-CHS3       SVTCLPGCFSMYRIKSPKGSDGYWVPVLANPDIVERYSDNVTNTLHKKNLLLLGEDRFLS
S288C-CHS3        SVTCLPGCFSMYRIKSPKGSDGYWVPVLANPDIVERYSDNVTNTLHKKNLLLLGEDRFLS
RO88-CHS3         SVTCLPGCFSMYRIKSPKGSDGYWVPVLANPDIVERYSDNVTNTLHKKNLLLLGEDRFLS
VIN13-CHS3        SVTCLPGCFSMYRIKSPKGSDGYWVPVLANPDIVERYSDNVTNTLHKKNLLLLGEDRFLS
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VL3-CHS3          SLMLKTFPKRKQVFVPKAACKTIAPDKFKVLLSQRRRWINSTVHNLFELVLIRDLCGTFC
YJM789-CHS3       SLMLKTFPKRKQVFVPKAACKTIAPDKFKVLLSQRRRWINSTVHNLFELVLIRDLCGTFC
S288C-CHS3        SLMLKTFPKRKQVFVPKAACKTIAPDKFKVLLSQRRRWINSTVHNLFELVLIRDLCGTFC
RO88-CHS3         SLMLKTFPKRKQVFVPKAACKTIAPDKFKVLLSQRRRWINSTVHNLFELVLIRDLCGTFC
VIN13-CHS3        SLMLKTFPKRKQVFVPKAACKTIAPDKFKVLLSQRRRWINSTVHNLFELVLIRDLCGTFC
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VL3-CHS3          FSMQFVIGIELIGTMVLPLAICFTIYVIIFAIVSKPTPVITLVLLAIILGLPGLIVVITA
YJM789-CHS3       FSMQFVIGIELIGTMVLPLAICFTIYVIIFAIVSKPTPVITLVLLAIILGLPGLIVVITA
S288C-CHS3        FSMQFVIGIELIGTMVLPLAICFTIYVIIFAIVSKPTPVITLVLLAIILGLPGLIVVITA
RO88-CHS3         FSMQFVIGIELIGTMVLPLAICFTIYVIIFAIVSKPTPVITLVLLAIILGLPGLIVVITA
VIN13-CHS3        FSMQFVIGIELIGTMVLPLAICFTIYVIIFAIVSKPTPVITLVLLAIILGLPGLIVVITA
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VL3-CHS3          TRWSYLWWMCVYICALPIWNFVLPSYAYWKFDDFSWGDTRTIAGGNKKAQDENEGEFDHS
YJM789-CHS3       TRWSYLWWMCVYICALPIWNFVLPSYAYWKFDDFSWGDTRTIAGGNKKAQDENEGEFDHS
S288C-CHS3        TRWSYLWWMCVYICALPIWNFVLPSYAYWKFDDFSWGDTRTIAGGNKKAQDENEGEFDHS
RO88-CHS3         TRWSYLWWMCVYICALPIWNFVLPSYAYWKFDDFSWGDTRTIAGGNKKAQDENEGEFDHS
VIN13-CHS3        TRWSYLWWMCVYICALPIWNFVLPSYAYWKFDDFSWGDTRTIAGGNKKAQDENEGEFDHS
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VL3-CHS3          KIKMRTWREFEREDILNRKEESDSFVA-
YJM789-CHS3       KIKMRTWREFEREDILNRKEESDSFVA-
S288C-CHS3        KIKMRTWREFEREDILNRKEESDSFVA-
RO88-CHS3         KIKMRTWREFEREDILNRKEESDSFVA-
VIN13-CHS3        KIKMRTWREFEREDILNRKEESDSFVA-
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Crh1p

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S288c-Crh1                          MKVLDLLTVLSASSLLSTFAAAESTATADSTTAASSTASCNPLKTTGCTP
VIN13-Crh1                          MKVLDLLTVLSASSLLSTFAAAESTATADSTTAASSTASCNPLKTTGCTP
RO88-Crh1                           MKVLDLLTVLSASSLLSTFAAAESTATADSTTAASSTASCNPLKTTGCTP
EC1118-Crh1                         MKVLDLLTVLSASSLLSTFAAAESTATADSTTAASSTASCNPLKTTGCTP
AWRI1631gi|207344997|gb|EDZ719      MKVLDLLTVLSASSLLSTFAAAESTATADSTTAASSTASCNPLKTTGCTP
FostersB-gi|323304785|gb|EGA58      MKVLDLLTVLSASSLLSTFAAAESTATADSTTAASSTASCNPLKTTGCTP
VL3-gi|323354858|gb|EGA86691.1      MKVLDLLTVLSASSLLSTFAAAESTATADSTTAASSTASCNPLKTTGCTP
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S288c-Crh1                      DTALATSFSEDFSSSSKWFTDLKHAGEIKYGSDGLSMTLAKRYDNPSLKS
VIN13-Crh1                      DTALATSFSEDFSSSSKWFTDLKHAGEIKYGSDGLSMTLAKRYDNPSLKS
RO88-Crh1                       DTALATSFSEDFSSSSKWFTDLKHAGEIKYGSDGLSMTLAKRYDNPSLKS
EC1118-Crh1                     DTALATSFSEDFSSSSKWFTDLKHAGEIKYGSDGLSMTLAKRYDNPSLKS
AWRI1631gi|207344997|gb|EDZ719  DTALATSFSEDFSSSSKWFTDLKHAGEIQYGSDGLSMTLAKRYDNPSLKS
FostersB-gi|323304785|gb|EGA58  DTALATSFSEDFSSSSKWFTDLKHAGEIKYGSDGLSMTLAKRYDNPSLKS
VL3-gi|323354858|gb|EGA86691.1  DTALATSFSEDFSSSSKWFTDLKHAGEIKYGSDGLSMTLAKRYDNPSLKS
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S288c-Crh1                      NFYIMYGKLEVILKAANGTGIVSSFYLQSDDLDEIDIEWVGGDNTQFQSN
VIN13-Crh1                      NFYIMYGKLEVILKAANGTGIVSSFYLQSDDLDEIDIEWVGGDNTQFQSN
RO88-Crh1                       NFYIMYGKLEVILKAANGTGIVSSFYLQSDDLDEIDIEWVGGDNTQFQSN
EC1118-Crh1                     NFYIMYGKLEVILKAANGTGIVSSFYLQSDDLDEIDIEWVGGDNTQFQSN
AWRI1631gi|207344997|gb|EDZ719  NFYIMYGKLEVILKAANGTGIVSSFYLQSDDLDEIDIEWVGGDNTQFQSN
FostersB-gi|323304785|gb|EGA58  NFYIMYGKLEVILKAANGTGIVSSFYLQSDDLDEIDIEWVGGDNTQFQSN
VL3-gi|323354858|gb|EGA86691.1  NFYIMYGKLEVILKAANGTGIVSSFYLQSDDLDEIDIEWVGGDNTQFQSN
                                **************************************************

S288c-Crh1                      FFSKGDTTTYDRGEFHGVDTPTDKFHNYTLDWAMDKTTWYLDGESVRVLS
VIN13-Crh1                      FFSKGDTTTYDRGEFHGVDTPTDKFHNYTLDWAMDKTTWYLDGESVRVLS
RO88-Crh1                       FFSKGDTTTYDRGEFHGVDTPTDKFHNYTLDWAMDKTTWYLDGESVRVLS
EC1118-Crh1                     FFSKGDTTTYDRGEFHGVDTPTDKFHNYTLDWAMDKTTWYLDGESVRVLS
AWRI1631gi|207344997|gb|EDZ719  FFSKGDTTTYDRGEFHGVDTPTDKFHNYTLDWAMDKTTWYLDGESVRVLS
FostersB-gi|323304785|gb|EGA58  FFSKGDTTTYDRGEFHGVDTPTDKFHNYTLDWAMDKTTWYLDGESVRVLS
VL3-gi|323354858|gb|EGA86691.1  FFSKGDTTTYDRGEFHGVDTPTDKFHNYTLDWAMDKTTWYLDGESVRVLS
                                **************************************************

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VIN13-Crh1                      NTSSEGYPQSPMYLMMGIWAGGDPDNAAGTIEWAGGETNYNDAPFTMYIE
RO88-Crh1                       NTSSEGYPQSPMYLMMGIWAGGDPDNAAGTIEWAGGETNYNDAPFTMYIE
EC1118-Crh1                     NTSSEGYPQSPMYLMMGIWAGGDPDNAAGTIEWAGGETNYNDAPFTMYIE
AWRI1631gi|207344997|gb|EDZ719  NTSSEGYPQSPMYLMMGIWAGGDPDNAAGTIEWAGGETNYNDAPFTMYIE
FostersB-gi|323304785|gb|EGA58  NTSSEGYPQSPMYLMMGIWAGGDPDNAAGTIEWAGGETNYNDAPFTMYIE
VL3-gi|323354858|gb|EGA86691.1  NTSSEGYPQSPMYLMMGIWAGGDPDNAAGTIEWAGGETNYNDAPFTMYIE
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RO88-Crh1                       KVIVTDYSTGKKYTYGDQSGSWESIEADGGSIYGRYDQAQEDFAVLANGG
EC1118-Crh1                     KVIVTDYSTGKKYTYGDQSGSWESIEADGGSIYGRYDQAQEDFAVLANGG
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FostersB-gi|323304785|gb|EGA58  KVIVTDYSTGKKYTYGDQSGSWESIEADGGSIYGRYDQAQEDFAVLANGG
VL3-gi|323354858|gb|EGA86691.1  KVIVTDYSTGKKYTYGDQSGSWESIEADGGSIYGRYDQAQEDFAVLANGG
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VIN13-Crh1                      SISSSSTSSSTVSSSASSTVSSTVSSSVSS------------TVSSSSSV
RO88-Crh1                       SISSSSTSSSTVSSSASSTVSSSVSSTVSSSASSTVSSSVSSTVSSSSSV
EC1118-Crh1                     SISSSSTSSSTVSSSASSTVSSTVSSSVSS------------TVSSSSSV
AWRI1631gi|207344997|gb|EDZ719  SISSSSTSSSTVSSSASSTVSSSVSSTVSS----------------SSSV
FostersB-gi|323304785|gb|EGA58  SISSSSTSSSTVSSSASSTVSSSVSSTVS---------------SSSSV
VL3-gi|323354858|gb|EGA86691.1  SISSSSTSSSTVSSSASSTVSSTVSSSVSS------------TVSSSSSV
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S288c-Crh1                      SSSSSTSPSSSTATSSKTLASSSVTTSSSISSFEKQSSSSSKKTVASSST
VIN13-Crh1                      SSSSSTSPSSSTATSSKTLASSSATTSSSISSLEKQSSSSSKKTVASSST
RO88-Crh1                       SSSSSTSPSSSTATSSKTLASSSVTTSSSISSFEKQSSSSSKKTVASSST
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FostersB-gi|323304785|gb|EGA58  SSSSSTSPSSSTATSSKTLASSSVTTSSSISSLEKQSSSSSKKTVASSST
VL3-gi|323354858|gb|EGA86691.1  SSSSSTSPSSSTATSSKTLASSSATTSSSISSLEKQSSSSSKKTVASSST
                                **********************.********:*****************

