Impact of the protease-secreting yeast *Metschnikowia pulcherrima* IWBT Y1123 on wine properties and response of protease production to nitrogen sources

by

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Declaration

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Summary

Yeast of oenological origin belong to various genera and harbour unique metabolic properties that may significantly impact aspects of wine processing and composition. Extracellular aspartic proteases, and their secretion by wine yeasts, have received much attention due to their protein degradative ability as a possible solution to the quality problem of wine turbidity. This fault is generally addressed by wine-makers with the use of bentonite, but this fining agent is associated with various technological and organoleptic issues. A key contributing factor to haze formation in wine is the presence of heat unstable grape proteins, and their removal by proteases therefore presents an attractive alternative to the use of bentonite. The yeast *Metschnikowia pulcherrima* IWBT Y1123 has been isolated from grape juice and secretes an extracellular aspartic protease named MpAPr1. This enzyme demonstrated activity against grape proteins and reduced wine haze-forming potential under winemaking conditions after 48 h incubation with a purified exogenous MpAPr1 preparation. However, inoculation of *M. pulcherrima* IWBT Y1123 as a co-starter culture to wine fermentation for the secretion of MpAPr1 directly into the matrix presents a possible time- and cost-effective alternative that would eliminate the need for enzyme purification steps. Nevertheless, understanding protease regulation by environmental conditions and relating protease secretion and activity to its impact on wine properties could prove useful when considering inoculation strategies for this yeast.

This study sought to establish the potential for developing co-inoculation strategies of *M. pulcherrima* IWBT Y1123 with the efficient fermenter *Saccharomyces cerevisiae*, by assessing the impact of nitrogen sources and protein availability on protease production and activity by *M. pulcherrima* IWBT Y1123, as well as the impact of protease production and activity in grape juice inoculated with *M. pulcherrima* IWBT Y1123 on grape protein content, haze formation potential and wine volatile aroma profile. Protease production was shown to be subject to nitrogen catabolite repression in the presence of preferred sources of nitrogen, as well as induction by proteins. Upon inoculation into grape juice, an up-regulation of *MpAPr1* gene expression could be observed, as well as protease production and activity. With regard to the impact of *M. pulcherrima* IWBT Y1123 co-inoculation with *S. cerevisiae* on wine properties at the end of fermentation, total protein content and haze forming potential were lower compared to controls and the volatile profile was altered. Future work should focus on enhancing protease production by *M. pulcherrima* IWBT Y1123 to improve its viability as a commercial protease-producing yeast strain. Nevertheless, the results generated in this study contribute to knowledge in both fundamental and biotechnological aspects of protease secretion by *M. pulcherrima* IWBT Y1123.

Opsomming

Gis van oenologiese oorsprong behoort aan verskeie genera en bevat unieke metaboliese eienskappe wat die aspekte van wynverwerking en samestelling aansienlik kan beïnvloed. Ekstrasellulêre aspartiensuur protease, en hul afskeiding deur wyngiste, het baie aandag gekry vanweë hul proteïenafbrekende vermoë as 'n moontlike oplossing vir die kwaliteitsprobleem van wyn wasigheid. Die probleem word oor die algemeen deur wynmakers aangespreek deur die gebruik van bentoniet, maar hierdie klei word geassosieer met verskeie tegnologiese en organoleptiese probleme. 'n Belangrike bydraende faktor vir die vorming van wasigheid in wyn is die teenwoordigheid van hitte-onstabiele druiweproteïene, en hul verwydering deur protease bied dus 'n aantreklike alternatief tot bentoniet. Die gis *Metschnikowia pulcherrima* IWBT Y1123 is van druiwesap geïsoleer, en skei 'n ekstrasellulêre aspartiensuur protease genaamd MpAPr1 af. Hierdie ensiem het voorheen aktiwiteit teen druiweproteïene gedemonstreer, en na 48 uur inkubasie onder wynmaak omstandighede het ‘n gesuiwerde MpAPr1 voorbereiding die potensiaal vir die vorming van wasigheid verminder. Die inenting van *M. pulcherrima* IWBT Y1123 as 'n mede-kultuur vir wynfermentasie vir die afskeiding van MpAPr1 direk in die matriks, bied egter 'n moontlike tyd- en koste-effektiewe alternatief wat die behoefte aan ensiem-suiweringstappe sal elimineer. Nietemin kan die kennis van protease regulasie deur omgewingsomstandighede, en proteasekresie en aktiwiteit met betrekking tot die impak daarvan op wyn eienskappe, nuttig wees as inentingstrategieë vir hierdie gis oorweeg word.

