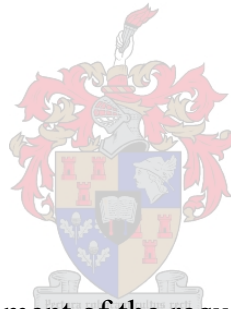# A MICROBIOLOGICAL SOLUTION TO VISIBLE WINE DEFECTS: pinking and protein haze formation

By

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated). The reproduction and publication thereof by the Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Summary

The wine industry is challenged with visible and nonvisible wine defects, which result in profit losses as consumers reject such wines. The clarity and color of the wine are essential to white wine consumers, as it is a measure of quality. Pinking of white wine and the formation of protein haze are some of the most frequent visual defects encountered in the industry. Pinking is a non-scientific term that describes the change in the natural white wine colour to a pink colour. Many speculations have been made concerning the causes of white wine pinking, but there is no conclusive explanation for the phenomenon yet. Protein haze in white wine is caused by the precipitation of pathogenesis-related proteins, namely thaumatin-like proteins, and chitinase.

While bentonite is commonly used as a fining agent to avoid protein haze, it has an adverse effect on wine quality. There is, therefore, a need for cost-effective alternatives aimed at preventing wines from both pinking and protein haze formation. Previous studies have reported that some yeast strains have the capability of reducing protein haze formation while no studies to date have reported the impact of yeast strains on wine pinking. In this study, a microbiological based solution was explored and the use of Saccharomyces cerevisiae and other wine-related yeast species as alternatives to chemically based fining agents was investigated. Monocultured and sequentially inoculated fermentations were carried out in both Sauvignon blanc must and synthetic grape must, and yeast cell wall chitin and mannoproteins levels were monitored during fermentation.

Interestingly, yeast cell wall chitin and mannoproteins levels decreased by more than half at the end of alcoholic fermentations from the initial day 1 level. A very promising correlation was obtained between chitin in the yeast cell wall and the binding of GFP-tagged chitinase to the cells. Different stains showed different binding affinities, which could be used to predict the haze protection of a particular strain. Some impact of yeast strains on pinking was also observed.

In conclusion, the data suggest that yeast strain selection may help reduce, if not in some cases eliminate the need for the use of bentonite as a fining agent for protein haze protection. The data also suggest that pinking can be somewhat reduced in similar ways.

Opsomming

Die wynbedryf word uitgedaag met sigbare en nie-sigbare wynafwykings, wat lei tot winsverliese as verbruikers sulke wyne verwerp. Die duidelikheid en kleur van wyn is noodsaaklik vir witwynverbruikers, aangesien dit 'n maatstaf van gehalte is. Die verpienking van witwyn en die vorming van proteïenwaas is van die mees algemene visuele defekte wat in die bedryf voorkom. Verpienking is 'n nie-wetenskaplike term wat die verandering in die natuurlike witwynkleur na 'n pienk kleur beskryf. Daar is baie spekulasies gemaak oor die oorsake van witwynpienk, maar daar is nog geen duidelike verklaring vir die verskynsel nie. Proteïenwaas in witwyn word veroorsaak deur die presipitasie van patogenese-verwante proteïene, naamlik thaumatien-agtige proteïene en chitinases.

Terwyl bentoniet algemeen gebruik word as 'n beboetmiddel om proteïenheur te vermy, het dit 'n negatiewe effek op wynkwaliteit. Daar is dus 'n behoefte aan koste-effektiewe alternatiewe wat daarop gemik is om wyne van beide pienk- en proteïen-waasvorming te voorkom. Vorige studies het gerapporteer dat sommige gisstamme die vermoë het om proteïenhaarvorming te verminder, terwyl geen studies tot dusver die impak van gisstamme op wynpynering aangemeld het nie. In hierdie studie is 'n mikrobiologiese gebaseerde oplossing ondersoek deur die gebruik van *Saccharomyces cerevisiae* en ander wynverwante gisspesies as alternatiewe vir chemies gebaseerde boete-middel. Monokultureerde en opeenvolgende geinokkuleerde fermentasies is uitgevoer in beide Sauvignon blanc-moes en sintetiese druiwe moes, en gisselmuurkitien- en mannoproteïenvlakke is tydens fermentasie gemonitor.

Interessant genoeg het gisselmuurkitien- en mannoproteïenvlakke met meer as die helfte aan die einde van alkoholiese fermentasies vanaf die aanvanklike dag 1-vlakke afgeneem. 'n Baie belowende korrelasie is verkry tussen chitien in die gisselwand en die binding van GFP-getikte chitinases aan die selle. Verskillende gisstamme het verskillende bindingsaffiniteite vertoon, wat gebruik kan word om die waasbeskerming van 'n bepaalde stam te voorspel. Daar is ook 'n paar impak van gisstamme op pienking waargeneem.

Ten slotte dui die data daarop dat die keuse van gisstamme kan help verminder, indien nie in sommige gevalle die behoefte aan die gebruik van bentoniet as 'n boete-agent vir proteïen-waasbeskerming uitskakel nie. Die data dui ook daarop dat pienking op soortgelyke maniere ietwat verminder kan word.

This thesis is dedicated to my late mother and brother.

To my dad and siblings thank you for the love, support and words of encouragement throughout the years of my studies.

Kuyasa nangomso, ngoba umzingisi akanashwa.

Biographical sketch

Amanda Kufa was born in Mount Ayliff, Eastern Cape on July 5, 1994. She attended Mount Ayliff Junior Secondary School. In 2008, she attended St John's College in Mthatha. In 2012, she started her studies at the University of Fort Hare, where she obtained Bachelor of Sciences in Biochemistry and Microbiology in 2015. Thereafter in 2016, she enrolled for a Hons-BSc-degree in Microbiology at the same institute. In 2017, she enrolled further enrolled for her postgraduate studies at the Institute for Wine Biotechnology to further her studies.

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Preface

This thesis is presented as a compilation of five chapters. Referencing is done to the style of the journal South African Journal of Enology and Viticulture.

Chapter 1   **General Introduction and project aims**

Chapter 2   **Literature review**

Reviewing the formation of haze and pinking potential of white wines as visible defects

Chapter 3   **Materials and Methods**

Chapter 4   **Research results**
Investigation the ability of *Saccharomyces cerevisiae* and wine-related yeast strains against the formation of protein haze and pinking potential in Sauvignon blanc.

Chapter 5   **General Discussions and Conclusions**

Table of content

Figure Index

Figure 2.1: The mechanism is adopted from van Sluyter et al. (2015). This is the revised mechanism of haze formation, which occurs through three sequential steps. The mechanism is now recognized as a multifactorial process. The first step is the denaturation of the wine proteins exposing their hydrophobic binding sites, followed by the second step where the proteins use the exposed hydrophobic binding sites to self-aggregate and the third step is a further aggregation of the aggregate resulting in haze.

Figure 2.2: Composition and structure of the yeast cell wall adapted at (Schrueder et al., 1996). The figure shows the connection of the yeast cell wall polysaccharides and proteins and where component is located in the cell wall.

Figure 4.1. Monoculture and sequentially inoculated fermentations in synthetic media and real grape must. Fermentations carried out in 80 mL of must, and fermentation curves were generated from the weight loss during fermentations. **(A)** mono-culture inoculation and **(B)** sequentially inoculated with *S. cerevisiae BM45* at 48 hours of the grape must fermentations. **(C)** and **(D)** are sequentially inoculated with *S. cerevisiae BM45* at 48 hours of the synthetic grape must fermentations

Figure 4.2: The fluorescence levels of the chitin and mannoproteins were measured using the flow cytometry. Calcofluor white and Concanavalin A (Alexa 647), fluorescent dyes were used to bind to the chitin and mannose in the yeast cell wall respectively. Quantification based on 50,000 cell per strain. **(A)** Shows the chitin fluorescence levels obtained for each strain and **(B)** Shows the mannoproteins levels based on the mannose, monomer of mannoproteins

Figure 4.3: The relative binding of crude grape GFP-chitinase to chitin in the yeast cell wall. The binding assay was done on day three and day five of the fermentations. **(A)** Day 3 and **(B)** Day 5 of the fermentations.

Figure 4.4: Haze levels of Sauvignon blanc. **(A)** Shows the Haze levels in mono-cultured and **(B)** Haze levels in sequentially cultured fermentation. Bentonite was added in 5 different dosages. In addition, none was added onto the control.

Figure 4.5: Haze levels measured in synthetic grape must fermentations. The haze was induced with the addition of 0.5 g/L of BSA and potassium sulphate. **(A)** Mono-cultured fermentations and **(B)** Sequentially inoculated fermentations.

Figure 4.6: Pinking potential of Sauvignon blanc. The pinking was induced by adding 0.072% of hydrogen peroxide onto the wines. **(A)** Shows the pinking potential in mono-cultured and **(B)** pinking potential in sequentially cultured fermentation. Bentonite was added in 5 different dosages. In addition, none was added onto the control.

Table Index

Abbreviations

BSA	Bovine serum albumin
FITC	Fluorescein isothiocyanate
GDP	Gross domestic product
GFP	Green fluorescence protein
HPF	Haze mannoproteins factor
Hpf1p	Haze protective mannoproteins factor gene
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB (agar and broth)	Lauria Bertani
MS300	Synthetic grape must
MWCO	Molecular weight cut off (Daltons)
PBS	Phosphate buffered saline
PR proteins	Pathogenesis-related proteins
PVPP	Polyvinylpolypyrrolidone
TLPs	Thaumatin-like proteins
YPD agar	Yeast peptone dextrose

Chapter One

General introduction and project aims

1. Introduction

Wine contributes significantly to the world economy, and in 2017, it is reported that wine export contributed R36.1 billion to the South African GDP (U.S$ 349.4 billion) (SAWIS, 2017). Consumers expect a product in line with their preferences. People "taste with their eyes," and white wines that show visual defects affecting colour or clarity such as haziness and pinking are disqualified from commercialization. Wine producers want to guarantee defect-free wines to ensure high consumer liking scores and market success, and hazy wines appear unappetizing to casual drinkers. The presence of precipitates in wines can be a result of three factors: microbial instability, tartrate instability and protein heat instability (Ribereau-Gayon et al., 2006). The phenomenon of protein haziness in wine is a result of precipitation of protein in bottled wines (Van Sluyter et al., 2015). Pinking, on the other hand, is a term that is used to describe the discoloration of white wine to a pinkish-blush colour as specified by Lamuela-Raventos et al. (2001).

Regarding protein haze formation, many studies have provided information on the proteins that are responsible for its establishment in wines (Van Sluyter et al., 2015). One group of proteins has in particular been identified as being primarily responsible for the formation of haze, the grape pathogenesis-related proteins such as thaumatin-like protein and chitinases (Tian et al., 2015). These proteins survive the fermentation process and form part of the final wine (Deytieux et al., 2007; Monteiro et al., 2007; Tian et al., 2015; Tian et al., 2017; Ndlovu et al. 2018).

Data show that in general the concentration of pathogenesis-related proteins increases and their diversity decreases as the grape berries ripen (Pocock et al., 2000; Deytieux et al., 2007; Giribaldi et al., 2007; Monteiro et al., 2007). , and these include UV exposure (Tian et al., 2018), fungal infections (Tian et al., 2015) and antifungal activity (Laurindo et al., 2018). After bottling, the transportation and storage conditions also have an impact on haze formation, which is affected by inappropriate handling and shipping conditions (Van Sluyter et al., 2015; Lankhorst et al., 2017). The temperature that is higher than 25°C has been shown to result in the formation of sediments in the wines and the formation of haze (Butzke et al., 2012).

The causes of white wine pinking are not fully understood and several hypotheses that have been proposed regarding the origin of pinking. Jones (1989), suggested that pinking could be a result of combinations or reactions between more than ten different compounds and polymeric compounds,

including anthocyanins, oxygen and sulphur dioxide. However, other studies have disputed that anthocyanins may be a cause of white wine pinking (Lamuela-Raventos et al., 2001). It was also reported that the pink chromophore that results in the colour change in white wines might be a derivative of 2-S-glutathionyl-caftaric acid (van Wyk et al., 1996). The matter of what causes pinking is based on speculations, and there is no scientific literature available.

To prevent and protect the wines from protein haze, wine producers use fining agents. These include bentonite, enzymes such as acid protease (Theron et al., 2017), magnetic removal of pathogenesis-related proteins (Mierczyaska-Vasilev et al., 2017), ultrafiltration's and adsorbents (Van Sluyter et al., 2015). Several authors have explored the effectiveness of proteolytic enzymes, in free and immobilized forms, for reducing of haze formation in white wines (Marangon et al., 2011; Younes et al., 2013; Liburdi et al., 2010; Benucci et al., 2014). However, none of the methods have been entirely accepted by the industry, and they are not cost-effective to serve as replacements for bentonite (Mierczyaska-Vasilev et al., 2017).

Bentonite is still the most commonly used fining agent for commercial wineries (Waters et al., 2005; McRae et al., 2018). The mechanism of action of bentonite is based on the binding of proteins to the clay and the formation of sediments referred to as lees that will settle at the bottom of the tank (Van Sluyter et al., 2015). The use of bentonite to clarify wines may however also result in changes to wine quality because it is not specific and can bind to other wine components (Sanborn et al., 2011; Tomasino et al., 2012; Muhlack et al., 2016). The amount to be added depends on the concentration of proteins to be removed (Pocock et al., 2011). Previous studies proposed that measuring key wine components might provide a more accurate method for predicting haze protein concentration in Sauvignon blanc wine (de Bruijn et al., 2014). Bentonite treatment has negative side-effects including, the total volume loss of wine of about 3-10% of the first wine (Waters et al., 2005) cost linked to being around the U.S. $ 0.5-1 billion per year (Majewski et al., 2011; Van Sluyter et al., 2015). Other issues that are related to the use of bentonite include the tank downtime and health-related topics such as inhalation by the workers (Salazar et al., 2007; Majewski et al., 2011; Van Sluyter et al., 2015). The ability of commercial chitin and the role of mannoproteins in reducing the formation of haze in white (Dupin et al., 2000; Vincenzi et al., 2005; Cilindre et al., 2008; GómezPastor et al., 2010), has been studied as well as the role of yeast cell wall chitin (Ndlovu, 2012; Ndlovu et al. 2018).

For pinking potential, polyvinylpolypyrrolidone (PVPP), a synthetic polymer that is used to reduce the content polyphenols that are associated with browning and astringency in white wine (Ribreau-Gayon et al., 2000; Bowyer, 2008), has been suggested as a tool to remove a pink colour and pinking precursor compounds from white wines (Iland et al., 2000). PVPP does also badly influence the wine aroma when compared to the other fining agents; however, the use of PVPP is expensive, and treatment efficiency is not guaranteed (Lamuela-Raventos et al., 2001).

A study by Ndlovu et al. (2018) reported that certain yeast strains of the species *Saccharomyces paradoxus* and *Saccharomyces cerevisiae* were able to protect the wines from haze formation. The study suggested that yeast cell wall chitin was responsible for this result since a strong correlation between the chitin levels and the reduction of haze. Also, mannoproteins secreted by yeast were also shown to reduce the formation of haze in studies that were conducted by Ledoux et al. (1992), Waters et al. (1994), and Dupin et al. (2000). Over the past years, other species of wine-related yeasts have been investigated for their potential contribution towards wine quality and some of these strains have since been commercialized (Jolly et al., 2014; Masneuf-Pomarade et al., 2016). These include species from genera such as *Candida, Kloeckera, Kluyveromyces, Debaryomyces, Hanseniaspora, Pichia, Metschinikowia, Schizosaccharomyces, Saccharomyces, Starmerella, Torulaspora, Cryptococcus or Rhodotorula* (Copozzi et al., 2015; Alessandra et al., 2015). Some of these species have also shown the ability to survive until the end of the fermentation (Pina et al., 2004; Combina et al., 2005; Viana et al., 2008).