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RO88-Crh1                       SESIISSTKTPATVS-------STTRSTVAPTTQQSSVSSDSPVQDKGGV
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FostersB-gi|323304785|gb|EGA58  SESIISSTKTPATVS-------STTRSTVAPTTQQSSVSSDSPVQDKGGV
VL3-gi|323354858|gb|EGA86691.1  SESIISSTKTPXTVSSTPATVSSTTRSTVAPNYPTIFRIIGQSCTR----
                                *********** ***       *********.        ...

S288c-Crh1                      ATSSNDVTSSTTQISSKYTSTIQSSSSEASSTNSVQISNGADLAQSLPRE
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RO88-Crh1                       ATSSNDVTSSTTQISSKYTSTIQSSSSEASSTNSVQISNGADLAQSLPRE
EC1118-Crh1                     ATSSNDVTSSTTQISSKYTSTIQSSSSEASSTNSVQISNGADLAQSLPRE
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FostersB-gi|323304785|gb|EGA58  ATSSNDVTSSTTQISSKYTSTIQSSSSEASSTNSVQISNGADLAQSLPRE
VL3-gi|323354858|gb|EGA86691.1  --------------------------------------------------
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RO88-Crh1                           GKLFSVLVALLALL-
EC1118-Crh1                         GKLFSVLVALLALL-
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FostersB-gi|323304785|gb|EGA58      GKLFSVLVALLALL-
VL3-gi|323354858|gb|EGA86691.1      ---------------
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Supplementary Figure 4.2: Alignment sequences using ClustalW and ClustalX version 2 (2007)

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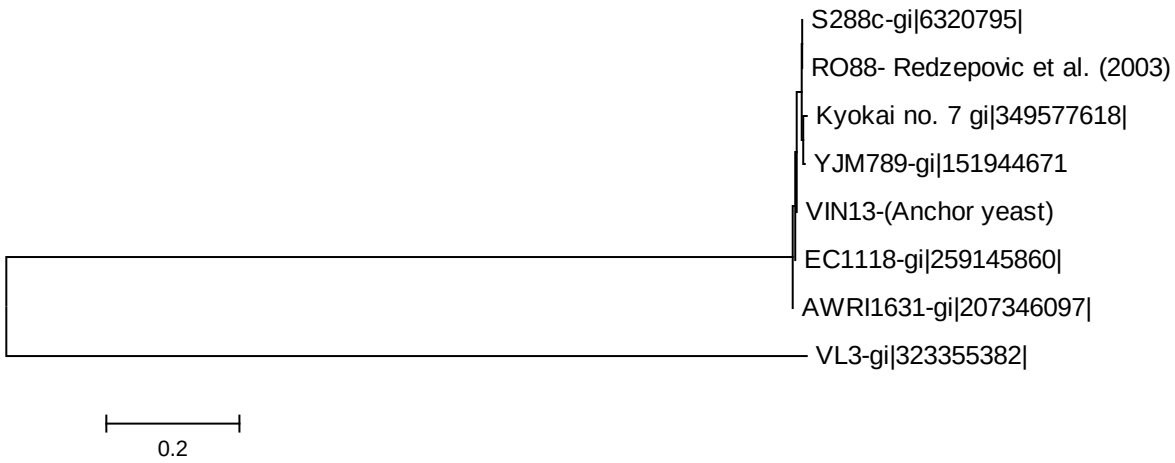

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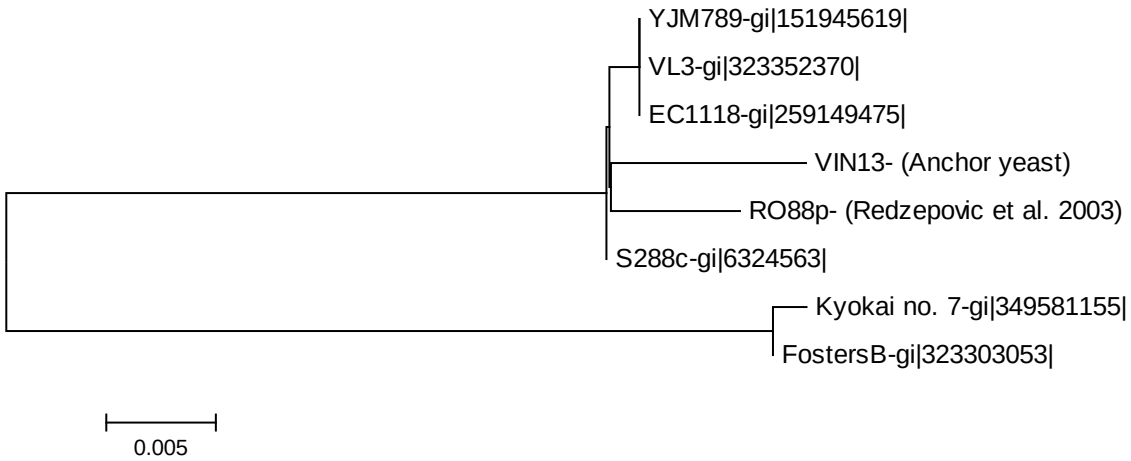

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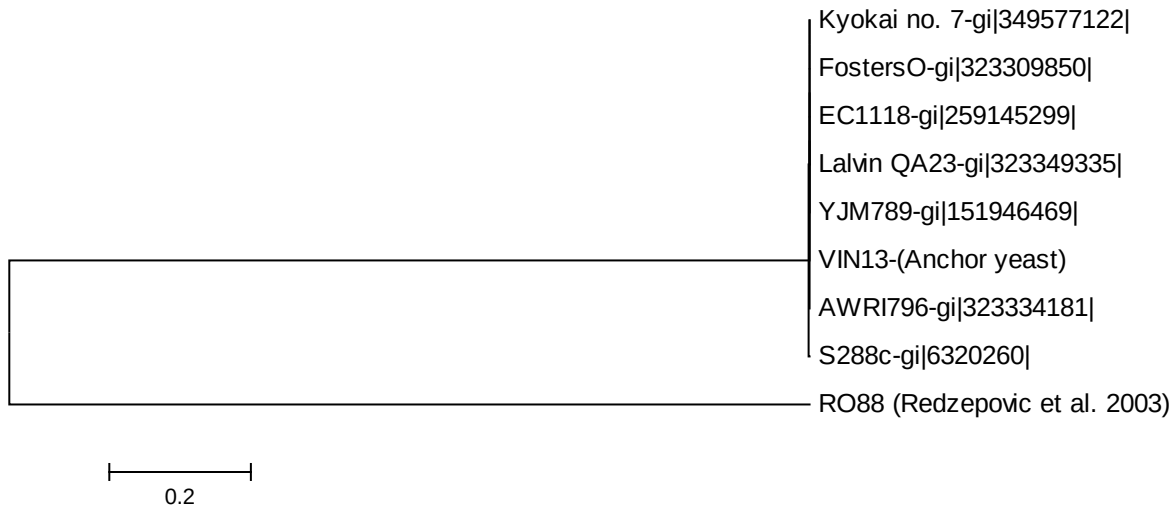

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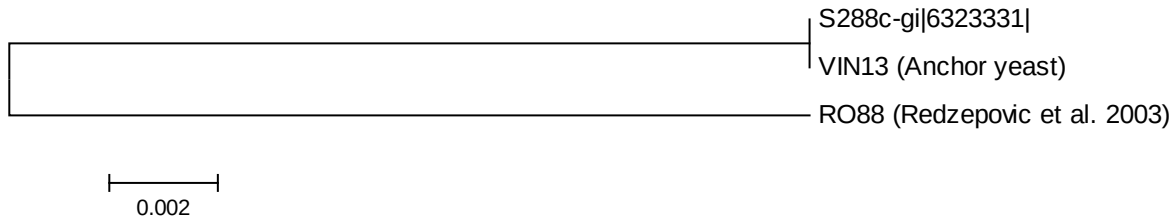

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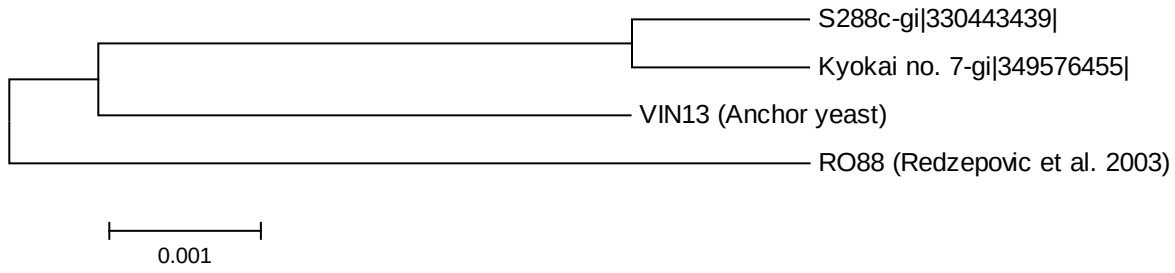