Hierdie studie het gepoog om die potensiaal vir die ontwikkeling van gesamentlike inentingstrategieë van *M. pulcherrima* IWBT Y1123 met die effektiewe fermentor *Saccharomyces cerevisiae*, te bepaal deur die impak van stikstofbronne en proteïenbeskikbaarheid op protease produksie en aktiwiteit deur *M. pulcherrima* IWBT Y1123 te evalueer, sowel as die impak van protease produksie en aktiwiteit in druiwesap met *M. pulcherrima* IWBT Y1123 ingeënt op druiweproteïeninhoud, wasigheids-vormingspotensiaal en aroma-profiel. Protease produksie het getoon dat dit onderhewig is aan stikstof kataboliet-onderdrukking in die teenwoordigheid van voorkeurbronne van stikstof, sowel as induksie deur proteïene. Met inenting in druiwesap kan 'n opwaartse regulering van MpAPr1-gene-uitdrukking waargeneem word, sowel as proteaseproduksie en -aktiwiteit. Met betrekking tot die impak van *M. pulcherrima* IWBT Y1123 mede-inenting met *S. cerevisiae* op wyn eienskappe aan die einde van fermentasie, was die totale proteïeninhoud en wasigheidsvormende potensiaal laer in vergelyking met kontrole, en die vlugtige profiel is verander. Toekomstige werk moet fokus op die verbetering van protease produksie deur *M. pulcherrima* IWBT Y1123 om die lewensvatbaarheid daarvan as kommersiële protease-produserende gissoort te verbeter. Nietemin dra die resultate wat in hierdie studie

gegenereer word, by tot kennis in beide fundamentele en biotegnologiese aspekte van proteasekresie deur *M. pulcherrima* IWBT Y1123.

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Vir Pa was kennis en geleerdheid ‘n manier van deelneem.

Biographical sketch

Carla Snyman was born in Pretoria, South Africa, on 9 December 1993 and was raised in the Western Cape. She attended Parel Vallei High School, where she matriculated in 2011. In 2013 she enrolled for a BSc degree in Molecular Biology and Biotechnology at Stellenbosch University. After obtaining her undergraduate degree in 2015, she commenced with a BSc Honours degree in Wine Biotechnology at the Institute for Wine Biotechnology, Stellenbosch University. She graduated with her Honours degree in 2016 and the following year continued with an MSc in Wine Biotechnology.

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Preface

This thesis is presented as a compilation of 5 chapters.

Chapter 1 **General introduction and project aims**

Chapter 2 **Literature review**
Regulation of extracellular protease gene expression in yeast and filamentous fungi

Chapter 3 **Research results**
Investigating the expression, secretion and activity of the aspartic protease MpAPr1 by *Metschnikowia pulcherrima* IWBT Y1123

Chapter 4 **Research results**
Investigating the impact of the protease-secreting yeast *Metschnikowia pulcherrima* IWBT Y1123 on wine properties