Most of these strains, however, lack competitiveness under oenological conditions such when compared to the traditional wine yeast strains (Beyl et al., 2013; Corderdao-Bueso et al., 2013; Maturan et al., 2015; Myiona et al., 2016). They are therefore used in combination with the S. cerevisiae (Canonico et al., 2016; Medina-Trujillo et al., 2017; Whitener et al., 2017) (Schuller and Caal, 2005; Comitini et al., 2011; Milanovic et al., 2012; Contreras et al., 2015; Morales et al., 2015; Lencioni et al,. 2016; Ciani et al., 2016 a&b; Varela, 2016; Wang et al., 2016). Studies have shown the contribution of wine-related in solving other wine defects (Ciani et al., 2016a; Pérez-Torrado et al., 2017 a&b) but there is limited information on the contribution of wine-related strains towards reducing the formation haze and pinking of white wines as a possibility. There is not enough information concerning pinking as its causes and treatment are still a matter of speculation and hypotheses. This study will, therefore, explore the potential of several wine-related yeast strains from several species to prevent visual defects.

Problem Statement.

Wine protein haze formation and pinking are common visual defects that remain a challenge to winemakers and wine consumers. Wine-related Saccharomyces yeast strains have previously been shown to significantly impact on protein haze formation in white wines (Ndlovu et al., 2018). No data, however, exist on whether other wine-relevant non-Saccharomyces yeast might have similar impacts. Also, there is little information about whether these yeast species or strains can affect the pinking of white wines.

1.1 Objectives of the study

Two objectives of this study are:

1. To screen wine-related yeast strain's ability in reducing pinking and wine protein haze at the end of alcoholic fermentation and in wines.

2. To investigate chitin and mannoproteins content in yeast cell wall during fermentations

5

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Chapter Two

Literature Review

Visual defects of white wines: Protein haze and pinking

Introduction

In the wine industry, it is imperative for a wine to be appealing to consumers (Van Sluyter et al., 2015). Visual defects such as pinking and protein haze are significant problems and lead to consumer rejection of affected wines. White wine pinking is defined as an alteration in colour from the typical white wine colour of yellow-white to a pinkish blush and is hypothesized to be due to sudden exposure to oxygen (Bennett et al., 2011; Lamuela-Raventos et al., 2001). The scientific literature that exists is based on speculations and hypotheses pinking, but there is nothing conclusive (Andrea-Silva et al., 2014). Wine protein haze is due to the precipitation of protein aggregates in the wine resulting in the formation of visible haze in mostly white wines (Batista et al., 2009; Tabilo-Munizaga et al., 2014; Van Slutyer et al., 2015). The proteins that are responsible for the formation of haze will be further described in the literature. The literature review will describe the strategies that have been used to treat wine protein haze, and white wine pinking and evaluate alternatives of the chemical fining agents that are currently used by wine producers.

1. White wine pinking

Pinking is the alteration of the natural colour of white wines to a pinkish red colour (Simpson, 1977; Lamuela-Raventos et al., 2001). Pinking has been observed and reported in wines produced from Vitis vinifera L. grapevine varieties such as Sauvignon blanc, Colombard, Chenin blanc, Chardonnay, Viognier, Crouchen, Muscat Gordo Blanco, Palomino, Riesling, Semillon, Sultana, and Thompson Seedless (Simpson, 1977; du Toit et al., 2006; Marias, 1998; Lund et al., 2009). Those mentioned above also shows extreme sensitivity to oxygen exposure and contain a variety of aroma compounds sensitive to oxidation, including excessive amounts of the light-sensitive aroma compound 2-methoxy-3alkylpyrazines, also known as methoxypyrazines (Marais, 2005; Scheiner et al., 2009; Andrea-Silva et al., 2014; Wendorff, 2006). Methoxypyrazines causes grapes and the resulting wine to be more susceptible to oxidation (Marais, 2005). Oxygen-sensitive wines may oxidize very rapidly following the slightest uptake of oxygen; the wines therefore become pink. Pinking does not affect other sensorial characteristics such as aroma and flavor (Andrea-Silva et al., 2014). The phenomenon nevertheless has a severe impact on wine consumers as stated by Simpson (1977). Jones (1989) notes that in worst cases of pinking, leading to commercially rejection.

1.1 Factors that influence white wine pinking

There is very little literature on wine pinking, while the available data is still inconclusive about the actual causes of this phenomenon (Lamuela-Ravento et al., 2001; Andrea-Silva et al., 2014). Some

chemical compounds and other factors have been proposed to be responsible for white wine pinking (Lamuela-Raventos et al., 2001). Different compounds and polymeric materials (Jones, 1989; Andrea-Silva et al., 2014) may cause the colouration of the wine. Oenological conditions affecting pinking:

Oxidation: One hypothesis proposes that white wine pinking is linked to browning, which generally is a result of oxidation of the wine (Simpson, 1977; Vaimakis and Roussis, 1993; Lamuela-Raventos et al., 2001; Escudero et al., 2002; Clark et al., 2008). Browning and pinking are two different occurrences. Both occur because of contact with or exposure to air or atmospheric oxygen; however, browning may occur in wine in the absence of pinking (Simpson et al., 1983; Lamuela-Raventos et al., 2001). In some white wines, where both phenomena have been observed, pinking is observed after slight exposure to oxygen, whereas browning is occurring after further oxidation (Singleton, 1987). Therefore, the precursor of the pink chromophore is the first component to oxidize and does so before browning. It has been shown that pinking may also occur without the subsequent browning of wines (du Toit et al., 2006; Andrea-Silva et al., 2014). The exposure of wines to oxygen results in the absorption of relatively high amounts of oxygen by mainly phenolic compounds (Waterhouse and Laurie, 2003; Oliveira et al., 2011). It has been shown that Sauvignon blanc wines that are prepared under no controlled maceration have increased polyphenols levels compared to wines that are prepared under controlled maceration techniques (Olejar et al., 2015). These include cryogenic maceration, which minimizes the loss of aroma compounds (Hernanz et al., 2007), and semi-cryogenic maceration (Gawel et al., 2014).

Phenolic compounds: Pinking is thought to be caused by the fast reaction of flavens to red flavylium salts in the presence of oxygen (Zoecklein et al., 1998). Andrea-Silva et al. (2014) highlighted malvidin3-O-glucoside as the most predominant anthocyanin in pinked wines derived from Siría grapes. Literature suggests that Siría white grapes can synthesize anthocyanins in their skin even though they are synthesized in relatively small amounts as compared to red grape varieties (Fournier-Level et al., 2010; Clifford, 2000; Wu et al., 2014). This observation, however, does not apply to all white grape cultivars (Andrea-Silva et al., 2014).

Other factors: Ascorbic acid reacts with oxygen in the wine resulting in the production of H2O2 (Skouroumounis et al., 2005). The H2O2 and ortho-quinones are known to participate in further reactions that may cause a detrimental impact on the colour, and aroma of white wine (Singleton, 1987; Peng et al., 1998; Bradshaw et al., 2003). Hydrogen peroxide is used to induce pinking in white wine when

determining the pinking potential of wine according to Simpson et al. (1983), the pinking potential of wines is dependent on the concentration of free sulphur dioxide in the wine, and it is therefore imperative that adequate levels of free sulphur dioxide be maintained. To detect and induce the pinking potential of hydrogen peroxide is used to induce the pinking potential as described by Iland et al. (2000). The test allows for a quantitative measure of a possibility of pinking in the wines upon the addition of hydrogen peroxide.

2. Wine protein haze

Wine proteins tend to become insoluble and precipitate which leads to a visible haze in white wines during storage (Van Sluyter et al., 2015; McRae et al., 2018). Residual proteins are essential during the production of wines but also responsible for commercially unacceptable haze or deposits during the bottling of wine (Ferreira et al., 2002; Waters et al., 2005). These proteins are referred to as pathogenesis-related (PR) proteins (Tian et al., 2015) and are responsible for haze (Van Sluyter et al., 2015; Theron et al., 2017). Even though the concentrations are relatively low, these proteins have significant relevance in winemaking and can contribute to protein haze formation (Sauvage et al., 2009; Blasco et al. 2011; Marangon et al., 2014). Although turbid wines do not impose health risks, they are visually unappealing to consumers and an indicator of inferior quality to producers (Marangon et al., 2011).

2.1 Factors influencing the formation of protein haze in white wines

The formation of haze is a well-defined and characterized process. The occurrence of protein haze is dependent on physicochemical parameters and non-proteinaceous factors of the wine such as alcohol levels metal ions, pH, sulphate, ionic strength, polysaccharides and phenolic compounds (Pocock et al., 2007; Batista et al., 2010; Marangon et al., 2011; Dufrechou et al., 2012; Gazzola et al., 2012; Pocock et al., 2007; Van Sluyter et al., 2015). Other factors include; pathogenic fungi such as Botrytis cinerea (Ribéreau-Gayon et al., 2006; Ferreira et al.,2002), grape cultivar (Hayasaka et al., 2001), vintage (Monteiro et al., 2003), disease pressure and even harvest seasons (Pocock et al., 1998). During fermentations, the fermenting microorganisms can result in a complex protein mixture (Kwon, 2004; Marangon et al., 2001; Vincenzi et al., 2011) that can cause haze if not well cleared after fermentations. Wine protein haze caused by proteins in wine remains a significant problem for the wine industry and requires costly treatments. With the vast literature on protein stability that is available, there still is not enough on the absolute protein concentrations at which wines will remain protein stable (McRae et al., 2018).

2.1.1 Proteins

The major proteins that are responsible for the formation of haze in white wines are the pathogenesis-related proteins (Vincenzi et al., 2005; Esteruelas et al., 2009; Sauvages et al., 2010; Tian et al., 2015). 2.2.1. Although the proteins and peptide form minor constituents of the wine, they contribute towards the quality of the final product (Flamini and De Rosso, 2006).

2.1.2 Pathogenesis-Related Proteins

Pathogenesis-related proteins are proteins in Vitis vinifera grape juices and wines and are present at low concentrations, between 10 mg/L and 500 mg/L (Waters et al., 2005). The pathogenesis-related proteins are essential to grape berries, as they are produced during pathogenic related stress as a defense mechanism. They can survive the harsh condition of fermentations and remain soluble in wine, and at later stages, they result in the formation haze (Van Sluyter et al., 2015). Pathogenesis-related proteins are secreted by different plants and their differences from one another but thaumatin-like proteins (TLP) and chitinases found in grape berries, skin and pulp they are of importance towards the formation of haze (Tian et al., 2015). They are also induced in non-pathogenic stress-related conditions such as the cytoplasm separation and elevated concentration of plant hormones (Wagih and Coutts, 1981; Antoniw et al., 1981). The concentration of these proteins in the grape differ. They exist in different forms, and not all PR-proteins are found in the Vitis grapes, the table above summarizes the groups and their characteristics.

Table 2.1: Characteristics of pathogenesis-related proteins found in Vitis grapes (Enoki and Suzuki,

Family	Properties	Function/ target site	References
PR-1	Antifungal Unknown		Bertsch *et al.,* 2003
PR-2	β-1,3-Glucanase	Cell wall (β-1,3-glucan)	Mauch *et al.,* 1988; Fujimori *et al.,* 2016; Akiyama *et al.,* 2004
PR-3	Chitinase (types I, II, IV, V, VI, and VII)	Cell wall (chitin)	Enoki and Suzuki, 2016
PR-4	Chitinase (types I and II)	Cell wall (chitin)	Enoki and Suzuki, 2016
PR-5	Thaumatin-like	Plasma membrane	Jacobs *et al.,* 1999; Stintzi *et al.,* 1991; Roberts *et al.,* 1990; Jayasankar *et al.,* 2003
PR-10	Ribonuclease (like)	RNA	Liu *et al.,* 2006; He *et al.,* 2013; Fujimoto *et al.,* 1998; Gonneau *et al.,* 2001; Jelloili *et al.,* 2010

PR-14	Lipid-transfer protein	Involvement in defense signaling pathway	Grant and Lamb (2006); Buhot et al., 2004; Girault et al., 2008
PR-15	Oxalate oxidase	Production of H_2O_2 with	
PR-16	Oxalate oxidase-like protein	Antimicrobial activity	Godfrey et al., 2007

A study by Tian et al. (2015) quantified the distribution of the PR proteins in Sauvignon blanc grapes berries using HPLC and the concentrations and was observed that the skin contains more proteins as opposed to the pulp of the berry. That results obtained were higher than the ones that were obtained from a study by Pocock et al. (1998), but the trend was the same. However, the concentration of the PR protein in juice is determined by their concentration in the pulp (Tian et al., 2015) because the skins are removed during the first steps of winemaking

Table 2.2: Quantification of PR proteins in Sauvignon blanc (Tian *et al.,* 2015)

Sample	TLP (mg/L)	Chitinases (mg/L)
Skin	581.8	442.4
Pulp	275.1	248.2

2.1.2.1 Chitinases and Thaumatin like proteins

Chitinases are proteins with a low molecular weight, such as glycosyl hydrolases with molecular weight ranges of 20 kDa to about 90 kDa (Bhattacharya et al., 2007; Falconer et al., 2010) and are sensitive to changes in temperature and pH (Dufrechou et al., 2013). Chitinases is a glycosyl hydrolase enzyme that hydrolyzes chitin (Ong et al., 2017). Chitinases impart a significant role in wine haze since they can easily precipitate and a linear correlation between chitinases content in wine and haze formation was found as described by (Marangon et al., 2010; Marangon et al., 2011b; Ndlovu et al., 2018). In grapevines, there at least 13 different chitinases isoforms found in different tissue forms, mostly located in the grape berries. (Tian, 2014). During storage, chitinases are the one that is responsible for haze formation in white wines (Gazzola et al., 2016; Vincenzi et al., 2015). The unfolding of chitinases is irreversible, once they do denature they cannot regain their structure again (Van Sluyter et al., 2015) and this results in the formation of precipitates that then lead to the formation of haze. The thaumatin-like protein family is characterized principally by its thermostability and by showing no significant conformational changes (Dufrechou et al., 2013). Toledo et al. (2016) in their study of the theoretical approach to understanding the haze phenomenon concludes that TLP complexation with phenolic

15

compounds is also assumed to result in the haze that is observed in bottled wines. An observation was made, that the proteins were only increasingly becoming stable as the wine pH was increased to 7.5 (Mesquita et al., 2001). The concentration of TLPs after fermentations was reported to decrease and only 60% from the initial juice concentration of PR proteins remain in Sauvignon Blanc wines (Manteau et al., 2003; Marangon et al., 2009; Le Bourse et al., 2011).

The difference of temperatures required by chitinases and TLPs to unfold, aggregate and precipitate is explained by Marangon et al. (2010). The TLPs have different isoforms that differ concerning unfolding temperatures resulting in other TLPs being heat-unstable while some are heat-stable and forming haze wines (Gazzola et al., 2012; Marangon et al., 2014). In the wine, the classes of haze-forming proteins have different temperatures at which instability occurs. It has been that the TLPs are more stable than the chitinases, their unfolding temperatures differ (61-62°C and 55°C) respectively (Falconer et al., 2010). In a study by Tian et al. (2017) an observation was made that the decrease of chitinases was more significant that of TLPs due to irreversible denaturation of chitinases. According to Falconer et al. (2010), at temperatures higher than 40°C, the chitinases can only remain stable for hours while TLPS can remain stable for at least 20 days. Both the chitinases and TLPs have different aggregate characteristic and tendencies. It was also observed that the stable TLPs that have a reversible unfolding and this is not true for unstable TLP isoform will remain denatures and they are responsible for the formation of haze (Gazzola et al., 2012; Dufrechou et al., 2013; Marangon et al., 2014).

Table 2.3: Summary of general properties of chitinases and TLPs (van Sluyter *et al.,* 2015).