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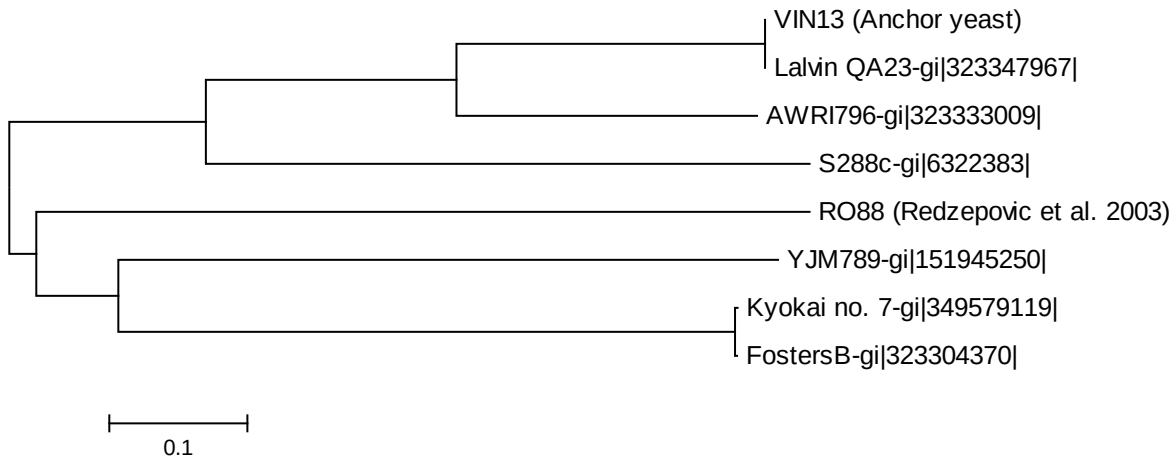
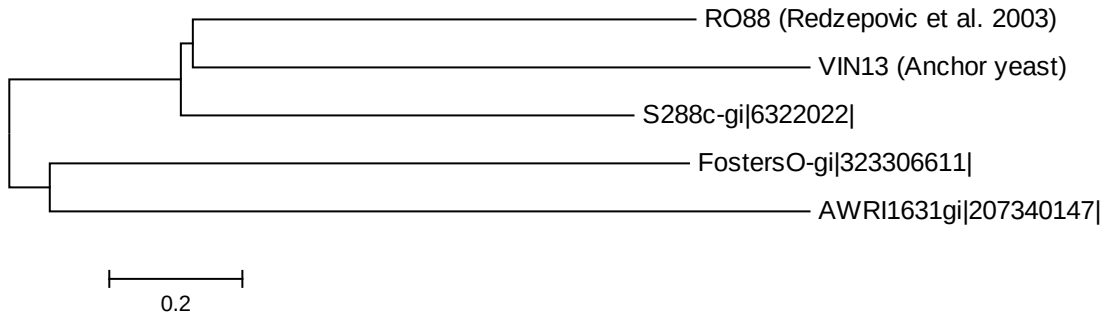


g. *HPF1'*



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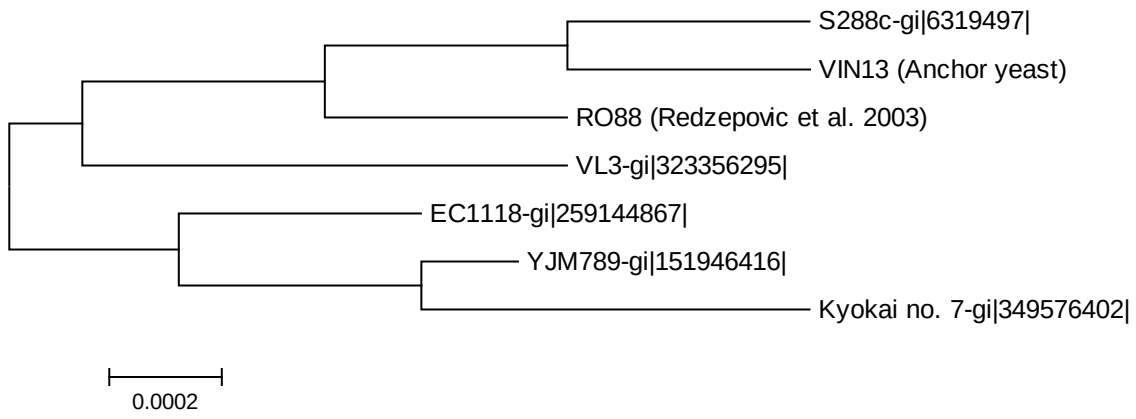
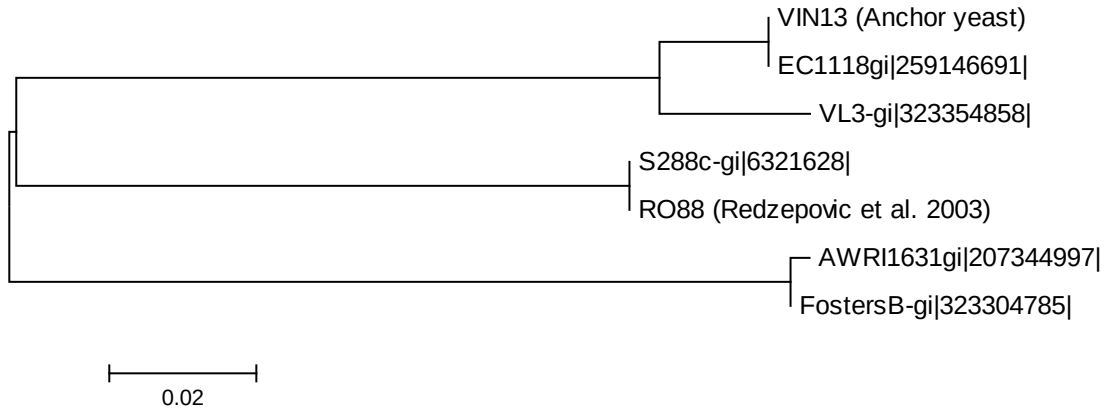


i. *CRH1*



Supplementary Figure 4.3a-i: Evolutionary relationships of taxa. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.,* 2011). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Poisson correction model (Zuckerkandl and Pauling, 1965) and are in units of number of amino acid substitutions per site.

Chapter 5

Research results III

S. paradoxus strains reduce wine haze formation through higher cell wall chitin levels

Patent filled: METHOD OF INHIBITING HAZE FORMATION IN WINE

Ref No: P2355ZA00, South Africa.

This manuscript will be submitted for publication in **International Journal of Food Microbiology**

S. paradoxus strains reduce wine haze formation in part through higher cell wall chitin

5. Abstract

Of all proteins found in wine, grape chitinases have recently been shown to likely be the main contributors to wine haze formation. Our studies have shown that *S. paradoxus* yeast strains are significantly more efficient in protecting wine from protein haze formation than traditional *S. cerevisiae* wine yeast strains (Chapter 3). The data also suggest that differences in the *S. paradoxus* and *S. cerevisiae* exoproteomes (chapter 4) may in part be responsible for the differences in haze protection ability but that they are unlikely to be solely responsible for these differences. Some of the proteins found in different concentrations in the exoproteomes of the two species are related to chitin biosynthesis, a major constituent of yeast cell walls. Furthermore, chitin has in the past been shown to efficiently reduce wine haze formation when used as a clarifying agent. In this study we screened yeast cell walls for cell wall chitin levels. The data show that cell walls of *S. paradoxus* contain higher concentrations of chitin than *S. cerevisiae* strains. Furthermore, both *Escherichia coli*-produced grape chitinases and commercial chitinases bind more efficiently to the *S. paradoxus* cell wall than to the *S. cerevisiae* cell wall, thus suggesting a mechanism by which *S. paradoxus* cells may selectively remove these haze forming proteins from wine.

5.1 Introduction

Bottled white wines can develop haze due to the precipitation of grape pathogenesis-related (PR) proteins that 'survive' the fermentation process due to their stability under high ethanol conditions, low pH levels, and the proteolytic activity of grape and yeast proteases. While some contradictory findings have been made in the past regarding the exact causes of wine protein haze formation (Marangon *et al.,* 2010a; Siebert *et al.,* 2009; Batista *et al.*, 2010), several recent publications convincingly demonstrate that grape chitinases, and to a lesser degree thaumatin like proteins, are the major contributors to the phenomenon (Esteruelas *et al.,* 2009; Batista *et al.,* 2009; Falconer *et al.,* 2010; Marangon *et al.,* 2011). Other proteins such as β-1,3-glucanases and ripening-related protein Grip22 (Esteruelas *et al.,* 2009) have also been identified in wine haze particles, but appear not to be causally responsible for its formation, or to be minor contributors at most (Waters *et al.,* 1996).

The evidence for chitinases as a primary causative agent for protein wine haze are based on thermal unfolding studies of grape thaumatin-like protein and chitinase and on using differential scanning calorimetry. Falconer *et al.* (2010) demonstrated that grape chitinase were the major players in heat-induced haze in unfined wines as they have a low melting temperature. Moreover, the last stage of chitinase aggregation was observed to be irreversible and strongly associated with visible haze formation when compared to invertase and thaumatin-like protein. A linear correlation was also found to exist between chitinase content in wine and haze intensity (Marangon *et al.,* 2010b; Marangon *et al.,* 2011). Moreover, Palmisano *et al.* (2010) observed that the glycoproteins identified in wine haze had hydrolase activity (38%) and chitinase activity (13%). Studies by Marangon *et al.* (2010a) in simple model solutions suggested that chitinases have a half-life in wine of six minutes at 55°C, thus extrapolating down to a half-life of three days at 35°C or two years at 25°C when compared to the thaumatin-like protein that melted at 62°C, with a calculated half-life of 300 years at 25°C.