Chapter 5 **General discussion and conclusions**

Table of Contents

Chapter 1

General introduction and project aims

Chapter 1 - General introduction and project aims

1.1 Introduction

The myriad of ways in which the wine fermentation matrix is influenced by the growth and activity of yeasts other than the strong fermenter *Saccharomyces cerevisiae*, and how they may be exploited to enhance wine quality and processing, is just beginning to be explored (Escribano et al. 2017). Non-*Saccharomyces* yeasts of oenological origin are frequently inoculated as co-starter cultures with *S. cerevisiae* to improve aspects of wine flavour and aroma (Oro et al. 2014). This co-inoculation strategy has proved valuable in making use of indigenous resources to attain sought-after organoleptic characteristics in wine, while maintaining the fermentative properties conferred by *S. cerevisiae* (Comitini et al. 2011). However, there is limited information regarding the action by which non-*Saccharomyces* yeasts impact wine properties and how this action is regulated within the yeasts (Maturano et al. 2015). Such knowledge could allow for targeted inoculation strategies performed under conditions designed for the optimal exploitation of co-starter cultures in improving wine quality. Furthermore, facets of wine quality that could be positively affected by the use of specific non-*Saccharomyces* yeasts may extend beyond the improvement of its flavour and aroma profile.

One aspect of wine quality, specifically in white and rosé wines, generally regarded as a fault by consumers and which is therefore an important target when considering methods for improvement is that of haziness (Waters et al. 2005). A key contributor of turbidity is protein heat instability and methods for the elimination of wine haze have therefore focused primarily on the removal of grape proteins (Van Sluyter et al. 2015). Bentonite is most commonly employed by wine-makers as a fining agent, but the disadvantages associated with the use of this clay has led to the quest for alternative stabilisation strategies (Van Sluyter et al. 2015).

The use of proteolytic enzymes for their protein degradative action is a particularly appealing alternative to bentonite as this strategy minimizes wine volume loss and aroma stripping (Van Sluyter et al. 2015). In fact, the extracellular aspartic protease, MpAPr1, from a non-*Saccharomyces* yeast of oenological origin, *Metschnikowia pulcherrima* IWBT Y1123, has shown activity against grape proteins and efficacy in reducing wine haze when used as an exogenous enzyme application under winemaking conditions (Theron et al. 2018). This application furthermore led to changes in the wine volatile aroma profile, which is a potential added benefit of the amino acids released as degradation products of protease activity when metabolised by fermenting yeasts via the Ehrlich pathway.

However, inoculation of this yeast as a co-starter culture to wine fermentation for the purpose of protease secretion directly into the fermentation matrix has yet to be investigated for its ability to reduce wine haze and impact wine properties, which is what this study sought to do. Inoculation of *M. pulcherrima* IWBT Y1123 would theoretically eliminate the need for enzyme purification steps, thereby presenting a time- and cost-effective strategy that does not suffer from the same legal restrictions that should be considered for enzyme preparations (Schlander et al. 2016). Nevertheless, understanding protease regulation in *M. pulcherrima* IWBT Y1123 and those factors which control MpAPr1 production, as well as the relation between protease activity in mixed cultures of *M. pulcherrima* IWBT Y1123 and *S. cerevisiae* to its actual impact on wine properties, could prove invaluable for developing effective inoculation strategies for the reduction of wine haze.

Environmental factors that have been shown to play a regulatory role in the gene expression of extracellular proteases in yeasts and filamentous fungi include pH, temperature, sources of nitrogen, carbon and sulphur, as well as the presence of proteins (Hanson and Marzluf 1973; Gonzalez-Lopez et al. 2002; Dabas and Morschhäuser 2008; Katz et al. 2008). These are all factors relevant to the oenological environment which may therefore play a role in MpAPr1 regulation during a *M. pulcherrima* IWBT Y1123 and *S. cerevisiae* mixed culture fermentation. Due to the preference of fungi for low molecular weight sources of nitrogen such as ammonium and amino acids for nutrition, elements such as proteases that are involved in the utilization of alternative nitrogen sources, such as protein, are often under control of mechanisms subject to nitrogen catabolite repression. Thus, the presence of preferred sources repress protease activity, which is derepressed and induced in the absence of preferred sources and presence of protein, respectively, as observed in the pathogenic yeast *Candida albicans* (Dabas and Morschhäuser 2008). In this study, the impact of nitrogen sources and protein availability on protease production and activity by *M. pulcherrima* IWBT Y1123 was investigated.