Properties	Chitinases	Stable TLPs	Unstable TLPs
1. Unfolding temperatures	55°C [1]	61-62°C [1]	*56°C [1]*
2. Aggregate characteristics	Visible aggregates (≥1µm) [2]	Micro aggregation (≥150nm) [2,3]	Visible aggregates (≥1µm) [1,5]
3. Aggregation tendencies	Self-aggregate [3,2]	Cross-linked with other wine components [2,4]	Self-aggregate [5]

[1] Falconer *et al.,* 2010
[2] Marangon *et al.,* 2011
[3] Marangon *et al.,* 2012
[4] Gazzola *et al.,* 2012
[5] Marangon *et al.,* 2014

2.2 Mechanism of protein haze formation in white wine

The mechanism of the protein haze formation in the past decade was described as a two-step mechanism; where proteins are denatured by heat followed by the aggregation of denatured proteins (Pocock et al., 2007; Dufrechou et al., 2010). Batista et al. (2009) indicated that there are two mechanisms responsible for the heat-induced precipitation of wine proteins; (i) step one occurs to high pH values, resulting in reduced protein solubility at its pI and (ii) others occur at lower pH values also at other values, depending on the X factor, sulphate (Batista et al., 2010). Pocock et al. (2006) & Marangon et al. (2010a) proposed the theory of wine haze formation and later revised by Van Sluyter et al. (2015).

The mechanism of haze formation is now recognized as a multifactorial process that follows three steps that proceed as follows: During storage under relatively high temperatures. The haze was forming protein get denatured, the hydrophobic binding sites hidden in the core of the protein structure are, therefore, exposed (Marangon et al., 2010). According to Marangon et al. (2014), this step is correct for the thaumatin-like proteins, which have been deemed responsible for the protein haze in wines. Self-aggregation of proteins, during the second step: the proteins begin to aggregate via the exposed hydrophobic binding sites and the ionic strength of the wine is modified further promoting the aggregation of proteins (Marangon et al., 2010). The last step; the aggregates become cross-linked through sulphites, salts, metals, and phenolics (Van Sluyter et al., 2015). Chitinases and unstable TLPs each can self-aggregate via exposed binding sites, which then leads to the formation of visible aggregates (Falconer et al., 2010; Marangon et al., 2011; Marangon et al., 2012; Marangon et al., 2014). The protein will gradually continue to aggregate and reach a size of (>1µm) that is visible and start precipitate and resulting in a hazy wine (Marangon et al., 2011; Marangon et al., 2012; Dufrechou et al., 2013).
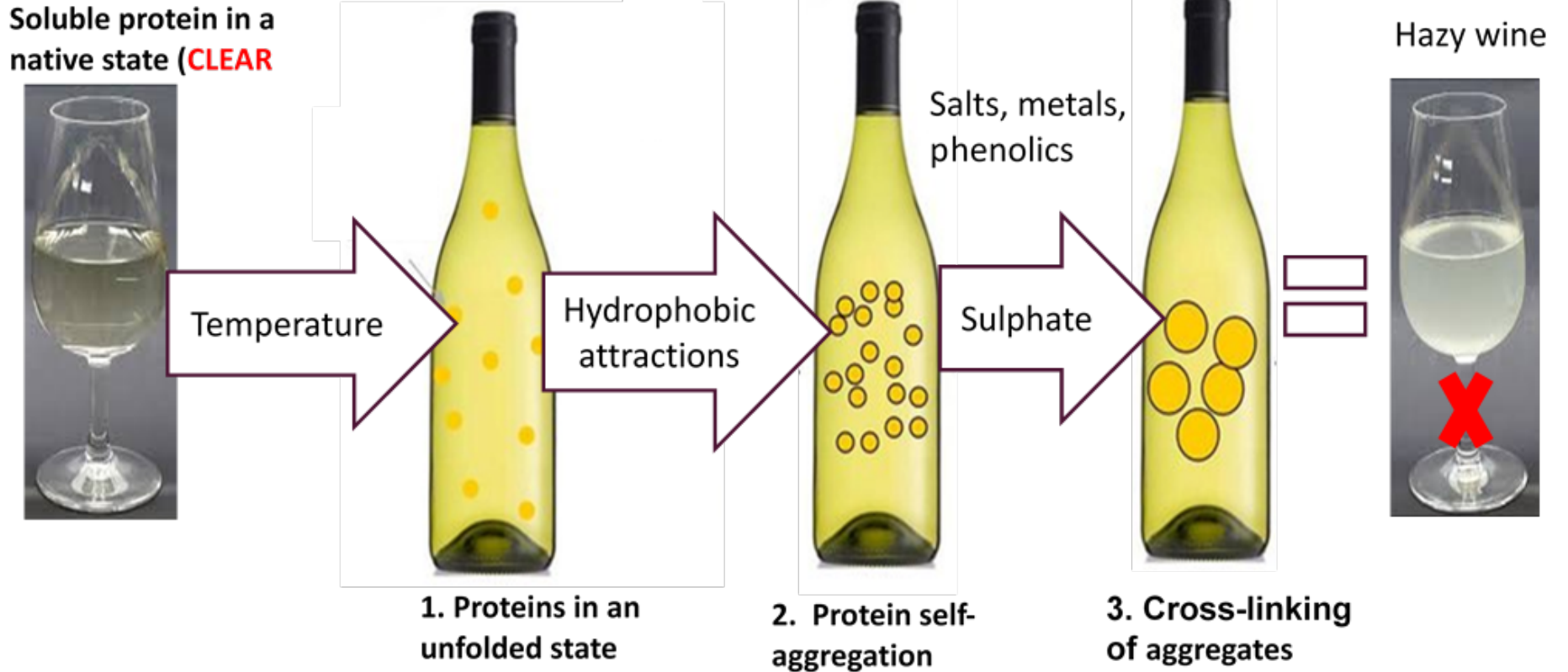
Figure 2.1: The mechanism is adopted from van Sluyter *et al.* (2015). This is the revised mechanism of haze formation, which occurs through three sequential steps. The mechanism is now recognized as a multifactorial process. The first step is the denaturation of the wine proteins exposing their hydrophobic binding sites, followed by the second step where the proteins use the exposed hydrophobic binding sites to self-aggregate and the third step is a further aggregation of the aggregate resulting in haze.

2.3   Currently used treatments for wine pinking and haze formation

Protein stability is achieved through the addition of bentonite, which is a fining agent (Vincenzi et al., 2005b). Bentonite is the most commonly used fining agents, and there is vast literature that is available based on its mechanisms and conditions. Other measures have also been explored as alternatives to bentonite. These include the use of, enzymes such as acid protease (Theron et al., 2017). The yeast cell wall properties have also been used to be explored for clarity and colour stabilization (Vincenzi et al., 2005; Cilindre et al., 2008; Gómez-Pastor et al., 2010), overexpression of the haze protective factors such as mannoproteins (Feuillat, 2003; Waters et al., 2005; Brown, 2007; Palmisano et al., 2010; Tabilo-Munizaga et al., 2014). The role of yeast cell wall chitin (Ndlovu, 2012; Ndlovu et al. 2018). Reported pre- or post-pinking treatments for the removal of pink materials and their precursors in must/wine utilize fining agents (such as nylon, casein, and PVPP). Antioxidants (such as ascorbic acid), or chelating agents (such as diethyldithiocarbamate), as well as mannoproteins, and dehydrated yeast cells (Lamuela-Raventos et al., 2001; Andrea-Silva et al., 2014).

2.3.1 Proteolytic enzymes

Pocock et al. (2003) demonstrated that after proteins have been denatured at 90oC, they are prone to degradation by proteolytic enzymes; the enzymes have little to no negative impact on sensory properties of wine are minor. Marangon et al. (2012) used an enzyme aspergillopepsin (I) and (II) to degrade white wine haze proteins, which can tolerate acidic pH and the fermentation process. For better enzyme, clarification the grape must be flash pasteurized for 1 minute at 75oC before excellent results could be obtained. A study that was conducted in synthetic grape must by Theron et al. (2017) using extracellular aspartic protease (MpAPr1) secreted by *Metschinikowia pulcherrima,* showed to be activity against chitinase without flash pasteurization. The enzyme was able to reduce the levels of haze, which showed that aspartic protease could be an alternative for bentonite. It is recommended that the enzyme should be added to the grape must prior fermentations, so that the fermentation and enzymatic reactions could take place simultaneously (Moreno Arribas and Polo, 2005). However, this is not a cost-effective alternative for bentonite and requires detailed kinetic characteristics (Schlender et al., 2017; Theron et al., 2017; Theron et al., 2018). Those as mentioned above still set limitations for the wine producers due to the conditions that the authors employed while conducting the experiments, they were not conducted under conditions that mimicked the wine producers set up. The study is based on not changing the matrix of the grape must and following the same procedures that are followed by the winemaker.

2.3.2 Polyvinylpolypyrrolidone

Polyvinylpolypyrrolidone (PVPP), a water-based insoluble polymer that is used to remove browning and possible pinking in pinked wines (Tobe, 1983; Lamuela-Raventos et al., 2001; Andrea-Silva et al., 2014). The mode of action of PVPP is through hydrophobic binding that selectively binds polyphenols, flavens and mono-and dimeric phenolics from wines. The mode of action of PVPP concurs with the speculation of possible causes of pinking, previously stated, the fast reaction of flavens to flavylium. The removal of flavens would, therefore; results in reduced pinking potential in wines. The amount of PVPP used is dependent upon the number of phenolics to be removed (Donel et al., 1993; Spagna et al., 2000; Andrea-Silva et al. 2014). It has been shown that an average amount of 10-40g/L and a maximum of 80g/L can be used for clearing white wine pinking (Rankine, 2004; Ribereau-Gayon et al., 2006). It was also found that a combination of PVPP with ascorbic acid could be used for reducing white wine pinking and at concentrations of 45 mg/L (Lamuela-Raventos et al., 2001).

2.3.3 Bentonite fining

Bentonite comprises of 70–80% of montmorillonite, hydrated aluminium silica with exchangeable cationic components: $(Al, Fe, and Mg)Si_4O_{10}$ (Trigueiro et al., 2018). Montmorillonite clay has a multilayer structure of alumina hydro silicate forming platelets (Sauvage et al., 2010; Makhoukhi et al., 2009). The addition of bentonite is universally used in the winemaking industry to prevent the formation of protein haze in wines (Hoj et al., 2001; Ferreira et al., 2002; Trigueiro et al., 2018). The process is achieved through cationic exchange capacity of bentonite clay (Sauvage et al., 2010; Lambri et al., 2012; Hung et al., 2013; Jaeckels et al., 2015; Dordoni et al., 2015). The convalescing of wine from the bentonite lees by filtration results in more disadvantages such as the loss of flavors leading to inferior quality wines (Lambri et al., 2010; Ndlovu, 2012).

Besides, the use of bentonite for correcting protein haze results in loss of wine volume of about 310% as bentonite lees (Brown et al., 2007). The loss of wine with bentonite is estimated to cost the global wine industry around $1 billion per year (Majeweski et al., 2011). Other risks include costs involved in tank downtime during the treatment, occupational health risk associated with the inhalation of bentonite and slips hazards induced by bentonite slurry spills (Armada and Falque, 2006; Salazar et al., 2007). It was found that bentonite was not selective and removed all proteins and other wine components that contribute to wine aroma (Ferreira et al., 2002; Moio et al., 2004; Lambri, 2010). Lambri et al., (2012) by using five different types of sodium bentonite showed that different labels of bentonite could be used

to remove the proteins responsible for wine turbidity selectively. However, bentonite is still the most commonly used clarifying agent despite its drawbacks that it imposes on the wine quality.

2.4   Microbiological solution: Wine-related yeast strains

The presence of wine-related yeast strains has been reported in alcoholic fermentations (Jolly et al., 2014; Borneman et al., 2013; Taillandier et al., 2014; Jolly et al., 2017); however, they to lack competitiveness under oenological conditions compared to S. cerevisiae. The importance of the wine-related yeast strains have been well studied (Ciani et al., 2010; Domizio et al., 2014; Ciani et al., 2016; Ong et al., 2017), they mainly contribute during the early stages of fermentation (Fleet, 2008). The wine-related yeast strains are of importance to wine producers due to their metabolic properties whose features differ from S. cerevisiae (Romano et al., 1997; Fleet, 2008; Medina et al., 2018). These include enzymes that are secreted by the yeasts; the secondary metabolite that interacts with the components of the grape must (Medina et al., 2018).

It has been shown that the wine-related yeast strains can stabilize the colour of wine (Morata et al., 2012; Bennito et al., 2014). The few studies that have been conducted with the focus of reducing haze formation and colour stabilization in wine have only focused on the use of *S. cerevisiae* strains. These include studies by; Brown et al. (2003), Vicenzi et al. (2005a&b), Brown et al. (2007), Ndlovu (2012) and Ndlovu et al. (2018). Studies by Theron et al. (2017), Schalnder et al. (2017) and Theron et al. (2018) explored the importance of extracellular enzymes (proteases) secreted by wine-related yeast strains, such as *Metschinikowia pulcherrima* and *Wickerhamomyces anomalus* in degrading protein that forms a haze in wines through hydrolysis, where the proteins are cleaved by aspartic proteases resulting in reduced haze. The importance of yeast cell wall derived polysaccharides have been shown to improve wine clarity through (Comitini et al., 2011; Giovani et al., 2012; Domizio et al., 2014) and colour stabilization (Benito et al., 2011; Morata et al., 2012; Benito et al., 2014). The stabilization of colour has been shown through the interactions of polyphenols and mannoproteins in red wine, but none has been shown in white wine. The interactions will further be discussed under mannoproteins.

The contribution wine-related yeast strains towards improved wine quality based on the yeast cell wall properties is not explicitly described. The microbiological approach is motivated by the difference between *S. cerevisiae* and wine-related yeast strains that have shown that wine-related yeast strains produce high polysaccharides as compared to *S. cerevisiae* strains (Romani et al., 2010), which suggested that they are differences between the yeast cell wall of *S. cerevisiae* and wine-related strains. Another

study by Moore et al. (2015) concurs, supported these finding, and reported on the differences, the polysaccharide content of the wine-related yeasts. It, therefore, serves the purpose of the study to explore the use of the wine-related yeast strains for removal of chitinases and thaumatin-like proteins while also reducing the pinking potential of white wines based on the previously stated speculations. Very little information that is involved in the use of wine-related yeast cell wall polysaccharide for haze formation and pinking potential of white wine. The polymeric wine material includes polysaccharides, protein, and phenolic compounds which interact with anthocyanins (Gonçalves et al., 2012; Gonçalves et al., 2018). Therefore, the following section of the literature investigates the contribution of the yeast cell wall polysaccharides.

2.4.1   The yeast cell wall architecture

The cell wall is a dynamic structure whose integrity adapts based on the availability of carbon source, nutrients, oxygen availability, temperatures and external pH conditions (Aguilar-Uscanga and Francois, 2003; Schiavone et al., 2014). The cell wall of Saccharomyces cerevisiae comprises of 85% polysaccharides and 15% proteins, composed of $(1\rightarrow3)$-β-glucans containing branches of $(1\rightarrow6)$-βlinked D-glucans, chitin, α-mannoproteins, and proteins (Giese et al., 2016). The composition of the yeast cell wall is strain dependent, (Domiizio et al., 2014). Under stress conditions, the yeast cell wall is remodeled (Klis et al., 2006). The remodeling include an increase in chitin fractions (Popolo et al., 2001) an increase in the amount of other several cell wall proteins transient re-distribution of β-1,6-glucan synthase complex the cell resulting in changes in the cross-links between cell wall polymers (Jung and Levin, 1999; De Nobel et al., 2000).