In the previous chapter, we showed that several types of proteins were secreted at higher levels by a *S. paradoxus* strain which provided a higher level of haze protection than the *S. cerevisiae* strains. Such proteins included enzymes involved in the remodeling of cell wall chitin. Interestingly, chitin addition to unfined wine has been shown to reduce wine haze by up to 80% of total haze induced by the heat test (Vincezi *et al.,* 2005). Wine haze reduction observed with the use of chitin as a fining agent is based on the hypothesis that grape chitinases possess a chitin-binding domain different from the catalytic site (Graham and Sticklen, 1994). Here we investigated the possibility that yeast cell wall chitin might be involved in haze reduction by allowing binding of grape chitinases to the yeast cell wall. The data indeed show that (i) grape chitinases bind to yeast cell walls, that (ii) yeast cells with higher chitin levels bind higher levels of chitinases, that (iii) *S. paradoxus* cell walls appear to contain higher levels of chitin and that *S. paradoxus* has a significantly higher capacity to eliminate chitinases from a solution than *S. cerevisiae*. Taken together, the data suggest that the haze reducing activity of *S. paradoxus* can at least in part be linked to the levels of chitin found in the cell wall. These findings therefore suggest a novel strategy for reducing wine haze by using *S. paradoxus* yeast strains.

5.2 Materials and methods

5.2.1 Fermentation

Fermentations were carried out in triplicate at 21°C in 120-ml bottles with a 50 ml working volume without agitation and fitted with air traps. Commercial wine strains (Table 5.1), were used to ferment chemically defined synthetic MS300 medium (Bely *et al*., 1990) and Chardonnay grape juice to dryness. Residual glucose and fructose concentrations were less than 5 g/l as measured using a D-glucose/fructose enzymatic kit (Amersham).

Table 5.1: *S. cerevisiae*[a], *S. paradoxus*[b] and hybrid[ab] yeast strains used in this study

Strain	Description	Source
BM45[a]	Industrial wine yeast strain	Lallemand Inc. (Montreal, Canada)
VIN13[a]	Commercial yeast strain	Anchor Yeast (Cape Town, South Africa)
L2323[a]	Industrial wine yeast strain	Lallemand Inc.
WE372[a]	Industrial wine yeast strain	Anchor Yeast
N96[a]	Commercial yeast strain	Anchor Yeast
NT50[a]	Commercial yeast strain	Anchor Yeast
L2226[a]	Commercial yeast strain	Lallemand Inc.
Fermicru XL[a]	Commercial yeast strain	DSM Food Specialties B.V. (Flemingham, Netherlands)
NT112[a]	Commercial yeast strain	Anchor Yeast
SC22[a]	Commercial yeast strain	University of California (Davis, USA)
D254[a]	Commercial yeast strain	Lallemand Inc. (Montreal, Canada)
EC1118[a]	Commercial yeast strain	Lallemand Inc. (Montreal, Canada)
Exotic[ab]	Commercial yeast strain VIN13×RO88 hybrid	Mocke (2005)
P 01-167[b]	Industrial wine yeast strain	Phaff Yeast Collection (University of California, Davis, CA, USA)
P 01-208[b]	Isolated from olive brine	Phaff Yeast Collection
P 01-146[b]	Isolated from Luca Cocolin, Italy	Phaff Yeast Collection
RO88[b]	Industrial wine yeast strain	Redžepović *et al.* (2003)

5.2.2 Heat stability test

The heat stability of wine samples was determined as described by Pocock and Waters (2006) with all measurements made in triplicate with the appropriate controls. Briefly, the assay was carried out by centrifuging fermented chardonnay grape must at 5,000 rpm for 5 min to remove cells. After taking absorbance readings at 520 nm, the wine sample was heated at 80°C for 2 h and then cooled to 4°C for 16 h. A_{520nm} was measured after acclimatization at room temperature

for 30 min. Haze was measured by calculating the difference in absorbance before and after heating of the wine sample (Waters *et al.*, 1992).

5.2.3 Calcofluor white staining, fluorescence microscopy and flow cytometry

Calcofluor white staining of cells and fluorescence microscopy was adapted from de Groot *et al.* (2001). Cells grown overnight at 30^{o}C in YPD were either directly used for microscopy or inoculated into fresh YPD medium. To enhance the detection of cell wall-related phenotypes, the cells inoculated in the fresh medium were further incubated for 5 h at 37^{o}C. About 200µl of the cell culture was centrifuged and the cells were washed with PBS (phosphate buffered saline) buffer saline (pH 7.4). PBS buffer was made by adding the following (g/l): 8.01 NaCl, 0.20 KCl, 1.78 $Na_2HPO_4.2H_2O$ and 0.27 KH_2PO_4. Cells were stained with about 10 µl of calcofluor white stain after addition of 10 µl KOH (10%) following the manufacturer's instructions (Fluka Analytical, Sigma-Aldrich, 18909-100ml). Image acquisition was performed on an Olympus Cell® system attached to an IX 81 inverted fluorescence microscope equipped with an F-view-II cooled CCD camera (Soft Imaging Systems). The excitation laser used was the violet laser with 407 nm wavelength and the emission filter used was the Pacific Blue channel with a 450/40 band pass filter. Images were processed and background-subtracted using the Cell® software, and presented in a maximum intensity projection.

For the quantification of chitin levels using the flow cytometry, a BD FACS Aria flow cytometer was used. BD FACS Diva v.6.1.3 software was used for the data capture. The excitation and emission filter laser used were the same as described above. 50, 000 cells were used for the quantification of chitin levels.

5.2.4 Overexpression of GFP-tagged grape *Vitis vinifera* chitinase in *Escherichia coli*

General molecular biology techniques used in this study were following protocol described by Sambrook *et al.* (1989). *E. coli* DH5α (Gibco BRL/Life Technologies, Rockville, MD) was used as a host for all plasmid amplifications. Total grape berry RNA was isolated as previously described by Schmitt *et al.* (1990) and DNase I (Roche diagnostics) treatment was used to

eliminate DNA contamination. One µg of the extracted RNA was used as template for cDNA synthesis using the ImProm-II™ reverse transcription system according to the manufacturer instructions (Promega). Highly expressed grape chitinase gene was amplified from grape berry cDNA using chitinase forward primer 5' CATATGGCAGCCAAGCTACTAACAGTC 3' (*Nde*I) and chitinase reverse primer 5' CTCGAGGCAAGTGAGGTTGTCACCA 3' (*Xho*I). The amplified chitinase class ivD fragment (Accession number: AF532966.1) was cloned into a shuttle vector pJet1.2/blunt (CloneJet™ PCR Cloning kit, Fermentas, Inqaba Biotech, SA) following the protocol described by the manufacturer before cloning into the pET14b vector following the pET system manual. Green fluorescent protein was PCR amplified from pKEN mut 2 vector using 5' CTCGAGATGAGTAAAGGAGAAGAACTTTTCAC 3' (*Xho*I) as the forward primer and 5' GATCGGATCCTTATTTGTATAGTTCATCCATGCC' 3 (*Bam H*I) as the reverse primer, cloned first into a shuttle vector pGEM-T-easy (Promega Promega Corporation, Madison, USA) before cloning into the pET vector. High-fidelity DNA polymerase Phusion® (Finnzymes, catalogue # F530L) was used for all the PCR reactions. Finnzymes Phusion® High-Fidelity DNA Polymerase manual was followed for the PCR program and an annealing Tm of 60^{o}C was used for all the primers. The cloned grape chitinase tagged to GFP in pET vector (pET-GFP-Chi) was sequenced to confirm the correct sequence identity.

A protocol described by Lee and Coleman (2006) was used to overexpress the grape chitinase protein in *E. coli* Rosetta 2(DE3) pLysS. For the overexpression of GFP-tagged grape *Vitis vinifera* chitinase in *E. coli*, pET-GFP-Chi vector was transformed into *E. coli* Rosetta 2(DE3) pLysS. *E. coli* Rosetta 2(DE3) pLysS containing pET-GFP-Chi was grown in LB broth at 37 °C until cell density of ~0.6 (OD 600 nm) was reached. The cell culture was cooled to 25 °C and the grape chitinase expression was induced with 0.4 mM IPTG at 25 °C overnight. The cell pellet from the overnight expressed cell culture was freeze-thawed once to rupture the bacterial cell membrane and allow the lysozyme, produced by *E. coli* Rosetta 2(DE3) pLysS, to degrade the bacterial cell wall. Lysozyme 10 mg/ml was added to further lyse the cells. The cell pellet was re-suspended in 50 mM potassium phosphate cell lysis buffer, pH 8.0, containing 300 mM KCl and 10% glycerol, based on 7 mL lysis buffer per gram of wet cell pellet. 0.1% (v/v) DNase

and 0.1% (v/v) RNase were added to reduce the viscosity of the cell lysate. The soluble protein fraction (crude cell lysate) was separated from the cell debris by centrifugation at 14,000 rpm. The crude protein extract was concentrated using Amicon Ultra-15 Centrifugal Filter columns (Millipore™, Merck, Ireland, catalogue # UFC901096) with a cut-off of 10 kDa. The concentrated enzyme was dissolved in enzyme storage buffer (50 mM potassium phosphate buffer, pH 7.0, containing 150 mM KCl, 1 mM DTT, 1 mM EDTA, and 10% glycerol).