This study furthermore sought to relate protease activity in mixed cultures of *M. pulcherrima* IWBT Y1123 and *S. cerevisiae* in grape juice to its impact on the grape protein content of the wine, haze formation potential, and wine volatile aroma profile. Such information would greatly assist the development of effective inoculation strategies based on the desired properties of the final wine, taking into account the effect of secreted enzymatic activities and microbial interactions during fermentation.

1.2 Project aims

The aim of this study was to generate a deeper understanding of the interaction of the protease-producing yeast *M. pulcherrima* IWBT Y1123 with its environment, regarding the

influence of some environmental factors on protease production and activity, as well as the impact of protease production and activity on the oenological environment and, ultimately, wine properties.

The specific objectives of the study were as follows:

1. To assess the influence of low molecular weight nitrogen sources in the presence and absence of protein on protease production and activity (and vice versa) when *M. pulcherrima* IWBT Y1123 was grown in minimal medium or synthetic grape juice.
2. To monitor *MpAPr1* expression and protease activity in grape juice inoculated with *M. pulcherrima* IWBT Y1123.
3. To relate protease activity in mixed cultures of *M. pulcherrima* IWBT Y1123 and *S. cerevisiae* in grape juice to its impact on the grape protein content of the wine, haze formation potential, and wine volatile aroma profile.

This thesis is composed of a review of the literature surrounding the current knowledge on extracellular protease regulation in yeasts and filamentous fungi, followed by two research chapters. Objectives 1 and 2, which involve aspects of protease regulation and gene expression, are addressed in Chapter 3, whereas Chapter 4 is focused on the impact of *M. pulcherrima* IWBT Y1123 on wine properties as stipulated by Objective 3.

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Chapter 2

Literature review

Regulation of extracellular protease gene expression in yeast and filamentous fungi

Chapter 2 - Regulation of extracellular protease gene expression in yeast and filamentous fungi

2.1 Introduction

Proteases are widely distributed across all forms of life including vertebrates, plants, bacteria, fungi, as well as retroviruses (Davies 1990). They constitute a large group of enzymes which act as catalysts in the hydrolysis of peptide bonds in proteins (Rao et al. 1998). Whereas extracellular proteases generally lead to the degradation of exogenous protein sources into smaller molecules for subsequent absorption by the cell, intracellular proteases are integral to processes of metabolic regulation. Microbial extracellular proteases in particular have been studied extensively due to their biotechnological potential in various industrial and pharmaceutical applications, as well as their implications in contributing to the virulence of some pathogenic organisms (de Souza et al. 2015).

Fungi are often the preferred source for the production of exogenous protease preparations due to various technical and economic advantages, such as ease of biomass filtration from the culture supernatant and the speed of their growth (Saran et al. 2007). Such preparations are of great commercial value to the food, beverage, leather, pharmaceutical, medical and detergent industries (Theron and Divol 2014). Moreover, these protease-producing yeast and filamentous fungi also find applications as starter cultures in industries of fermented food, beverage, and bioprocessing, where they secrete their hydrolytic enzyme directly into the matrix that would benefit from its activity without the need for additional enzyme recovery steps (Kitano et al. 2002; Singh 2002; Breuer and Harms 2006).

Whether extracellular fungal proteases are being produced for exogenous enzyme preparations, or secreted by an inoculant directly into the medium for which the proteolytic action is intended, the regulatory mechanisms which govern protease production play a role. These pathways guide the response of fungi to external environmental conditions and changes, including that of protease expression and secretion (McCotter et al. 2016). It is therefore greatly beneficial for industrial applications of fungal proteases to understand these mechanisms and how they influence protease yield. Factors such as nutrient limitation of nitrogen, carbon and sulphur sources, the presence of exogenous protein, as well as temperature and pH all play a role in regulating extracellular protease production (Hanson and Marzluf 1973; Ogrydziak 1993; Peñalva and Arst 2004; Dabas and Morschhäuser 2008; Katz et al. 2008). They do so at the transcriptional level, using molecular pathways with regulatory elements that are often conserved between yeast and filamentous fungi.