Table 2.4: Composition of the yeast cell wall (Aguilar-Uscanga and Francois, 2003; Klis *et al.,* 2002, 2006)

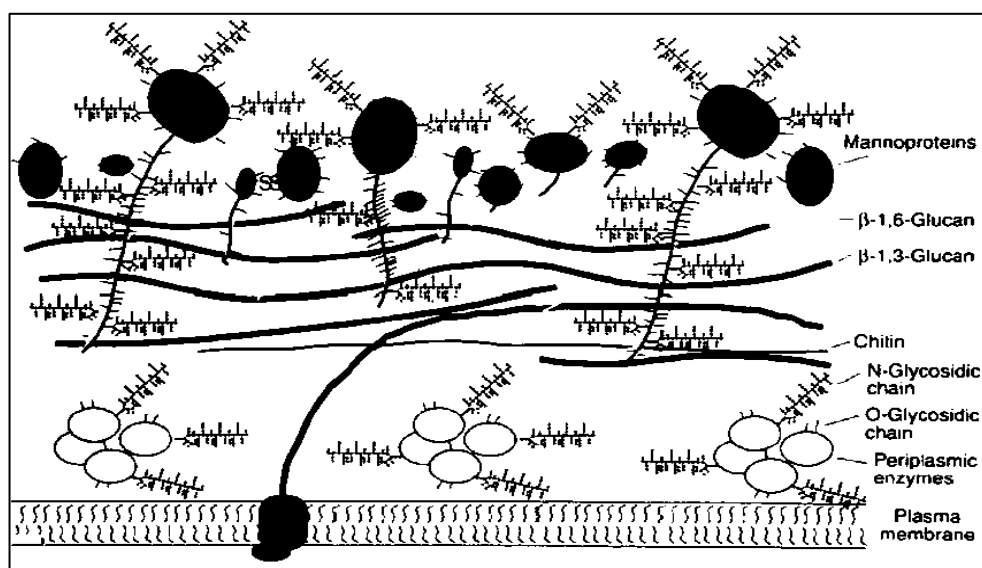| Macromolecules	% of cell wall dry weight	Degree of polymerization	Average Molecular weight	Level of branching
Mannoproteins	30-50	Highly variable	Highly variable	high
B(1,3)	5-10	150	24	moderate
B(1,4)	30-45	1500	240	high
Chitin	1.5-6	120	25	linear

Figure 2.2: Composition and structure of the yeast cell wall adapted at (Schrueder *et al.,* 1996). The figure shows the connection of the yeast cell wall polysaccharides and proteins and where component is located in the cell wall.

2.4.2   Chitin

Chitin is a polysaccharide, linear and poly-β - (1, 4)-N-acetyl-D-glucosamine (Vani and Stanley, 2013; Melida et al., 2015). Chitin is the second most abundant biopolymer on earth after cellulose (Xu et al., 2013, Hamed et al., 2016). Chitin has an acetamide group (NH-CO-CH3) at the C-2 (Thirunavukkarasu et al., 2011; Muthukrishnan et al., 2018). It is found in the various source, and various microorganisms produce chitin in the cell wall (Jothi et al., 2012; Sharp, 2013; Hamed et al., 2016). Moreover, chitin is a substrate for the action of chitinases, which has a chitin-binding domain, which allows it to be able to bind directly to the polymer. This study has been explored, focusing on the crucial role of chitinases in the formation of haze (Waters et al., 1996). Both chitin and chitosan have been receiving attention as valuable biopolymers due to their contributions in biotechnology (Philibert et al., 2016).   Vincenzi et al. (2005b) then explored the properties of chitin; they used commercial chitin to reduce the formation of haze in wines. The study showed that the addition of 20 g\L of chitin reduced about 80% of the total haze in unrefined wines, while 1 g/L only reduced 50% of the haze formed. The study was then further compared to bentonite; a dosage of 0.5 g/L can eliminate haze while a very high dosage of chitin can only partially remove the haze. (Vincenzi et al., 2005).

In a study that was conducted by Ndlovu et al. (2018) focused on the possibility that the yeast cell wall chitin may also be able to bind the grape chitinases and reduce the formation of haze. The data that was obtained from the study suggested novelty as the yeast cell wall that had high chitin levels were able

23

to reduce the formation of haze. The study was conducted using yeast from the genus Saccharomyces, and the relation between the amount of chitin and haze reduction is not expected to hold for wine-related yeast strains due to the previously mentioned differences. There is very little to no evidence that indicates the possibility of thaumatin-like proteins having the same chitin-binding properties as opposed to chitinases (Vincenzi et al., 2005).

2.4.3   Mannoproteins

Mannoproteins are major polysaccharide groups (Feuillat, 2003) that are found in wines, have one to four residues of mannose linked by α-(1→2) or α-(1→3) linkages (Pérez-Serradilla and Luque de Castro, 2008), and form part Saccharomyces cerevisiae yeast cell wall proteins (Klis et al., 2002). Mannoproteins are released from the yeast cell wall during autolysis (Blasco et al., 2011; Vincenzi et al., 2014) and are they vary between species and even strains (Martinez-Rodringuez and Pueyo, 2009; Blasco et al., 2011; Capese et al., 2018). The concentration of mannoproteins produced by yeast during wine production is relatively low to have any significance for commercially use (Dupin et al., 2000b; Feuillat, 2003) and their concentration ranges from 100-150 mg/L (Chalier et al., 2007). Mannoproteins compete with wine components and protein for aggregate formation with denatured protein to either form haze or reduce haze (Giese et al., 2016).

An establishment of the protective effect of mannoproteins was by Ledoux et al. (1992) who demonstrated that the fragments of mannoproteins isolated from yeast invertase reduced the incident of haze in white wine (Waters et al., 1993, 1994a; Dupin et al., 2000). The impact of adding commercial mannoproteins in wine has been explored as a technological adjuvant to improve the quality of wine (Feuillat, 2003; Caridi, 2006). Commercial mannoproteins have the same effect and improve the phenolic composition and organoleptic properties of the wine (Ramos-Pineda et al., 2018). The commercial mannoproteins have been shown to reduce haze in white wines. Waters et al. (1994a) isolated the haze protective factors (HPF) in mannoproteins isolated from Carignan Noir wine and were referred to as Hpf1p and Hpf2p (Stockdale, 2000; Brown, 2003) (haze protecting factors).

The action of the haze protecting factors was shown to be active towards the reduction of haze formation in wine. Brown et al. (2007) cloned and overexpressed YOL155c and YDR055w in laboratory *S. cerevisiae* strains, they encoded the haze protecting factors Hpf1p and Hpf2p respectively, and the use of Hpf2p for reduction of haze showed a reduction of 40%. Mannoproteins are known for reducing haze in white wines (Waters et al., 1994a) and for interacting with some wine aromas such as polyphenols

(Gunuta et al., 2007). The competition between and proteins results in polyphenol stabilization in red wines. The mannoproteins encapsulate polyphenols formation complexes via the hydrophobic pockets created by mannoproteins (Mateus et al., 2017).

The polarity nature of hydrophilic and hydrophobic nature of the yeast cell wall allows the interaction and absorption of the wine components, such as polyphenols and pigments (Lubber et al., 2003; Bzducha-Wróbel et al., 2018; Li, & Karboune, 2018). Studies have shown possible importance of the interaction of mannoproteins and polyphenols concerning colour stability in wines (Guadalupe & Ayestarán, 2008; Guadalupe et al., 2007, 2010). During the vinification process of the wines, yeast strains play an important role and influence the profile of anthocyanins and their derivatives (Medina et al., 2005; Monagas et al., 2007; Valentao et al., 2007). Andre-Silva et al. (2014) suggested that anthocyanins are present in white wines, however, in low amounts, which suggests the possibility of reduction of pinking through mannoproteins.

Conclusion and Future prospects

The counteraction of wine defects, haze formation, and pinking are essential to a winemaker. There is still very little known about the cause of white wine pinking, but many hypotheses have been suggested. The study has been motivated by the developments towards the use of wine-related yeast strains towards improved wine quality. The winemakers use fining agents such as bentonite and or PVPP to prevent or reduce the formation of haze and pinking in white wines. However, the use of these agents harms the quality of the wine. Studies have been equally distributed in improving the quality of the wine, through exploring possible alternatives that can be used for winemaking (Van Sluyter et al., 2015; Ciani et al., 2016; Tian et al., 2017; McRae et al., 2018; Ndlovu et al., 2018). Wine-related yeast strains have shown to contribute significantly towards improved wine quality through the contribution towards other aspects (Jolly et al., 2014; Jolly et al., 2017).

There is still no scientific literature review about the wine-related yeast and their contribution towards haze formation. Based on the existing literature that has been reviewed the study will employ techniques and biological methods that will show how the yeast cell wall can be used alternative for bentonite. The yeast cells walls ability to bind to the precursors of the above discussed visual defects will be evaluated. The evaluating will predict a possible influence on haze reduction and possible on the prevention and reduction of the pinking potential. The yeast cell wall properties that are involved in reducing haze formation and possible pinking will therefore also be evaluated during fermentations. At

the end of the fermentations, the influence of both the chitin and mannoproteins will be evaluated to not any differences. This information will then be linked to either the strains can be used as an alternative or cannot be used. The study is set up in a manner that mimics the conditions of an industrial set up to prevent any exposing the must to any changes. The study aims to explore the properties of the wine-related yeast cell wall from different species as alternatives to the use of fining agents aimed at improving wine clarity and preventing pinking of white wines. A total of 62 strains were screened for their influence but the study will only give the strains that showed a positive influence on the visual defects. The following Chapter Three, shows all the microbiological techniques that have been used to evaluate these strains.

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28

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Chapter Three

Materials and Methods

3.1   Strains and inoculation preparations

The wine related-yeast strains that were used from the study were obtained from the Institute of Wine Biotechnology, Stellenbosch University yeast culture collection (Table **3.1**). Strains that were used were selected the IWBT strains collection after they had been screened based on their chitin levels using the flow cytometry BD FACS Aria.

(a) Screening for strain with minimal chitin levels

Various wine-related yeast strain from the IWBT yeast collection were screened using a method adapted from de Groot et al. (2001) and Ndlovu (2012). The yeast cells were grown in yeast extract peptone dextrose (YPD) agar plates and incubated at 30^0C for 24 hours. Cells were inoculated in a 5 mL YPD broth and pre-cultured in a 5 mL YPD broth at 30^0C for 5 hours and collected by centrifugation at 11000 rpm/ 4^0C/ 10 min, washed with 500 µL of 1× PBS with pH 7.4 and the yeast cells were suspended in 350 µL of PBS. PBS contained 8.01(g/L) NaCl, 0.20 KCl, 1.44 $Na_2HPO_4.2H_2O$ and 0.27 KH_2PO_4 all in (g/L). Following the manufacturer's instruction, the cells were stained with approximately 20 µl of Calcofluor white stain (catalog number18909, 100 ml; Fluka Analytical, Sigma-Aldrich, composition calcofluor white M2R 1 g/L and Evans blue 0.5 g/L) after the addition of 20 µL of 10% KOH. The strains that were selected based on the screening were therefore used for the fermentations. Calcofluor white was used to selectively bind the chitin the yeast cell wall and followed by flow cytometry that allowed for a quantification of the fluorescence of calcofluor white stain, predicting the total chitin of the yeasts.

The total of strains that were screen was 62 yeasts, 60 wine-related yeast strains, and 2 *S. cerevisiae* yeast strains. The initial screening of the yeast strains amount of chitin and following the method started in 3.3. This allowed a clear prediction of prediction of the influence of strains towards haze formation and possible pinking. The quantification of total chitin and mannoproteins levels was at a maximum of 50,000 cells, and the quantification was done using BD FACS Aria Cell Sorter Flow cytometry equipped with BD FACS DiVa v8.1 software that captured the data. The fluorescence of stains is reported from the flow cytometry as arbitrary values in triplicates, the average is calculated, and the averages represented the total fluorescence. The excitation laser used was the solid-state sapphire laser at a wavelength of 405 nm for chitin, for mannoproteins 633 nm and the emission filters used was the FITC channel, with a 505 long pass and 530/30 for chitin and mannoproteins 660/20 bandpass filter.

Table 3.1: *Saccharomyces cerevisiae* and wine-related yeast strains used.

Species	*Species*	*Strain (IWBT codes)*	*Reference*
Saccharomyces	*S. cerevisiae*	BM45	Lallemand Inc. (Montreal, Canada)
Wine-related yeast strains			
Candida	*C. prunicola*	Y1162	IWBT Yeast Culture collection
	C. zemplinina	Y1082	IWBT Yeast Culture collection
Metschinikowia	*M. pulcherrima*	Y1063	IWBT Yeast Culture collection
	M. fructicola	Y1005	IWBT Yeast Culture collection
	M. pulcherrima	Y1094	IWBT Yeast Culture collection
Rhodotorula	*R. mucilaginosa*	Y1027	IWBT Yeast Culture collection
Cryptococcus	*Cr. oriensis*	Y872	IWBT Yeast Culture collection

3.2 **Expression and purification of vV Chitinases ivD in *E.coli***

The procedure was adopted from (Lee & Colman, 2007; Ndlovu et al., 2018). Transformed *E.coli* Rossetta 2(DE3) pLys with pET-chivD-GFP (Ndlovu et al., 2018)3.4 vector was used for the expression and purification of GFP protein. Luria Bertani agar (LB agar) and Luria Bertani broth (LB broth) were supplemented with 100 mg/mL of ampicillin and 34 mg/mL of chloramphenicol unless stated otherwise. All the incubations were carried out on a shaking incubator unless stated otherwise. The transformed E.coli Rossetta 2(DE3) pLys with pET-chivD-GFP vector was streaked on LB agar plates, and the plates were incubated at 37°C for 24 hours. From the plates a single colony was picked and inoculated on fresh 5 mL LB broth and incubated at 37°C, the culture is incubated to 5 hours and transferred to a fresh 100 mL of LB broth and incubated at 37°C for 24 hours. After 24 hours, the E.coli cell was harvested by centrifuging the culture at 6000 rpm for 10 minutes, and the pellet was retained and re-suspended into a fresh 500 mL of LB broth and incubated at 37°C. The culture was allowed to incubate for 5 hours until an OD600nm of 0.4 to 0.6 was reached. The culture was allowed to cool down to 25°C and 0.4M of IPTG dioxane-free (Isopropylβ-D thiogalactopyranoside) (Thermo Scientific®) is added to the culture to induce the expression of cloned genes and incubated at 25°C for 24 hours.

To obtain the protein; the culture was centrifuged at 6000 rpm for 20-30 minutes, the supernatant was discarded, and the pellet was retained. The pellet was weighed and frozen at -80°C and thawed at 37°C, the step was repeated two times. During the second time, the pellet was let to thaw until a slime was formed completely. A volume of 7 mL/g of E.coli lysis buffer with 0.1% Triton-X100 was added to the wet-slimy pellet. The lysis buffer contained 50 mM potassium phosphate, 300 mM KCl, 10% glycerol,

1 mg/mL DNase and 10 mg/mL RNase. The pellet was incubated at 37^{0}C for 1-2 hours. After a slimy yellowish, solution formed was centrifuged at 14000 rpm for 14 minutes at 4^{0}C. The supernatant was retained, and the pellet was discarded. The protein was concentrated to its original volume using Amicon Ultra centrifugal filter units Ultra-15, MWCO 10 kDa (Millipore®). Millipore™, Merck, 395 Ireland, catalog # UFC901096.

3.3   GFP-tagged crude grape chitinase- yeast cell wall binding

A method described by de Groot et al. (2001) was used to culture yeast cell. The optical densities (ODs) at 600 nm of the cells were used to makes sure that the equal amounts of the cells per culture were used. Volumes equivalent to the ODs were then centrifuged and washed with 1× PBS buffer. A volume 100 µL of the crude GFP-tagged-grape chitinase was added to a cell that had been suspended in 200 µL PBS buffer. The cells were incubated at 37^{0}C for 2 hours with shaking after incubation, and the cells were spun down and washed with 200 µL and suspended in 200 µL. The GFP-chitinase bound to the yeast cell was quantified using the flow cytometry at a maximum of 50,000 cells, and the quantification was done using BD FACS Aria Cell Sorter Flow cytometry equipped with BD FACS DiVa v8.1 software that captured the data. The excitation laser used was the solid-state sapphire laser at a wavelength of 488 nm and the emission filters used was the FITC channel, with a 505 long pass and 530/30 bandpass filter.