5.2.5 Chitinase assay

The extracted chitinase was assessed by carrying out a chitinase activity assay using different substrates. Chitinase activity assay kit (Sigma-aldrich, Missouri, United States of America, catalogue # CS0980) was used with 4-Nitrophenyl β-D-N,N',N''-triacetylchitotriose (a substrate suitable for endochitinase activity detection), 4-Nitrophenyl β-D-N,N',N''-triacetylchitotriose (a substrate suitable for endochitinase activity detection) and 4-Nitrophenyl N,N'-diacetyl-β-D-chitobioside (a substrate suitable for exochitinase activity detection (chitobiosidase activity) as instructed by the manufacturer.

5.2.6 GFP-tagged grape chitinase – yeast cell wall binding assays

Yeast cells were cultured following a method described by de Groot *et al*. (2001). Equal amount of cells based on OD measurement at 600 nm were washed using 1 x PBS buffer. 100µl of crude GFP tagged chitinase protein was added to the cells suspended in 200µl BPS buffer. These were incubated for 2 hours at either room temperature or 37^{o}C with shacking. The cells were centrifuged at 5000 rpm, washed twice with PBS buffer and visualized under a fluorescence microscopy. The excitation laser used was the solid state sapphire laser at wavelength of 488 nm and the emission filters used was the FITC channel, with a 502 long pass and 530/30 band pass filter. To quantify the GFP-tagged grape chitinase bound to the yeast cell wall, flow cytometry (BD FACS Aria flow) using the above described excitation and emission wavelengths. For the data analysis, FITC-Area Geometric mean of fluorescence intensity was used for the quantification of fluorescence produced by 50, 000 cells.

5.2.7 Yeast cell wall binding assays

Cells were grown in synthetic medium MS300 and equal amount of cells were centrifuged and washed with saline water (0.9 % NaCl). For the cell wall extract, yeast cells were grown in YPD broth and boiled for 15 min at 100^{o}C before washing with saline water. Chitinase enzyme (Tri*choderma viride*, Sigma-Aldrich, C8241) was dissolved in model wine solution of pH 3.3 containing 12% ethanol and 4 g/l tartaric acid to a final concentration of 0.5 mg/ml. The cell pellet was suspended in 1 ml of chitinase enzyme and incubated for 30 minutes with shaking at 37^{o}C. A negative control where no cells were added was also incubated. The suspension was centrifuged at 5, 000 rpm and 30 µl of the supernatant was used for further chitinase activity analysis. The chitinase assay kit (Sigma-aldrich, CS0980), was used to assay for the remaining chitinase activity as per manufacturer's instructions.

5.2.8 Effect of a commercial mannoprotein addition on wine haze reduction

Mannostab® (Laffort Oenologie, Bordeaux,France), a dried yeast cell wall extract found to also contain chitin was added into fermented MS 300 at varying concentrations ranging from 100 mg/l to 400 mg/l. Haze assays in which 1 g/l BSA and potassium sulphate were added to generate haze were carried out.

5.3 Results

5.3.1 Chitin levels: Fluorescence microscopy and Flow cytometer

To assess the difference in cell wall chitin levels between *S. paradoxus* and *S. cerevisiae,* cells from several strains were stained with calcofluor white. As shown in figure Figure 5.1a, a visual inspection suggested higher levels of fluorescence in *S. paradoxus* cells than in *S. cerevisiae* cells. This observation applied whatever the stage of growth or specific growth conditions of the strains. To confirm this observation, flow cytometry was used to quantify the chitin levels.

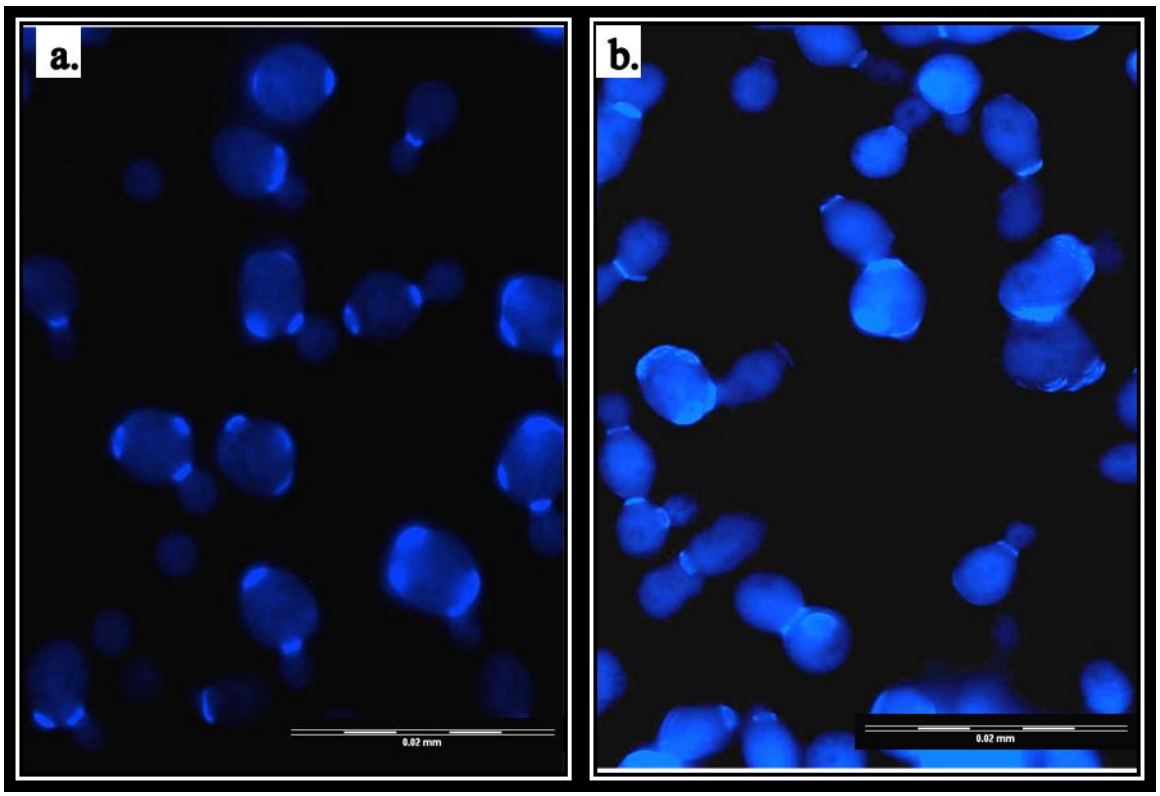


Figure 5.1a: *S. cerevisiae* (BM45) (a) and *S. paradoxus* (P01-208) (b) cells stained with calcofluor white stain. Cells were grown in YPD as described by de Groot *et al.* (2001) and responded in PBS buffer before staining and viewing under Olympus Cell® system attached to an IX 81 inverted fluorescence microscope equipped with an F-view-II cooled CCD camera (Soft Imaging Systems).

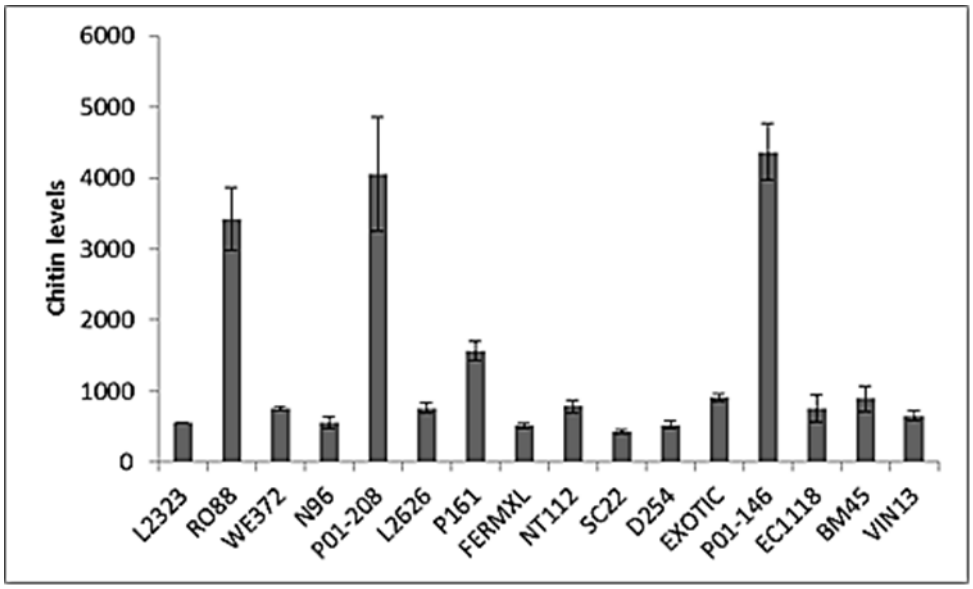


Figure 5.1b: Chitin levels quantified using flow cytometry after staining the cells with calcofluor white stain. Cells were grown overnight in YPD media and a tenth of the overnight culture pre-inoculated into fresh medium and grown for 5 h (de Groot *et al*., 2001) reaching an OD of ~7. Cells were stained with calcofluor white and further subjected to flow cytometry.

Figure 5.1b shows the differences in chitin levels between *S. cerevisiae* and *S. paradoxus* strains measured using flow cytometry. RO88, P01-146 and P01-208 had significantly higher chitin levels when compared to *S. cerevisiae* wine strains.

Chitin levels of Mannostab®, a commercial cell wall preparation responsible for reduction of tartrate instability were also measured. The amount of chitin measured from 0.1 g of Mannostab® was 454 (±184.8) and Mannostab® was shown to reduce wine haze formation in our conditions (Supplementary Figure 5.1).

5.3.2 Overexpression of GFP-tagged grape *Vitis vinifera* chitinase in *Escherichia coli*

In order to demonstrate the possibility that high chitin levels found in cell walls of *S. paradoxus* strains could be responsible for the reduction of protein instability in wine, we developed a grape chitinase - yeast cell wall binding assay. *V. vinifera* grape chitinase class IVD (*Chit*ivD) tagged to GFP was cloned in the pET14B vector and transformed in *E. coli* Rosetta 2(DE3) pLysS. To characterize the over-expressed grape chitinase protein, the extracted crude proteins were used for the chitinase enzyme activity assays. The data showed that chitinase activity in the extract was strongest when 4-Nitrophenyl β-D-N, N', N''-triacetylchitotriose as a substrate indicating that the overexpressed protein was an endochitinase (Figure 5.2).

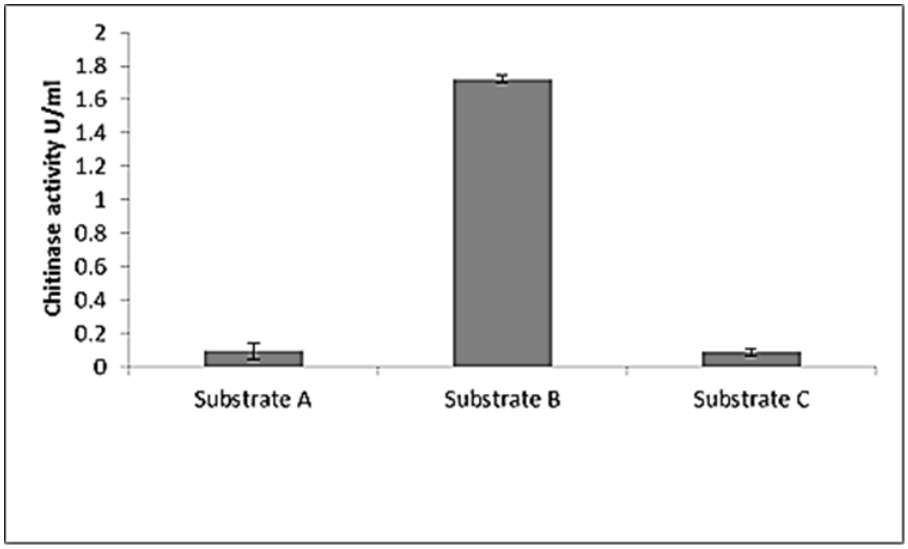


Figure 5.2: GFP-tagged chitinase activity from crude protein concentrate assayed in 3 different substrates (Key below) suitable for A: exochitinase, B: endochitinase and C: chitobiosidase activity detection supplied with the Chitinase assay kit (Sigma-aldrich, CS0980) following manufacturer's instructions.

Key: Substrates **Sample A**- 4-Nitrophenyl N-acetyl-β-D-glucosaminide – a substrate suitable for exochitinase activity detection (b-N-acetylglucosaminidase activity), **Sample B**- 4-Nitrophenyl β-D-N,N',N''-triacetylchitotriose – a substrate suitable for endochitinase activity detection and **Sample C** - 4-Nitrophenyl N,N'-diacetyl-b-D-chitobioside – a substrate suitable for exochitinase activity detection (chitobiosidase activity)

Chitinase binding assay was carried out using both GFP-tagged grape chitinase and a commercial chitinase from *Trichoderma viride*. Figure 5.3a shows bound GFP-tagged grape chitinase to the yeast cell while Figure 5.3b shows the differences in the fluorescence intensity quantified using a flow cytometer. It was observed that the *S. paradoxus* strains bound more grape chitinase in comparison to *S. cerevisiae* yeast cells.

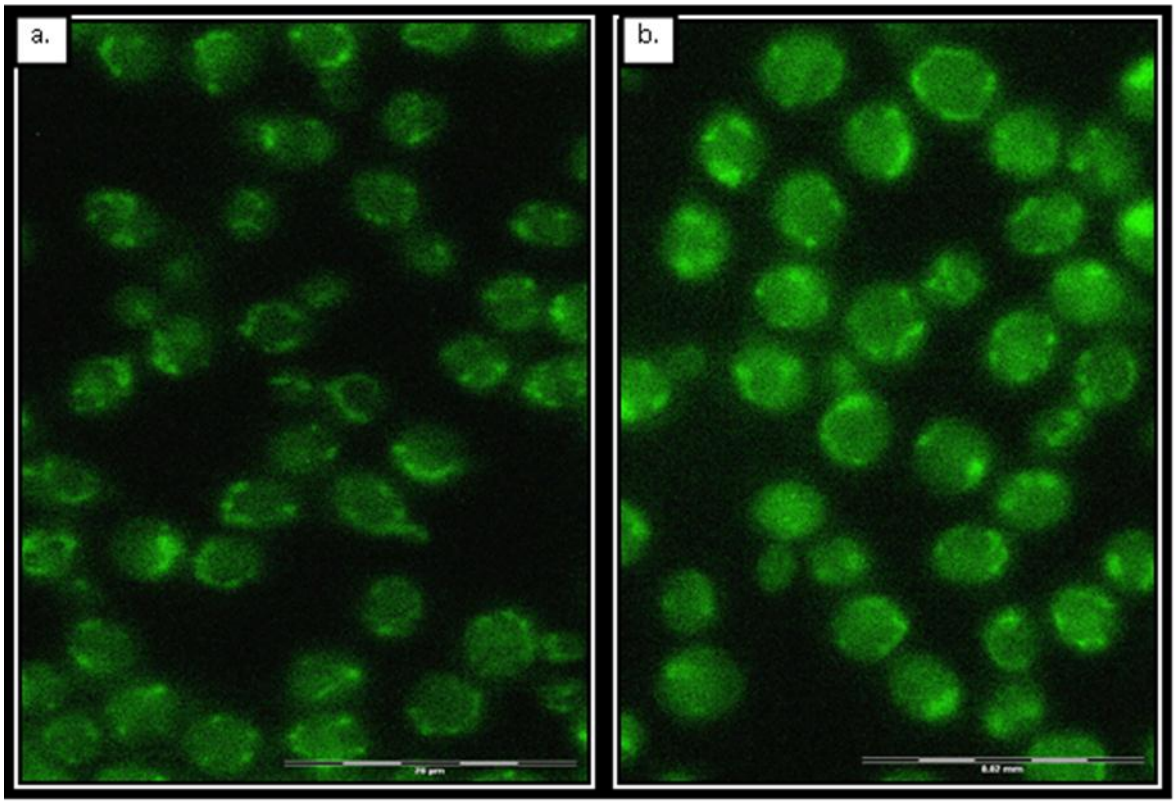


Figure 5.3a: GFP-tagged chitinase bound to the *S. cerevisiae* (BM45) and *S. paradoxus* (P01-208) yeast cells viewed under a fluorescence microscope. Cells were grown in YPD as described by de Groot *et al.* (2001) and equal amount of cells based on OD measurement at 600nm were washed and re-suspended in PBS buffer before adding GFP-tagged chitinase.

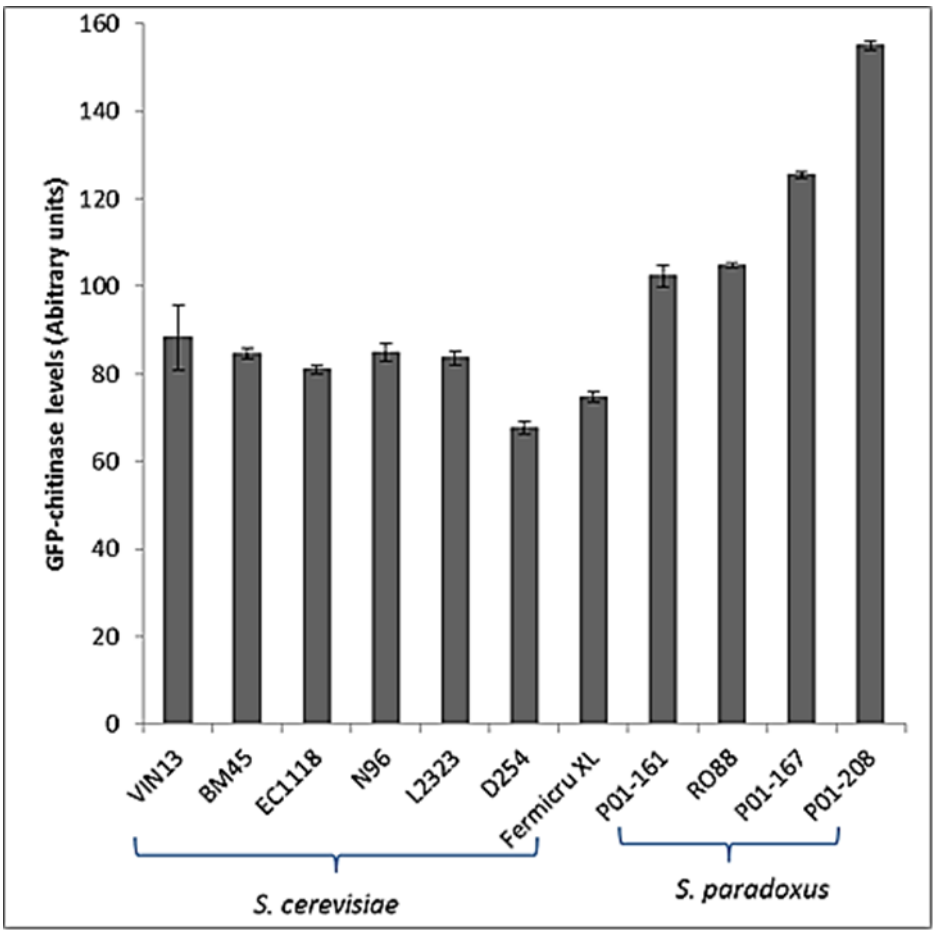


Figure 5.3b: GFP-chitinase levels bound to different yeast strains, quantified using BD FACS Aria flow cytometer. Cells were grown in YPD as described by de Groot *et al.* (2001) and equal amount of cells based on OD measurement at 600nm were washed and re-suspended in PBS buffer before adding GFP-tagged chitinase. Cells were further incubated for 2 hours at room temperature with shaking before washing and re-suspending in PBS buffer in preparation for quantification using a flow cytometer.