3.4   Calcofluor white and Concanavalin A Alexa Fluor 647 staining for fluorescence confocal microscopy and flow cytometry

The preparation, staining procedure of cells for flow cytometry and fluorescence microscopy was adopted from de Groot et al. (2001), Lomolino, and Curioni (2007) with modifications. Quantification of chitin levels of the yeast strains was done in triplicates for each strain. A volume of 2 mL was sampled from the synthetic must monoculture fermentation. The sampling days for chitin were (Day 0, 3, 5) and for mannoproteins (hour 0, 12, 24, 48) and the last day of fermentation for both chitin and mannoproteins. The last day of fermentation was 18th day, the day fermentations were terminated. The yeast cells from a 2 mL of synthetic must sampled and were harvest by centrifugation at 11000 rpm/ 4^{0}C/ 10 min, washed with 500 µL of 1× PBS with pH 7.4 and the yeast cells were suspended in 350 µL of PBS. PBS contained 8.01(g/L) NaCl, 0.20 KCl, 1.44 Na2HPO4.2H2O and 0.27 KH2PO4 all in (g/L).

The cells were stained with approximately 20 µl of calcofluor white after the addition of 20 µL of KOH for chitin and mannoproteins, and cells were stains with 20 µL of 5 mg/mL Concanavalin A Alexa Fluor 647 conjugate (catalog number 21421, ThermoFisher Scientific). Concanavalin A is conjugant that

intercalates in the yeast cell wall binding the mannose, the building blocks of mannoproteins. The Taking into account that the stain is light sensitive, everything was done in the presence of minimized light. The quantification of chitin and mannoproteins levels were at a maximum of 50,000 cells, and the quantification was done using BD FACS Aria Cell Sorter Flow cytometry equipped with BD FACS DiVa v8.1 software that captured the data. The excitation laser used was the solid-state sapphire laser at a wavelength of 405 nm for chitin, for mannoproteins 633 nm and the emission filters used was the FITC channel, with a 505 long pass and 530/30 for chitin and mannoproteins 660/20 bandpass filter.

For confocal microscopy, a volume of 10 µL of the cells was put on imaging chambers, and the image acquisition was performed using the Carl Zeiss Confocal LSM 780 Elyra S1 with SR-SIM super-resolution platform, and the images were processed using the Zen (2011). A software attached to the confocal microscopy and the images are presented in maximum intensity projection. The excitation laser used was the violet laser with a 500nm wavelength.

3.5   Fermentation conditions

The yeast cells were cultured in 5 mL of YPD broth; the cells were then pre-cultured in a 250 mL Erlenmeyer flask containing 100 mL of YPD broth and incubated at 30^0C on a rotary incubator. The yeast cells for inoculation were harvested at the end of an exponential phase by centrifuging at 11000 rpm at 4^0C for 10 minutes. The optical density of the cultures was read at 600 nm using a spectrophotometer, 106 cells/mL were inoculated into synthetic must (230 g/L; pH 3.3), and Sauvignon blanc grape must (236 g/L; pH 3.257). The fermentations were carried out in triplicates at 30^0C in 100 ml bottles with 70 mL working volume without agitation, and the bottles were fitted with airlock bubblers. The Cumulative weight loss (CO_2) was monitored daily throughout the fermentation period.

Table 3.2: The composition synthetic grape must media modified from Henschke and Jiranek (1993). and Bely *et al.* (1990) adjusted pH of 3.3 with KOH.

Base media pH 3.3		
	Compound	g/L
Carbon Source	D(+)-Glucose Anhydrous	115
	D(-)-Fructose	115
Acids	Potassium L-tartrate monobasic	2.5
	L-Malic Acid	3
	Citric Acid	0.44
Salts	K_2HPO_4	1.14
	$MgSO_4$ x $7H_2O$	1.23

43

	$CaCl_2$ x $2H_2O$	0.44
	NH_4Cl	0.46

Prepared in 1 L of 2% NaHCO3

		mg/L
Amino Acids	tryosin	18.33
	tryptophan	179.33
	isoleucine	32.73
	aspartic acid	44.51
	glutamic acid	120.43
	arginine	374.37
	leucine	48.43
	threonine	75.92
	glycine	18.33
	glutamate	505.27
	alanine	145.3
	valine	44.51
	methionine	31.42
	phenylalanine	37.96
	serine	78.54
	histidine	32.73
	lysine	17.02
	cystein	13.09
	proline	612.61
		g/L
Vitamins	Myo-inositol	10
	pyridoxine-HCL	0.2
	Nicotinic acid	0.2
	Calcium pantothenal	0.1
	Thiamine	0.05
	PABA-K	0.02
	Riboflavin	0.02
	Biotin	0.0125
	Folic acid	0.02
		mg/L
Trace Elements	$MnCl_2$ x $4H_2O$	20
	$ZnCl_2$	13.5
	$FeCl_2$	3
	$CuCl_2$	1.5
	BH_3O_3	0.5
	$Co(NO_3)_2$ x $6H_2O$	3
	$NaMoO_4$ x $2H_2O$	2.5
	KIO_3	1
		g/100mL
Lipids	Ergo sterol	1.5

Oleic	0.5

3.6   Bentonite addition

A 5% (w/v) of Bentonite slurry was prepared following a method by Boulton et al. (1996) and Bowyer (2008). The method described by Butzke (2009) was followed for the addition of bentonite dosages (12, 24, 36, 48 and 60 g/hL) to the wines. After the addition of bentonite, the wines were incubated at 25^{0}C in a shaking incubator for 30 minutes. The addition of bentonite was carried in triplicates. After incubation, the wines were centrifuged at 5000 rpm for 5 minutes. The wines were therefore subjected to the pinking potential assay and heat test.

3.7   Pinking potential

The pinking potential of the wines was performed according to the method described by Simpson (1977) and Iland et al. (2000), with a few modifications. Clear bottles were used for the potential pinking test. Wines after alcoholic fermentations were centrifuged to remove the yeast cells from fermentations at 11000 rpm/ 4^{0}C / for 10 minutes. A 1 mL of 0.072% (v/v) of hydrogen peroxide was added into 25 mL of the wine sample, and no hydrogen peroxide was added to the controls samples. For the controls, the bottles were filled to the brim with the wine sample (Iland et al., 2000). Both the control and the sample were placed in a dark cupboard overnight at room temperature. The absorbance of both the control and the samples with hydrogen peroxide were read using a spectrophotometer at 500 nm. The change (∆) in absorbance was calculated by subtracting the absorbance of the control from the absorbance of the sample and the wine was considered to have pinking potential if the difference is above 0.05 in absorbance units.

3.8   Heat test

To perform the heat stability assay, after fermentations of both the synthetic grape must and Sauvignon blanc grape juice. A concentration of 0.5 g/L of Bovine serum albumin (BSA) and 0.5 g/L potassium sulphate was added in the synthetic must MS300, and none was added to the Sauvignon blanc grape must as described by Pocock et al. (2007) before the heat test. The BSA is added as a model protein to the media as synthetic must do not comprise of proteins while potassium sulphate is a non-protein that also does aid in the formation of haze in wine. The addition of BSA and potassium have been shown to promote haze formation (Van Sluyter et al., 2015). The test was carried out as described by Pocock and Water (2006) with modifications, briefly, the MS300 and Sauvignon blanc were centrifuged at 11000 rpm /at 4^{0}C /for 10 min to remove excess yeast cells from the fermentations. The absorbance of the

45

supernatants from the fermented synthetic must (MS300), and Sauvignon blanc juice was read at 520 nm and was recorded as absorbance before the heat test. The absorbance after was read after heating the samples at 80^oC for 2 hours and followed by cooling at 4^oC for 16 hours. After the heat test, the absorbance of the samples was read after the acclimatization of the samples for 30 min to room temperature the absorbance was read at 520 nm. The difference in haze was calculated by adopting the method by Waters et al. (1992) and the instructions by Pocock and Waters et al. (2007) that samples are to be considered protein unstable when the absorbance difference between the heated and unheated samples is higher than 0.02 in absorbance units.

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Chapter Four

Research Results and Discussions.

A microbiological solution to visible wine defects: Haze formation and Pinking potential.

4.1   Fermentations kinetics.

Fermentations were carried out at 15°C in both real of Sauvignon blanc (Fig. **4.1A** and **B**) and synthetic grape must (Fig. **4.1C** and **D**). The fermentations were carried out in a manner that mimicked the industrial winemaking. To prevent alteration of the media complexity. The single culture (Fig. **4.1A** and C) and sequentially (Fig. **4.1B** and **D**) inoculated fermentations were monitored until weight loss ceased. *Saccharomyces cerevisiae* BM45 was sequentially inoculated after 48 hours. During the fermentations, the chitin and mannoproteins levels of the strains were monitored. At the end of the fermentations, the wines were subjected to heat test for determining haze levels and the potential pinking test.   The fermentation was terminated when the recording in weight loss remained constant with differences between 0.1-0.5 g. The wine-related yeast strains did not ferment to dryness, but S. cerevisiae did ferment to dryness.

The rates of weight loss through CO2 release differed slightly between different wine-related yeast strains (Fig. **4.1A and D**). In Sauvignon blanc monoculture fermentations, *C. prunicola* Y1162 had a slow weight lost through the release of CO2, and *M. pulcherrima* Y1094 the fastest when compared to all the other wine-related strains. As expected, only pure or sequential fermentations with *S. cerevisiae* BM45 fermented to dryness while the other wine-related strains did not complete the fermentations (Fig. **4.1C and D**). In both fermentations, sequential and monoculture fermentations *S. cerevisiae* BM45 consumed sugar faster as expected.
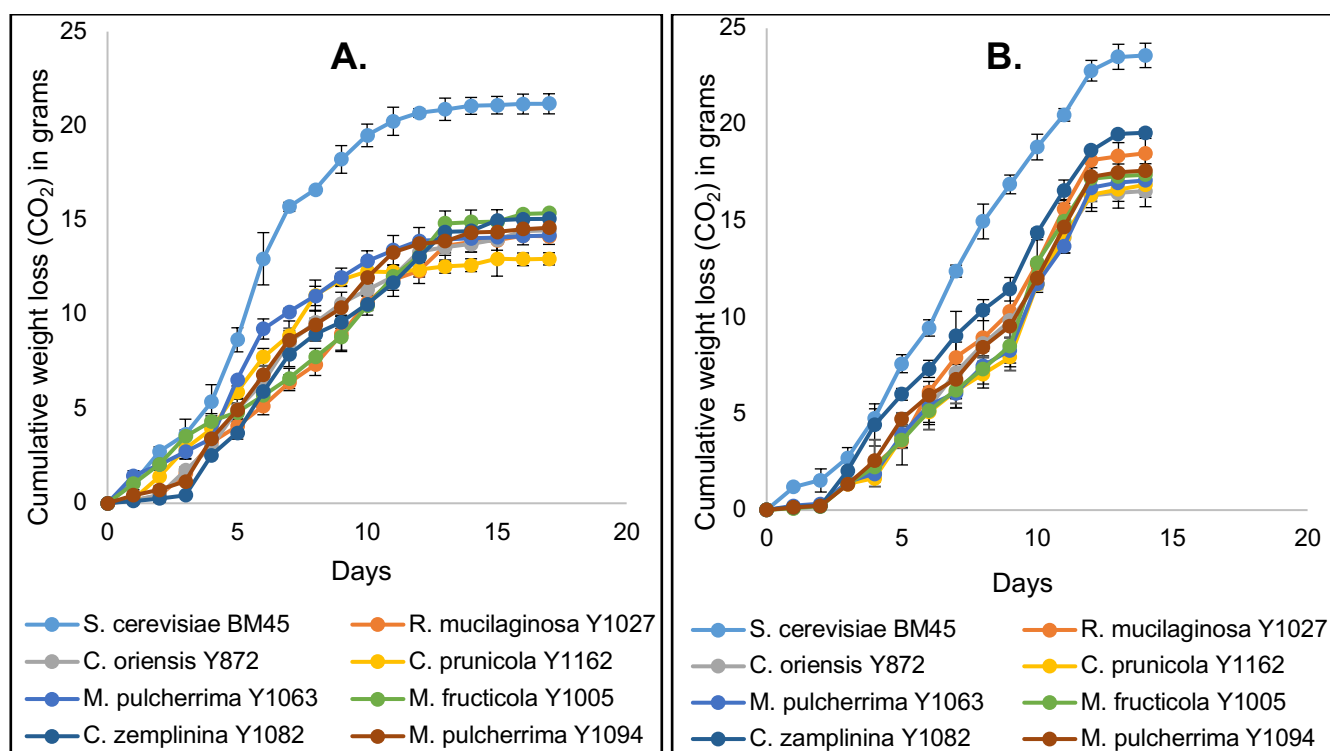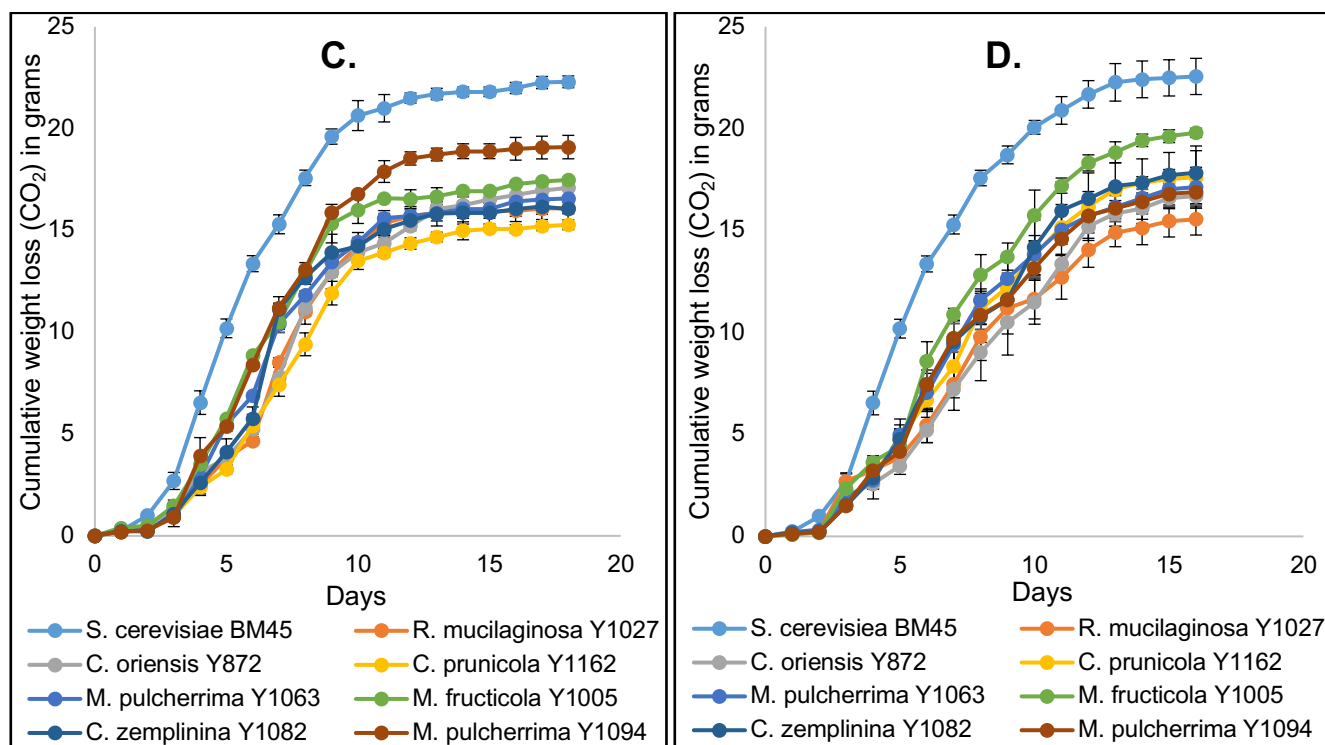
Figure 4.1. Monoculture and sequentially inoculated fermentations in synthetic media and real grape must. Fermentations carried out in 80 mL of must, and fermentation curves were generated from the weight loss during fermentations. **(A)** mono-culture inoculation and **(B)** sequentially inoculated with *S. cerevisiae* BM45 at 48 hours of the grape must fermentations. **(C)** and **(D)** are sequentially inoculated with *S. cerevisiae* BM45 at 48 hours of the synthetic grape must fermentations.

4.2   Monitoring chitin and mannoproteins levels during fermentations.

To monitor the changes in chitin and mannoproteins during fermentation, the flow cytometry was used and 50, 000 cells were used for quantification. The levels were monitored in synthetic must and not in grape must. A volume of 2 mL as stated at 3.4 was used for quantification of chitin and mannoproteins sampling times were different. Sampling for chitin was carried out on day 0, 3, 5 and at the end of fermentation and mannoproteins at 0, 12, 24, 48 hours and the end of the fermentations (Fig. **4.2A**). The end of fermentations referred day when fermentations were terminated, for monoculture fermentation the fermentations were terminated 18 days and sequential fermentations 16 days after fermentation. The data is presented in arbitrary units (AU). The data is relative and strain depended.

Chitin levels decreased significantly and in all species or strains between the initial day 0 and day 3, and remained mostly stable after that. Day 0 reflects the levels of chitin 1 hour after inoculation, suggesting that the pre-culture conditions must have led to a significant accumulation of chitin. Day 3 corresponds to an early stage of fermentation and exponential cellular growth. The days as mentioned

earlier were chosen based on literature to show a trend of chitin fold changes during any course stress related case towards the cells (Gomar-Alba et al., 2015). The fold changes of the chitin and mannoproteins levels that were observed differed per strains and the values obtained are strain depended and species depended. Therefore, strains cannot be compared based on how much of either chitin or mannoproteins does each strain contains. To calculate the fold changes the following changes were used for percentage increase and decrease:

1. % increase = increase in fluorescence ÷ old (hour day)florescence × 100
2. % decrease = decrease in fluorescence ÷ old (hour day)florescence × 100

The formulas were used for both chitin changes and mannoproteins changes. All the strains followed a similar trend throughout the fermentations and showed the highest chitin levels on day 0. The highest levels of chitin were observed in *Cr. oriensis* Y872, while S. cerevisiae had the lowest levels. Decreases in chitin ranged between 72 % in *C. zemplinina* Y1082 to 65% for *Cr. oriensis* and 39 % in *M. pulcherrima* Y1094 and *C. prunicola* Y1162. (Fig. **2B**) shows the differences in the mannoproteins levels of the wine-related yeast strains. At hour 0, the mannoproteins levels of the strains were higher than the levels recorded at the 12th hour of the fermentations.

There was no particular trend that all strains followed during fermentations. However, from the data, three strains followed a similar trend, the strains started with low mannoproteins levels, and the levels gradually increased at every time point from 12th, and a peak was observed at 24 hours and declined from 48 hours till the end of fermentations. These strains include *R. mucilaginosa* Y1027 and *C. prunicola* Y1162 and *M. pulcherrima* Y1094. While the following strains did the opposite of the strains as mentioned earlier. These strains started had a decrease in mannoproteins levels from the 12th hour to the end of the fermentations: *S. cerevisiae* BM45 (44 and 53 %), *M. pulcherrima* Y1063 (42 and 52 %), and *M. fructicola* Y1005 (28 and 47%), respectively. The following strains also followed a different trend from the trends as mentioned earlier. The mannoproteins levels for *M. fructicola* Y1005 and *Cr. oriensis* Y872 increased by 29.72 % during the 48th hour while for all the other strains the levels were decreasing.

The results obtained for chitin and mannoproteins agree with the theory by Klis et al. (2002), Aguilar-Uscanga and Francois (2003). The trend that was obtained was different from that of chitin; there was a particular trend that the mannoproteins levels followed during fermentations. This can be explained based

52

on the structural difference between chitin and mannoproteins, and their functions in the yeast cell wall. Chitin is secreted in a large amount in response to stress-related conditions, and this supports the increased levels of mannoproteins during the early hours of inoculation. While mannoproteins allow for the selective permeability of the yeast cells during fermentation. Hypothetically, the permeability of the yeast cell wall is supposed to increase, but that was not what was observed from the study. There are not a lot of studies that looked at the fold changes of chitin and mannoproteins on during fermentations.
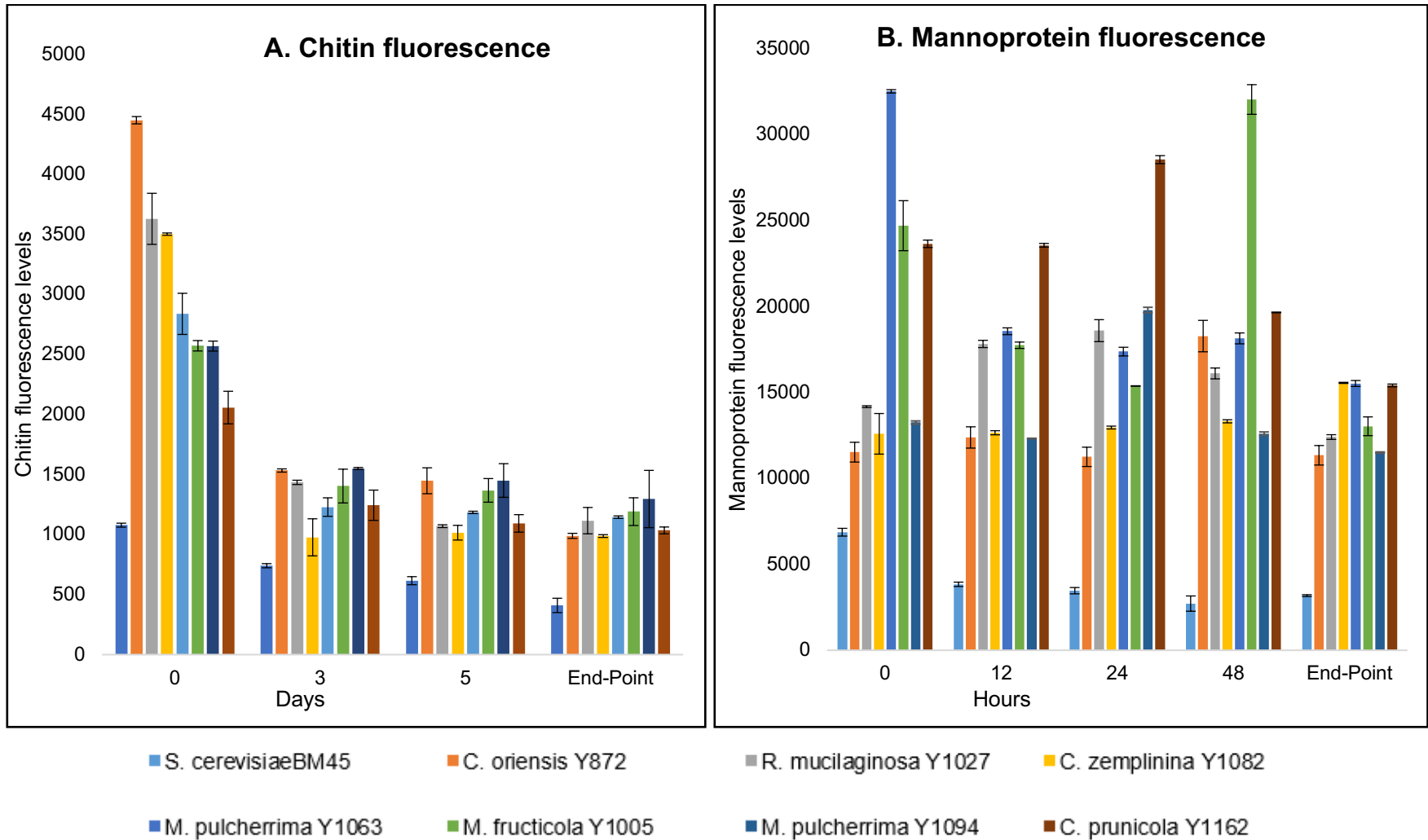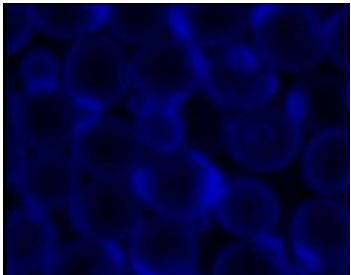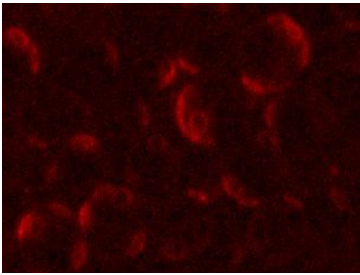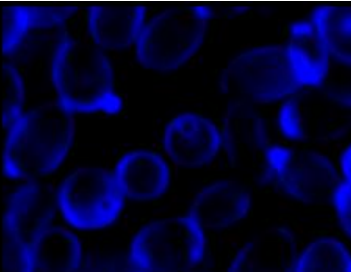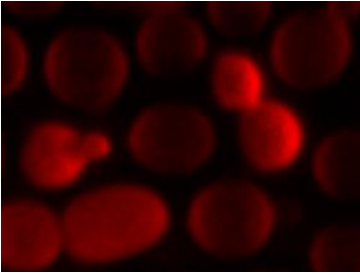
Figure 4.2: The fluorescence levels of chitin and mannoproteins were measured using the flow cytometry. Calcofluor white and Concanavalin A (Alexa 647), fluorescent fluorochromes were used to bind to the chitin and mannose in the yeast cell wall respectively. Quantification based on 50,000 cell per strain. **(A)** Shows the chitin fluorescence levels obtained for each strain and **(B)** Shows the mannoproteins levels based on the mannose, monomer of mannoproteins.
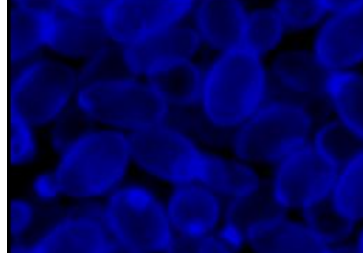
4.3   Fluorescence Microscopy.

Confocal microscopy and images observed cells were processed through the software ZEN 2011. Calcofluor white (blue) was used for chitin staining in yeast cell wall while Concanavalin A (Alexa 647) (red) used for labeling of mannose monomers of mannoproteins. The difference was observed as each species displayed different contents of both mannoproteins and chitin. Based on these difference, the location and distribution around the yeast cell wall, while the chitin is both around the scars and surface, mannoproteins are only located around the surface of the cells. (Gomar-Alba et al., 2015; Deguchi et al., 2015; Rizzetto et al., 2016) also observed similar differences. The highest concentration of chitin fluorescence was located at the bud scars of the cells with an exception to *M. fructicola* Y1005. The *C. prunicola* Y1162 and *C. zemplinina* Y1082 showed similarities in both the chitin and mannoproteins distribution fluorescence while *Cr. oriensis* Y872 was different from all the stains. For *Cr. oriensis* Y872, the fluorescence of chitin was mostly high around the surface of the cell compared to the bud scars. For all the strains from all the different groups of species showed a difference in the chitin while there was no difference in mannoproteins fluorescence.
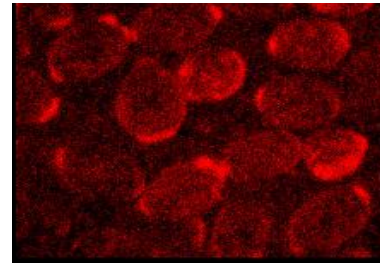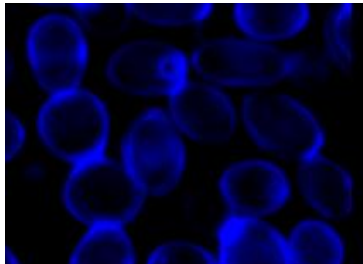
Table 4.1: Yeast cell wall images showing chitin and mannoproteins distributions in the yeast cell wall.

Yeast strains	*Calcofluor White*	*Concanavalin A (Alexa 647)*
Saccharomyces cerevisiae BM45		
Metschinikowia pulcherrima Y1063		

Candida prunicola Y1162		
Candida zemplinina Y1082		
Cryptococcus oriensis Y872		
Metschinikowia pulcherrima Y1094		
Metschinikowia fructicola Y1005		
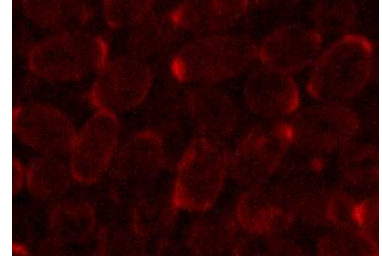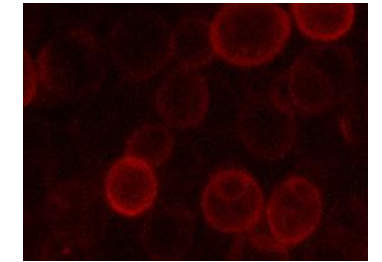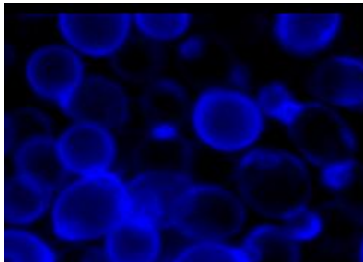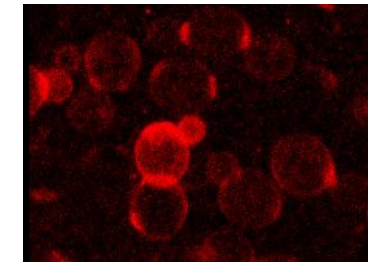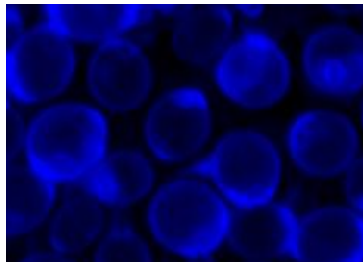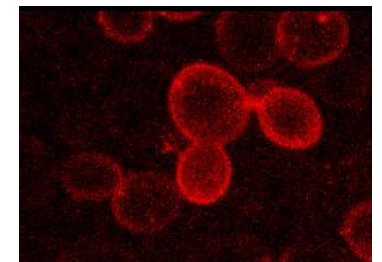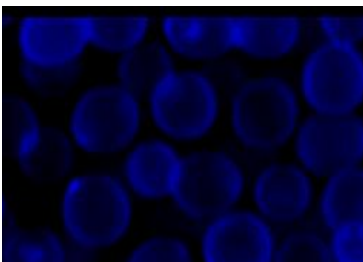
Rhodotorula mucilaginosa Y1027		
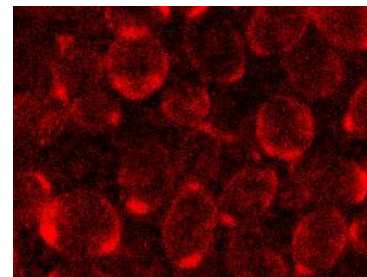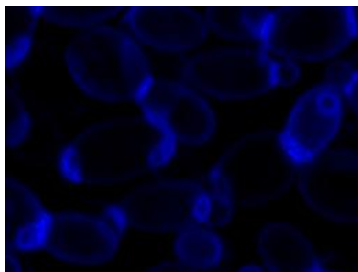
4.4   Crude grape GFP-chitinase binding assay.

The assay was carried to relate the number of chitin levels in each strain to the amount of GFP-chitinase that binds to each strain. Three biological repeats were used to experiment. The experiment showed that the binding of chitinase is strain dependent, as all the strains showed a different binding affinity to chitinase. The *S. cerevisiae* BM45 had the lowest levels of bound GFP-chitinase compared to all the wine-related yeast, which is broadly in line with the higher levels of chitin observed in these strains. However, while there appears to be some correlation between chitin levels and GFP-chitinase binding, the correlation did not hold correctly for all species. A relative correlation between the amount of chitinase and chitin was observed and this suggests a possibility of haze reduction. The correlation was observed on both days (3 and 5). A partial correlation can be drawn from a relation between Table (**4.1**) and the binding of chitinase to the yeast cell wall chitin.