Finally, a commercial chitinase was used for the binding assay to evaluate the binding of yeast cells to a commercial chitinase in a model wine solution. Commercial chitinase was dissolved in model wine solution containing 4 g/l tartaric acid and 12% ethanol at pH 3.3 to a final concentration of 250 mg/l. 1ml of the chitinase-model wine solution was added to equal amount of pelleted cells, mixed and incubated with shaking. The data showed that chitinase activity was reduced by 99.99 % when P01 and RO88 *S. paradoxus* live cells were used (Figure 5.4a).

To evaluate the possible use of cell wall material from *S. paradoxus* strains, the chitinase binding ability of cell wall extract was evaluated. Similar to live cells, cell wall extracts from the *S. paradoxus* strains especially RO88 revealed highly selective chitinase binding when

compared to *S. cerevisiae* strains (Figure 5.4b). However, yeast cell wall extract bound less chitinase when compared in comparison to live cells with the exception of VIN13 cells.

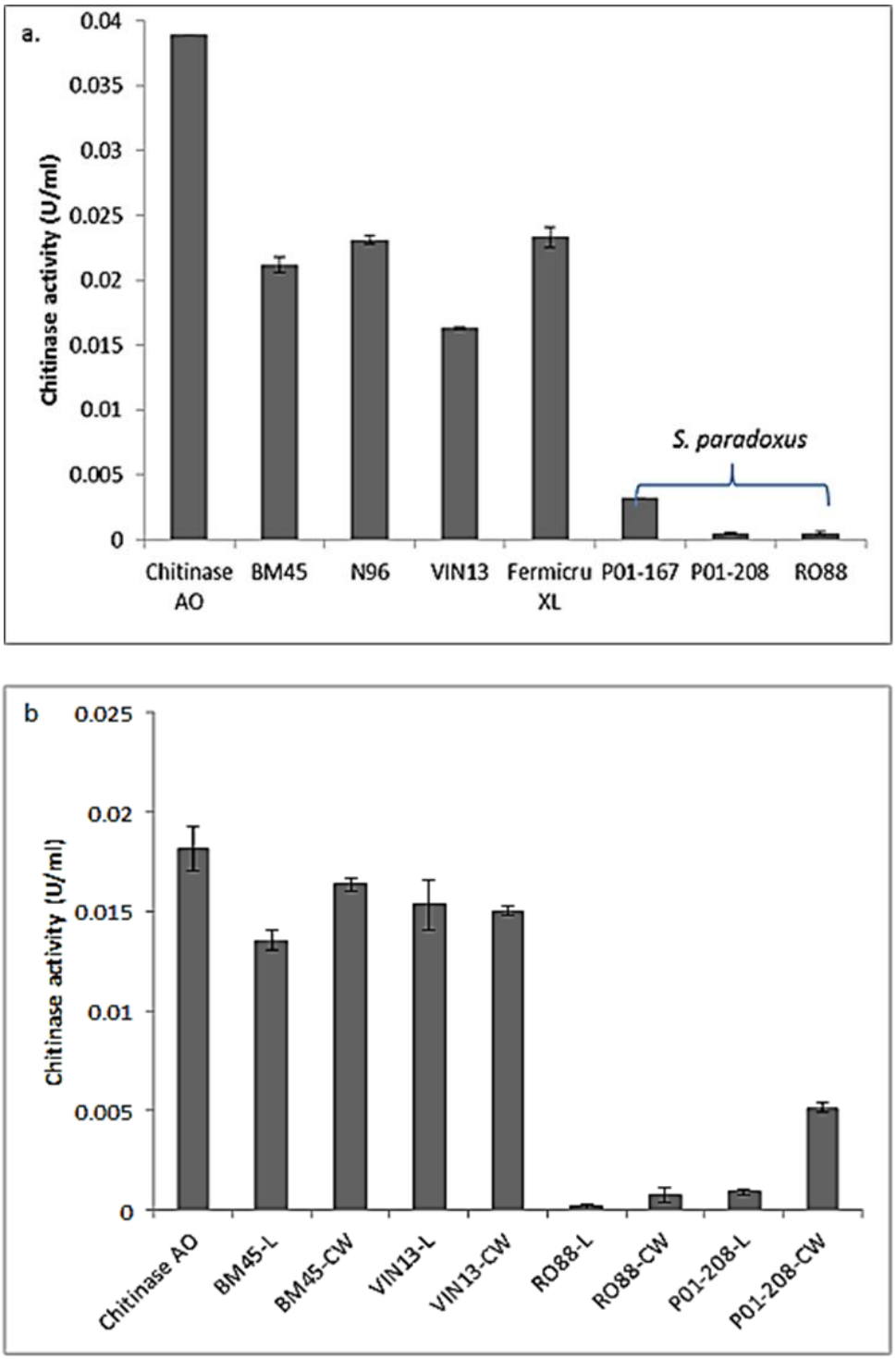


Figure 5a and b: a. Shows chitinase activity levels of chitinase remaining in model wine solution (12% ethanol, 4 g/l tartaric acid, pH 3.3) not bound to yeast cell wall after incubation with cells. b. Chitinase activity levels of chitinase not bound to yeast cell wall after incubation with live whole cells (L) and boiled cell wall extract (CW) from *S. paradoxus* and *S. cerevisiae* cells. Chitin levels were quantified using Chitinase assay kit (Sigma-Aldrich, CS0980) following manufacturer's instructions. Commercial chitinase (*Trichoderma viride*, Sigma-Aldrich, C8241) was used for yeast cell wall binding assay in this experiment.

5.4 Discussion

Considering the negative impact of fining agents on wine quality, alternative haze protection methods such as selection of yeast strains capable of reducing protein wine haze is warranted. Based on the haze protective nature of *S. paradoxus* strains in results obtained in chapter 3, we further investigated other haze protection factors that could be released by these strains besides the secreted exoproteome (results shown in chapter 4). Higher chitin levels were observed in *S. paradoxus* strains when compared to *S. cerevisiae* strains. Differences in chitin levels have been reported for certain cell wall mutants, but our data suggest that the *S. cerevisiae* wine yeast strains evaluated here all show very similar levels of cell wall chitin, and that all the *S. paradoxus* strains show significantly higher levels, suggesting that this is a species specific character. This is the first study to report higher levels of chitin in *S. paradoxus* strains than in *S. cerevisiae* strains. It is fascinating to note that some filamentous growing yeasts, such as *Candida albicans*, have been reported to have higher content of chitin which is an essential component of the cell wall of all pathogenic fungi (Lenardon *et al.,* 2010; Lee *et al.,* 2011), while chitin is absent from many other yeast species such as *Schizosaccharomyces pombe* a fission yeast (Bulawa *et al.,* 1986). Despite its small quantity (1-3%), chitin is essential for yeast survival, probably because of its central role in septation (Klis *et al.,* 2002).

Our data convincingly demonstrate that *S. paradoxus* cells are able to bind higher levels of chitinase than *S. cerevisiae* cells. Indeed, two independent assays, involving either *E. coli*-produced GFP-tagged grape chitinase or commercial chitinase, support this hypothesis. While we do not show that the chitinases are indeed bound to the cell wall chitin during wine making, the correlation between higher chitin levels and higher cell wall chitinase binding suggests that this interaction may indeed be a primary causative mechanism of wine haze reduction in wine during fermentation.

The reduced wine haze levels and high chitinase levels bound by the *S. paradoxus* strains also concur with observations made by Manteau *et al.* (2003). These authors observed the disappearance of chitinase from Champagne wine and attributed this observation to the

likelihood that the chitinase was fixed on the cell wall of *Saccharomyces cerevisiae* (yeast lees) after alcoholic fermentation and on the cell wall of the bacterium *Oenococcus oeni* after malolactic fermentation. Furthermore, the grape chitinases have been demonstrated to maintain their activity in wine at least for some months after alcoholic fermentation (Manteau *et al.,* 2003), therefore explaining the reduced haze levels observed during wine aging on lees (aging results shown in chapter 3).

In order to determine whether yeast cell wall extracts of *S. paradoxus* strains can be used as fining agents in cases where alcoholic fermentation is carried out using inoculated commercial *S. cerevisiae* strains, yeast cell wall binding assays were also carried out using yeast cell wall extracts. It was observed that similarly to live cells, the *S. paradoxus* cell wall extract bound higher levels of chitinase than *S. cerevisiae* strains. These results rule out the possibility of *S. paradoxus* strains producing yeast acidic proteases that may have hydrolyzed the grape chitinase (Manteau *et al.,* 2003).

In this study, it was further demonstrated that Mannostab®, a cell wall preparation with the ability of reducing wine haze formation contained not only mannoproteins but also chitin. These results may suggest that the chitin present in Mannostab® extracted from the yeast cell walls is in fact responsible for reducing protein wine haze formation possibly in conjunction with mannoproteins. It is also interesting to note that protein and mannoprotein profile of Mannostab was assessed in the previous Chapter 4 using SDS-PAGE gel (results not shown) where it was observed that the banding profile of mannostab is similar to that of *S. cerevisiae* strains and not of *S. paradoxus* strain.

It can also be further argued that in wine haze studies where cell wall deletion mutants are used for haze assays (Brown *et al.,* 2007; Gonzalez *et al.,* 2006; 2008; 2009), the observed wine haze reduction may not only be due to the higher release of mannoproteins, but may also be due to increases in chitin levels in at least some of these mutants. Deletion studies of certain

cell wall genes such as *GAS1* were indeed shown to result in high cell wall chitin levels (Popolo *et al.,* 1997).

In conclusion, our findings strongly indicate the possibility of using yeast strains displaying high chitin to reduce wine haze formation. *S. paradoxus* has been evaluated under wine making conditions and were found to possess good enological properties. For example RO88 has been shown to have high polygalaturonase activity (Mocke, 2005), influence the production of certain aroma compounds and contributed to the final quality of wine (Majdak *et al.,* 2002; Orliç *et al.,* 2007). *S. paradoxus* strains may therefore be considered to be used as starter cultures in wine fermentations and may also be used for future yeast breeding projects with the aim of reducing wine haze formation. Moreover this proposed method of wine haze protection is more sustainable in comparison to other used fining agents such as non-recyclable bentonite. In cases where *S. cerevisiae* are used for wine fermentations, *S. paradoxus* strains could possibly be used to make yeast hulls for use as clarifying agents.