The differences in the yeast cell wall chitin distribution can be linked the correlation observed in Fig (**4.3 A** and **B).** *M. pulcherrima* Y1063 and *C. zemplinina* Y1082, they show the most prominent difference on the fluorescence to yeast cell wall chitin, they had a uniform layer of fluorescence which could suggests pitiable accessibility to chitinase. However, this is a hypothesis, based on the fact that the chitinase binding is based on the total availability of the chitin. The data set obtained concurs with the data that was obtained from a study that was conducted by (Ndlovu *et al.,* 2018) had shown a very high level of correlation between chitin levels and binding of GFP-chitinase for *S. cerevisiae* mutants. It was therefore hypothesized that the differences in the capacity to bind chitinase are due to the differences in cell wall structure between species and that the percentage of chitin that is available for binding on the cell wall differs between each species. The data clearly shows that the showed that observed chitin levels do not correlate or predict how much chitinase would be bound to the chitin-binding domains, rather the binding of chitinase is depended on the structural differences on chitin.
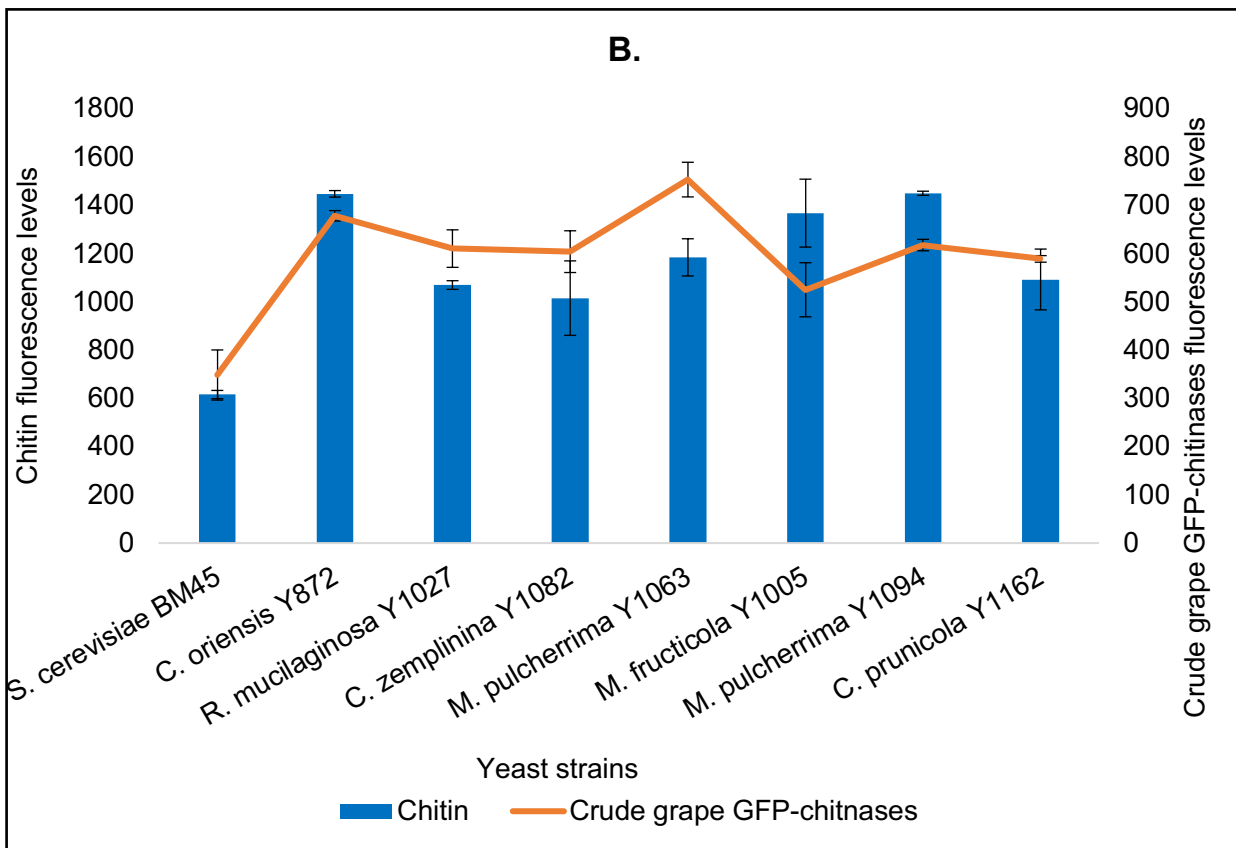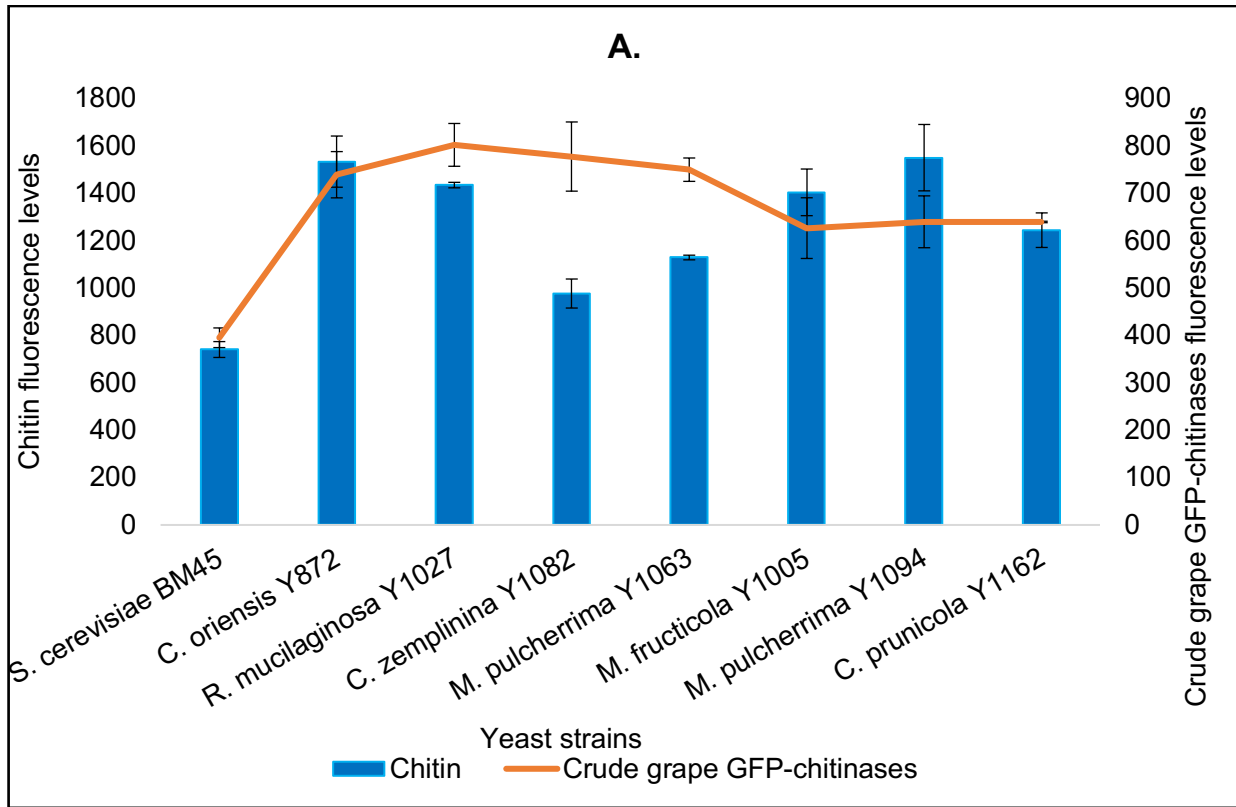
Figure 4.3: The relative binding of crude grape GFP-chitinase to chitin in the yeast cell wall. The binding assay was done on day three and day five of the fermentations. **(A)** Day 3 and **(B)** Day 5 of the fermentations.

4.5 Haze levels measured in synthetic grape must

To generate haze in the synthetic must. A concentration of 0.5 g/L of BSA and potassium sulphate were added to generate haze in synthetic must after alcoholic fermentation. Unlike in real grape must where the haze-forming proteins are already on the media. According to Pocock and Waters (2006) and Pocock et al. (2007), the wines were all unstable as the standard absorbance value of haziness is 0.02. The purpose of the experiment was to observe the prediction of how each stain will behave in real grape must. All the strains showed high haze levels in both monoculture and sequential fermentations, which is the reverse of what was observed in grape must fermentations haze levels (Fig. **5A and B**).

The data further suggest that yeast strains during fermentations release compounds that bind further to the haze forming protein and precipitate the proteins during fermentations While this not true for synthetic must fermentations where there are no other protein sources present other than those form the fermenting yeasts. The haze formed through the reaction of BSA and potassium sulphate will, therefore, be higher than haze formed naturally through haze-forming proteins. Ndlovu (2012) also observed a similar trend in the study. The addition of S. cerevisiae in synthetic must had an impact on the influence of strains to reduce haze formation which is in contrary to what was observed in Figure (4.4).

Table 4.4: Yeast strain reference for Hazel levels on **Figure 4.5.**

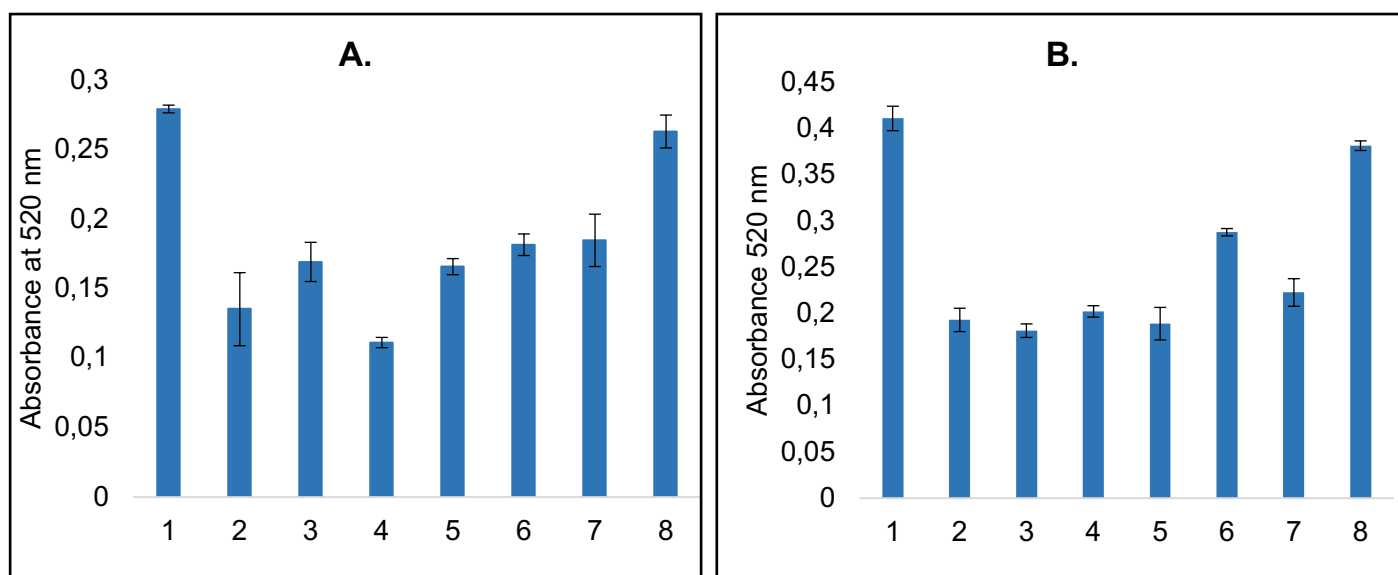| Yeast strains			
1. *S. cerevisiae* BM45	2. *R. mucilaginosa* 1027	3. *Cr. oriensis* Y872	4. *C. prunicola* Y1162
5. *M. pulcherrima* Y1063	6. *M. fructicola* Y1005	7. *C. zemplinina* Y1082	8. *M. pulcherrima* Y1094

59

Figure 4.5: Haze levels measured in synthetic grape must fermentations. The haze was induced with the addition of 0.5 g/L of BSA and potassium sulphate. (A) Mono-cultured fermentations and (B) Sequentially inoculated fermentations.

4.6   Haze levels measured in Sauvignon blanc

For comparison purposes and to determine the minimal dosage of bentonite required for each strain to obtain lower or no haze levels. Bentonite is a fining agent that is commonly used in the wine industry. Five dosages (12, 24, 36, 48, 60 g/hL) of bentonite were added to the wines after alcoholic fermentations. The haze levels were above the standard value of 0.02 in absorbance units as suggested by Pocock and

Waters (2006) and Pocock et al. (2007). Haze levels were measured after the addition of bentonite. Figure 4.4 shows that generally lower haze levels were observed in wines that had been fermented with non-Saccharomyces wine-related yeast strains compared to the ones fermented by *S. cerevisiae* BM45. In monoculture (Fig. **4.4A**) fermentations, *C. prunicola* Y1162 had no haze levels from dosages (36 - 60 g/hL) of bentonite while *Cr. oriensis* Y872 had no haze levels from (48 - 60 g/hL). However, for strains such as *C. zemplinina* Y1082 and *M. pulcherrima* Y1063 haze levels were measures throughout the different dosages. **Table 4.2** summarizes the data in (Fig. **4.4A**) to indicate which strains had haze and which ones did not have haze measured.

In sequential fermentations (Fig. 4.4B) with S. cerevisiae BM45, haze levels of the strains improved but the haze levels of the *C. prunicola* Y1162 changes were observed instability was only observed at 48 - 60 g/hL bentonite in sequential fermentations. *C. prunicola* Y1162 was stable at dosage 36 in monoculture fermentations. For both fermentations, 60 g/hL of bentonite was the maximum dosage that showed no haze formation. However, the strains still had no haze formation starting at different dosages, as each strain protects wines from haze formation differently and the affinity of removal of haze-forming proteins differs per strain. The tables below summarize the haze levels that were observed after the addition of bentonite, to indicate which dosages had no haze after the heat test. The tables are both for monoculture and sequential fermentations.

Table 4.2: Summarized haze levels of wine-related strains measured in real must monoculture fermentations after the addition of bentonite. Haze formation is indicated by (+) and no haze formation (-).

Strains	Bentonite concentrations (g/hL)				
	12	24	36	48	60
S. cerevisiae BM45	+	+	+	+	+
R. mucilaginosa Y1027	+	+	+	+	-
Cr. oriensis Y872	+	+	+	-	-

60

C. prunicola Y1162	+	+	-	-	-
M. pulcherrima Y1063	+	+	+	+	+
M. fructicola Y1005	+	+	+	+	-
C. zemplinina Y1082	+	+	+	+	+
M. pulcherrima Y1094	+	+	+	+	-

*Haze is indicated by (+) and no haze (-).

Table 4.3: Summarized haze levels measured in sequential fermentations after the addition of bentonite. Haze formation is indicated by (+) and no haze formation (-).