5.5 Acknowledgements

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We would like to thank Prof. M. du Toit for acquiring Mannostab® from Laffort Oenologie and Dr P. Young for supplying the *Vitis vinifera* grape cDNA.

Flow cytometry and fluorescence microscopy experiments were carried out at the Central analytical facility (CAF, Stellenbosch University) with the help of Dr. Ben loose and Ms. Lize Brewer.

5.6 References

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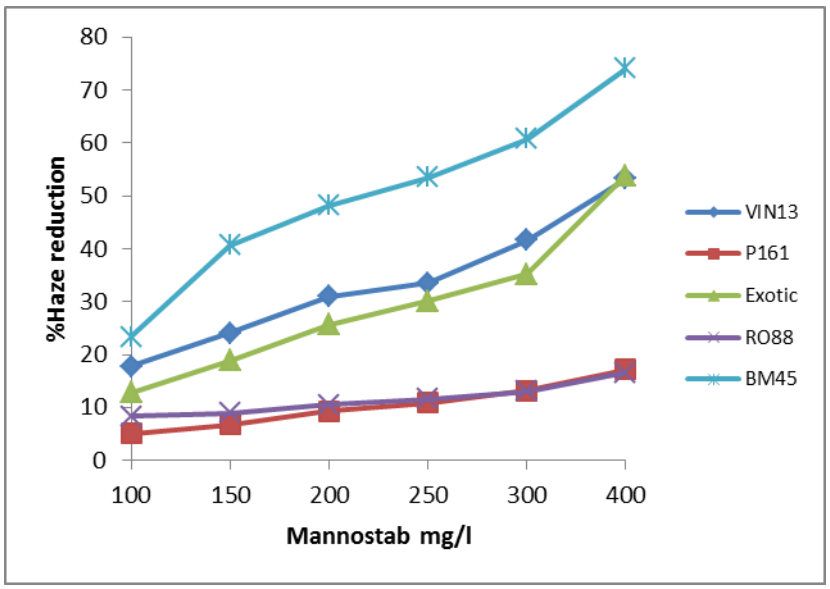
5.7 Supplementrary Figure



Supplementary figure 5.1: Effect of Mannostab® addition on haze formation in fermented MS300 expressed as a percentage decrease from the initial haze value (means of 3 analyses). The heat test was carried out at 80^{o}C for 2 h and 1 g/l of both BSA and sulphate were added to generate haze.

Chapter 6

General discussion and conclusion

6.1 General discussion and conclusion

Protein instability leading to wine haze formation is an economically significant problem in the global wine industry (Dupin *et al.,* 2000; Høj *et al.,* 2000; Lomolino and Curioni, 2007). In this study, we have been further investigating the possible role of wine yeast strains in helping address this problem. The data make a significant contribution to this field. Indeed, 6 major findings can be highlighted:

1. Results in the first research chapter of this thesis highlight the need of a standard assay to enable results to be comparable between studies and to help wine makers in decision making with regards to determining wine haze formation potential (Sarmento *et al*., 2000; Pocock and Waters, 2006; Esteruelas *et al.,* 2009). Moreover, in order to be in a position to develop ways of counteracting or preventing protein wine haze formation, further work is needed to develop a standard haze assay to enable the accurate assessment of the impact of the developed clarifying agent employed.

2. This study also demonstrated that strains overexpressing the flocculation mannoproteins, Flop did not have any impact on wine haze reduction indicating that only specific mannoproteins are prone to contribute to wine haziness and thus not all mannoproteins are able to reduce wine haze formation. It is therefore imperative to determine the identity of other mannoproteins capable of reducing wine haze formation besides the few already known haze protection factor proteins (hpf1p, hpf2p and hpf1'p) and invertase (Dupin et al., 2000; Brown *et al.,* 2007). This will enable the optimization processes for the use of mannoproteins in the wine industry therefore replacing the current costly clarifying agents employed in the industry.

3. Wine yeast strains of the species *S. paradoxus* show significant anti-protein wine haze activity. This finding opens a number of new avenues for future strain development. Indeed, the single *S. cerevisiae* / *S. paradoxus* hybrid (Mocke, 2005) wine yeast strain in this study showed intermediate wine haze protective ability, while some of the self diploidised spores showed even higher activity than the *S. paradoxus* wild type. We did not further assess whether these strains would be employable as wine yeast strains, but

the data clearly suggest that a breeding program with the aim of generating haze protective strains using *S. paradoxus* as a partner has a significant chance of success.

In addition, it cannot be excluded that it is possible that further analysis of a larger number of existing wine yeast strains would reveal some strains with higher haze protective activity.

4. The haze protective ability of *S. paradoxus* strains can at least in part be linked to the protein secretion properties of the strain. Our data indeed suggest that the *S. paradoxus* strain investigated here secretes higher levels of proteins with anti- haze formation activity. The study however has certain limitations, primarily because only two strains one S. cerevisiae and one S. paradoxus were investigated for the iTRAQ analysis. It is therefore not clear whether the reported protein secretion is representative of each species, or rather of the individual strains tested. It is also not clear how the specific growth conditions may impact on these properties. Furthermore, the iTRAQ methodology only provides relative quantification (Wiese *et al.,* 2007), and not absolute quantification of protein concentration. It therefore does not provide sufficient insights to firmly conclude that these differences in secretion levels are of relevance. Another limitation for this study was the fact that *S. cerevisiae* database was used to search for protein identities for proteins released by both *S. paradoxus* RO88 and *S. cerevisiae* VIN13. A high number of proteins secreted by RO88 were not annotated when compared to those of VIN13. Nevertheless, the fact that some of the proteins identified in the study did contribute to haze protection when over expressed in *S. cerevisiae* is encouraging, and strongly suggests that protein secretion by the RO88 strain is a contributing factor in the haze-protective phenotype. While sequence data showed some divergence between the sequences of the investigated genes between the *S. paradoxus* and *S. cerevisiae* strains, we did not further investigate whether these differences play a role in the specific role of these proteins or on their impact on haze protective activity,

5. Chitin levels of yeast cell walls are directly correlated with haze protective activity and these cell walls are capable of binding grape chitinases. In line with recent data suggesting that grape chitinases are in fact the major contributors to protein haze in wine (Batista *et al.,* 2009; Falconer *et al.,* 2010; Marangon *et al.,* 2011), our data indeed suggest that chitin levels in the yeast cell wall are a major contributing factor to the haze protective activity of the *S. paradoxus* strains. These findings provide also some explanation for the data in chapter 3 which suggested that *S. paradoxus* strains lead to higher haze formation in synthetic must when compared to the *S. cerevisiae* strains, while the opposite is observed in real must. Indeed, the synthetic must does not contain chitinases, and the positive impact of cell wall chitin would have been absent. Following these innovative results on *S. paradoxus* strains, a patent was filled for the use of the *S. paradoxus* strains in protecting wine from haze formation during this study under the title: Method of inhibiting haze formation in wine, Reference number P2355ZA00.

   It will also be worthwhile to determine whether the grape chitinases only binds to *S. paradoxus* cell wall chitin or whether chitin released into the medium may also play a role in the process of wine haze reduction. A further question that requires investigation is whether chitin levels alone or also chitin accessibility play a role in the process. Indeed, the chitinases bound to the cell wall did not specifically cluster around bud scars, suggesting that the binding occurred to the chitin that is distributed in a more diffuse manner. The chitin rings marking the bud scars appear to be less accessible. New strains may therefore not require increased chitin biosynthesis, but rather mutations that would lead to a redistribution of some of the chitin away from the bud scars.

6. *S. paradoxus* cell walls or live cells may constitute a new fining tool for the wine industry. The data obtained thus far indeed strongly suggest that by further optimizing the application and use of *S. paradoxus* cells, we may be able to fine wine sufficiently to make further clarification unnecessary. It is however necessary to further investigate the mechanisms that control chitin distribution and optimise growth conditions for increased chitin production. Besides gene deletions involved in cell wall biogenesis (Popolo *et al.,*

2007) several studies have demonstrated an increase in cell wall chitin in response to cell wall stress and cell wall perturbing compounds through the activation of the cell wall integrity pathway, CWI (Munro *et al.,* 2007; Reviewed in Klis *et al.,* 2006 and Levin, 2011).

Future prospect include investigating the following:

- Our data strongly suggest that the *S. paradoxus* strains have the capacity to reduce protein wine haze formation in comparison to *S. cerevisiae* strains. It will be interesting to determine the haze protection capacity of *S. paradoxus* strains to large scale fermentations similar to industrial fermentations.
- Live cells and cell wall extract of *S. paradoxus* were shown to bind more grape chitinases than *S. cerevisiae* strains possibly leading to reduced wine haze formation in wines fermented by the *S. paradoxus* strains. Further studies are needed to determine the effectiveness of yeast hulls/ cell wall extract from *S. paradoxus* strains in wine haze reduction capacity resulting in improved effectiveness of bentonite fining.
- Considering the positive impact of *S. paradoxus* strains on wine protein haze reduction, *S. paradoxus* strains may be considered for use in winemaking as starter cultures. Sensory analysis, mouth-feel and aroma compounds among other wine quality attributes of wines fermented using these strains needs to be evaluated.
- The effect of the *S. paradoxus* strains used in this study may be used to evaluate their impact on tartrate instability. Mannostab®, (Laffort Oenologie, Bordeaux, France) a commercial Mannoprotein used in improving wine tartrate instability was shown in this study to also reduce wine haze. It would therefore be interesting to observe if this also applies to cell wall extracts obtained from *S. paradoxus* strains.

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