Strains	Bentonite concentrations (g/hL)				
	12	24	36	48	60
S. cerevisiae BM45	+	+	+	+	+
R. mucilaginosa Y1027	+	+	+	-	-
Cr. oriensis Y872	+	+	-	-	-
C. prunicola Y1162	+	+	-	-	-
M. pulcherrima Y1063	+	+	+	-	-
M. fructicola Y1005	+	+	+	+	+
C. zemplinina Y1082	+	+	-	-	-
M. pulcherrima Y1094	+	+	+	+	-

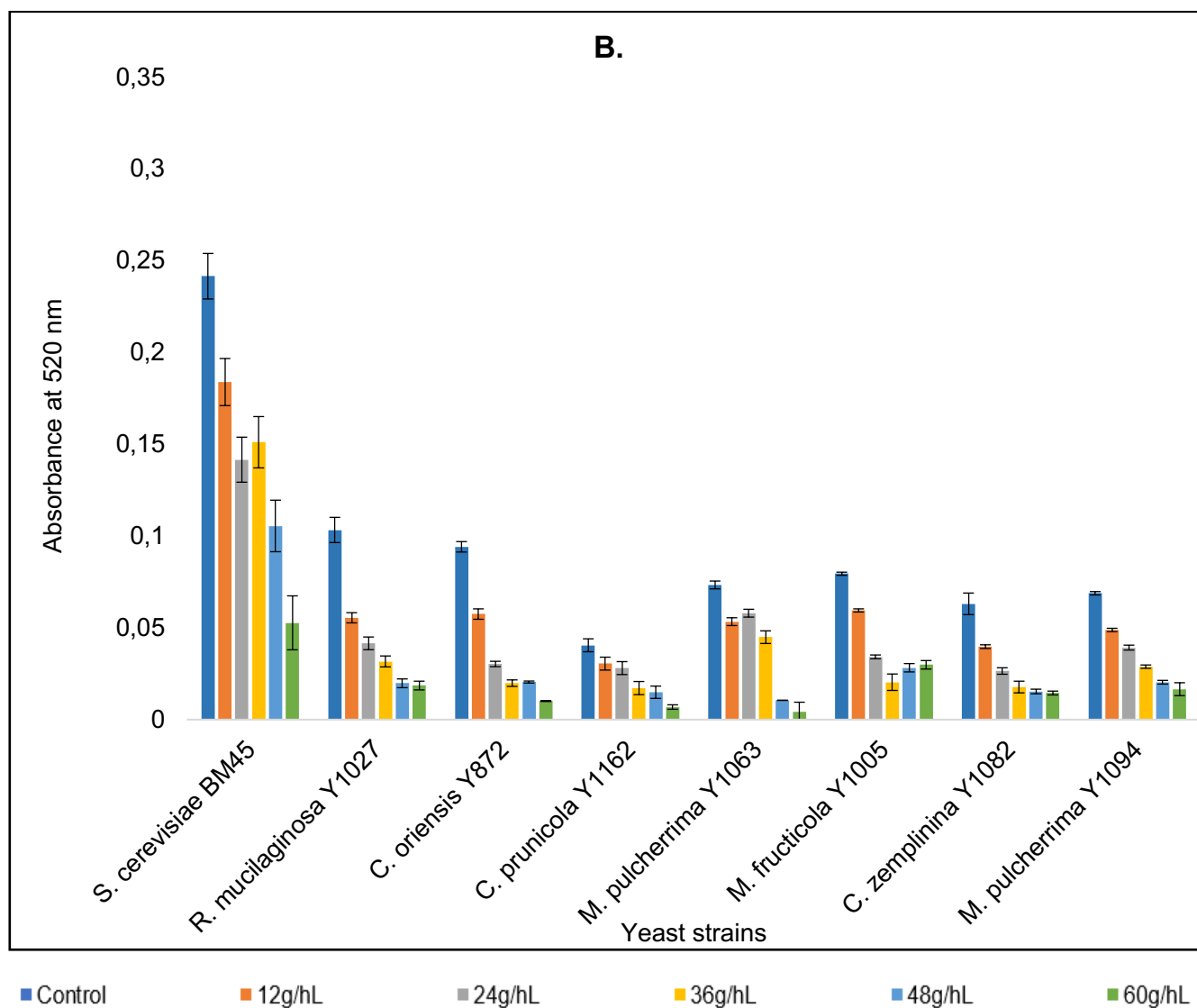*Haze is indicated by (+) and no haze (-).

A.

Figure 4.4: Haze levels of Sauvignon blanc. **(A)** Shows the haze levels in mono-cultured and **(B)** Haze levels in sequentially cultured fermentation. Bentonite was added in 5 different dosages. In addition, nothing was added onto the control. Haze levels are determined by the difference between heated and unheated wine samples.

4.7   Pinking potential of Sauvignon blanc.

The pinking potential was induced by the addition of 0.072% of hydrogen peroxide. To predict the pinking potential of the wines. The wines have a pinking potential if the absorbance value at 500 nm is above 0.05 in absorbance units (Fig. **6A and B**). In the monoculture fermentations, the pinking potential of wine-related yeast was lower than the pinking potential observed from *S. cerevisiae* BM45. S. cerevisiae BM45 fermented wines showed the highest pinking potential. The wine that was fermented with *M. pulcherrima* Y1094 and *C. prunicola* Y1162 had no pinking potential from 36 g/hL to 60 g/hL while pinking was observed in wines fermented with, *Cr. oriensis* Y872, and *C. zemplinina* Y1082 had no pinking potential from (48 - 60 g/hL). The following strains needed a higher dosage of bentonite, *R.*

mucilaginosa Y1027, *M. pulcherrima* Y1063 and *M. fructicola* Y1005 at 60 g/hL, summarized in (Table 4.5).

Table 4.5: Summarized pinking potential measured in monoculture fermentations after the addition. Pinking is indicated by (+) and no Pinking (-).

Strains	Bentonite concentrations (g/hL)				
	12	24	36	48	60
S. cerevisiae BM45	+	+	+	+	+
R. mucilaginosa Y1027	+	+	+	+	-
Cr. oriensis Y872	+	+	+	-	-
C. prunicola Y1162	+	+	-	-	-
M. pulcherrima Y1063	+	+	+	+	-
M. fructicola Y1005	+	+	+	+	-
C. zemplinina Y1082	+	+	+	-	-
M. pulcherrima Y1094	+	+	-	-	-

*Pinking is indicated by (+) and no Pinking (-).

Table 4.6: Summarized pinking potential measured in sequential fermentations after the addition. Pinking is indicated by (+) and no Pinking (-).

Strains	Bentonite concentrations (g/hL)				
	12	24	36	48	60
S. cerevisiae BM45	+	+	+	+	+
R. mucilaginosa Y1027	+	+	+	+	-
Cr. oriensis Y872	+	+	+	-	-
C. prunicola Y1162	+	+	-	-	-
M. pulcherrima Y1063	+	+	+	+	-
M. fructicola Y1005	+	+	+	+	-
C. zemplinina Y1082	+	+	+	-	-
M. pulcherrima Y1094	+	+	-	-	-

*Pinking is indicated by (+) and no Pinking (-).

The observation was that the addition of wine *S. cerevisiae* BM45 had a different impact on all the strains, an exciting trend was observed on *M. pulcherrima* Y1094. When in a monoculture fermentation, the strains performed better but the presence of *S. cerevisiae* BM45 decreased the pinking potential of the strain, but a slight change was observed on the dosages of bentonite to achieve no pinking in the wine. Literature suggests that the use of wine-related yeast strains as starter cultures for wine fermentations improved the aromatic profiles of the final wines while stabilizing the colour of wines (Jolly et al., 2017). The above mentioned concurs with the strains that show a decrease in pinking

potential with addition *S. cerevisiae* BM45. No change was observed for *C. prunicola* Y1162 had no pinking potential from 36 g/hL to 60 g/hL while strains *R. mucilaginosa* Y1027, *Cr. oriensis* Y872, *M. pulcherrima* Y1063, *C. zemplinina* Y1082 had no pinking potential from 48 g/hL to 60 g/hL (Table 4.6). The protection was sorely based on the differences in the yeast cell wall mannoproteins and how their efficiency in clearing the wine. The results from (Razmkhab et al., 2002) revealed that yeast cell walls have the absorption capacity that is efficient enough to absorb phenolic compounds (Razmkhab et al., 2002). It was therefore hypothesized that the strains that were able to protect the wines from pinking were the strains that had an efficient binding to capacity absorb and protect the wines from pinking.
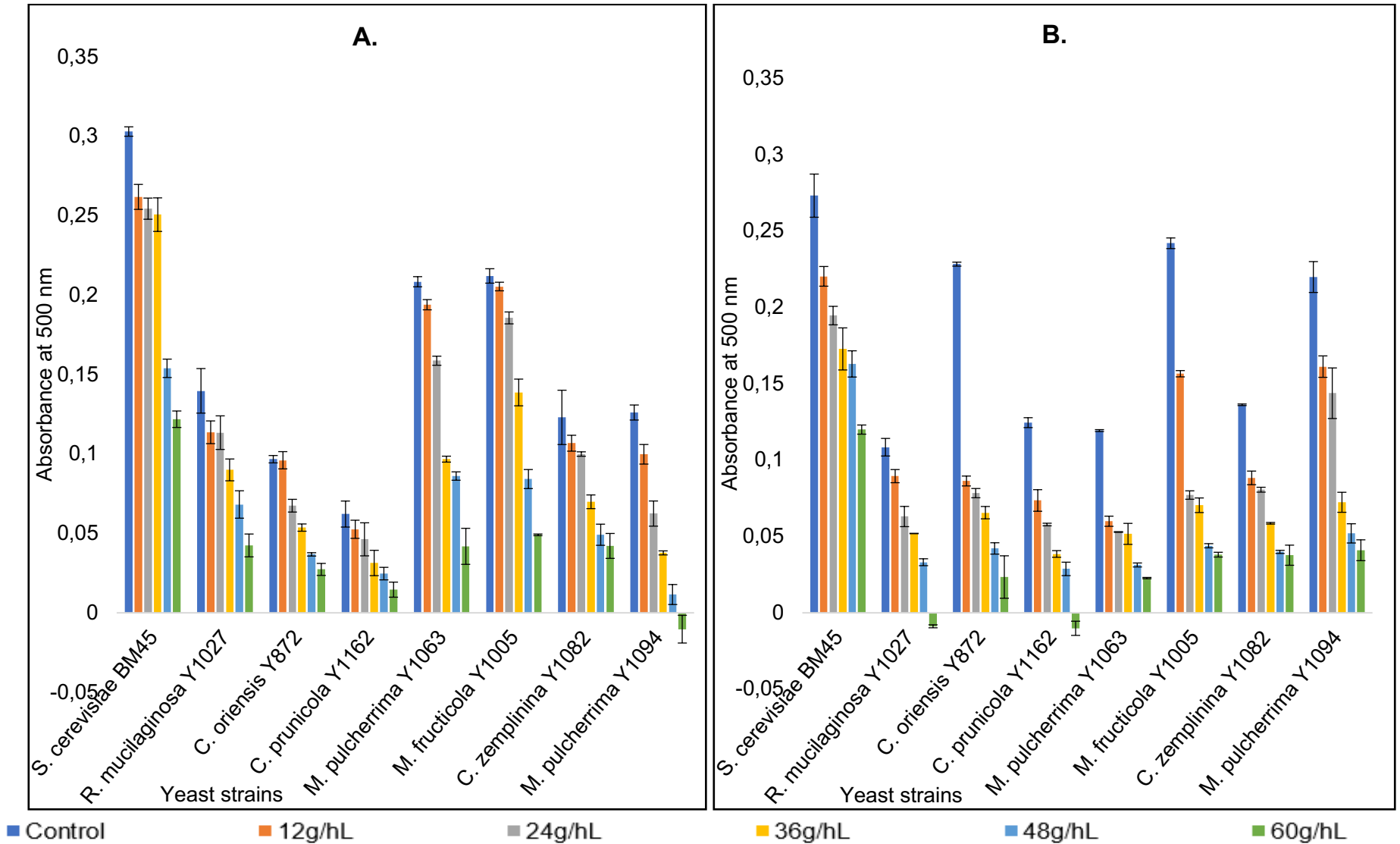
Figure 4.6: Pinking potential of Sauvignon blanc. The pinking was induced by adding 0.072% of hydrogen peroxide onto the wines. (A) Shows the pinking potential in mono-cultured and (B) pinking potential in sequentially cultured fermentation. Bentonite was added in 5 different dosages. In addition, none was added onto the control.

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Chapter Five

General Discussion and Conclusions

General Discussion

Haze formation and undesirable colour developments in commercially bottled white wines are considered visible wine defects. Consequently, in order to counteract the outcomes as mentioned earlier, the primary objective of this study was to assess whether wine-related yeast strains could provide an alternative to the traditional wine fining agents. Several yeast species are currently commercially used by the wine industry and were studied by exploring the yeast cell wall properties such as mannoprotein content and chitin concentrations.

The results obtained in the current study showed that for all yeast species chitin levels were higher at the initial stages of fermentations while at the end of the fermentations, chitin levels had decreased by half. This outcome was observed several times in all strains. Bulik et al. (2003) also reported a similar trend, while Choi et al. (1994) and Ziman et al. (1996) conducted a similar study using several stressful conditions (sorbitol, calcofluor white, and NaCl) and reported that chitin levels increased in response to stress, suggesting that the early high level of chitin might be linked to inoculation stresses.

On the other hand, concerning mannoproteins, no particular trend was followed by all of the studied strains. Concentrations varied for some strains at different time points while other strains maintained a constant profile.

The data showed that generally the ability of yeast strains to bind chitinase correlated well with the amount of chitin in the cell wall. This ability had previously been correlated with improved haze protection by Ndlovu et al. (2018). However, the correlation between chitin levels and chitinase binding was not perfect, suggesting that difference in cell wall structure between the different species investigated impacted the chitinase binding.

Strain C. prunicola Y1162 had relatively lower levels of chitin but were able to protect the wines from haze formation. This indicates that the amount of chitin in the yeast cell wall does not solely determine the ability of the strains to protect the wines from haze formation. It was then hypothesized that the critical contributing factors are the distribution of chitin in the yeast cell wall and the level of exposure of the chitinases binding sites for chitinase binding (regardless the amount of chitin). The fluorescence microscopy data showed that there are differences in the yeast cell wall chitin and mannoproteins distribution amongst the strains used in the current study. The chitin fluorescence is located both on the

bud scars and the surface of the yeast cell, while mannoproteins are only located around the yeast cell surface.

It was observed that the non-Saccharomyces wine-related yeast strains showed better protection for both haze formation and pinking potential compared to S. cerevisiae BM45. Additionally, the literature suggests that non-Saccharomyces wine-related yeast strains secrete proteins that improve wine quality (Mostert and Divol, 2014; Jolly et al., 2014; Lochbühler et al., 2015; Jolly et al., 2017; Escott et al., 2018). In this work, it was observed that some strains could protect the wines from only one defect and some strains from both defects. For example, M. fructicola Y1005 and M. pulcherrima Y1063 protected the wines from haze formation and not from pinking while Cr. oriensis Y872 and M. pulcherrima Y1094 protected the wines from only pinking. However, only C. prunicola Y1162 was able to protect the wines from both pinking and haze formation simultaneously.

Several previous studies reported that during winemaking the yeast strains can absorb the grape polyphenols (Minnar et al., 2018; Vernhet et al., 2018; Baiano et al., 2018; Checchi et al., 2018; Loira et al., 2018; Medina et al., 2018) which are hypothesized to be responsible for pinking. Polyphenols are positively charged while the yeast cell is negatively charged which influences absorption (Medina et al., 2005; Caridi, 2006; Guadalupe et al., 2007, 2010; Guadalupe & Ayestarán, 2008). Bentonite fining agent was used in the study to clarify wines at the end of alcoholic fermentations. A lab-scale method was followed, five dosages of bentonite (12, 24,36,48,60 g/hL) were used as previously mentioned. The strains used in the study had different minimal dosages of bentonite at which wine was protected from haze formation and pinking potential, but only one strain, C. prunicola Y1162, protected the wines without the addition the addition of bentonite.

The influence of yeast proteins on the wine clarity and wine colour was observed to be like that of commercial fining agents similar to observations by Charpentier et al. (2006) and Escribano-Viana et al. (2018) and Zhang et al. (2018). It is also worth noting that the addition of S. cerevisiae BM45 had a positive impact on the haze levels but a negative impact on the pinking potential of the wines. However, C. prunicola Y1162 was able to protect the wine from both defects with or without the addition of S. cerevisiae BM45. As previously, mentioned, high levels of chitin do not necessarily result in high protection of white wines from haze formation and pinking, which is an important attribute. This attribute of yeast strains provides a feasible alternative to bentonite for wine fining. Overall, the use of yeast strains

provides a natural alternative to chemicals such as bentonite, a desirable approach according to the industry and consumers point of view.

Conclusions and Recommendations

The study set a foundation of what can be expected from the use of wine-related yeast strains. The ideas developed in this study can, therefore, form the basis for some future studies. Such studies may include improving yeast screening procedures, the selection of strains based on their impact on both visual defects and further analysis of the strains to support their applicability in the industries. The study was conducted in a set up that mimicked the industrial winemaking conditions, and more real grape must data need to be generated. It is also recommended that the strains are tested in sterilized grape must to eliminate possible contributions from the wild microbiota and confirm the data set that was obtained from the study. It is also very essential that the population dynamics of the yeast be monitored in sequential fermentations.

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