Understanding how the protease is regulated and the properties of the regulated protease will aid in understanding how to optimise extracellular fungal protease production

and anticipate protease secretion under specific environmental conditions and the effects thereof on the dedicated matrix. This knowledge could furthermore be useful in determining targeted strategies for the improvement of extracellular protease production in fungi.

This review will focus on various elements of extracellular protease production by fungi. A brief overview of the ecological aspects and biological importance of protease production in nature will be provided, as well as a comparison between different yeast and fungi regarding their regulatory responses to various environmental factors in terms of protease production. The molecular mechanisms employed for protease regulation will be discussed for organisms that have been investigated to this extent, and a short summary of the biotechnological applications of protease-producing fungi as inoculants to food, beverage, and bioremedial fermentations is included.

2.2 Ecological aspects of protease-producing fungi

The ability of fungi to occupy many diverse environments, each governed by a set of conditions which dictate the unique obstacles to and opportunities for survival, have led to the development of numerous adaptive strategies utilised by fungi to exploit their specific surroundings and ensure proliferation therein (McCotter et al. 2016). The interaction of fungal organisms with the biotic and abiotic elements that characterise their environment, including the available compounds that may be utilised as nutrients as well as other organisms that could be exploited as hosts or symbionts, is often mediated by the properties and action of various proteins secreted by fungi into their environment (Krijger et al. 2014). The secretion of hydrolytic enzymes in particular plays a significant role in how fungi interact with their surroundings, for example, by making nutrients available to themselves and surrounding organisms or by causing damage to a host organism (McCotter et al. 2016).

2.2.1 Biological function

Extracellular proteases in particular play a critical role in many physiological and pathological processes, mediated by their degradative action on exogenous protein through catalysing the cleavage of peptide bonds (Rao et al. 1998). In general, the smaller peptide and amino acid molecules released as a result of this extracellular hydrolytic activity are absorbed by the cell as sources of nutrition, specifically of nitrogen, carbon and sulphur (Rao et al. 1998). This method of nutrient acquisition is therefore of great advantage to fungi under conditions limited in preferred sources of these nutrients, such as ammonia, glucose, and sulphate. Indeed, to avoid starvation fungi employ complex regulatory mechanisms involving the secretion of extracellular proteases to ensure the use of alternative nutrient sources, such as protein (Hensel et al. 1995).

Protease-secreting fungi therefore contribute extensively to ecosystem processes such as the decomposition of organic carbon and transformations of nitrogen (Sims and Wander 2002). Extracellular proteases have also been associated with sporulation and spore germination, specifically through the breakage of cell wall polypeptide linkages (Rao et al. 1998). An additional and important role of extracellular protease is in the virulence of some protease-secreting fungal pathogens. It has been described for several species including the human pathogens *Candida albicans* and *Aspergillus fumigatus* (Hensel et al. 1995). The mechanisms by which they contribute to disease have been shown to involve the hydrolysis of structural proteins in host cells, as well as factors of host immunity (Cassone et al. 2016).

2.2.2 Ecological niche

The different types of secreted fungal proteases vary in their properties and response to environmental conditions, and may play a role in determining where a specific protease-secreting organism occurs in nature. It is therefore unsurprising that the regulation of specific proteases in fungi often lead to their production under conditions which favour their activity (Gonzalez-Lopez et al. 2002). Secreted fungal proteases can be largely classified according to the position of the peptide bond that they cleave relative to the polypeptide chain, the nature of the amino acid at the enzyme active site, and the pH range in which optimal activity occurs (Theron and Divol 2014). They can thus be subdivided into exopeptidases which cleave near the termini of polypeptide chains, or endopeptidases which are defined by their tendency to act on the internal polypeptide chain. Exopeptidases are additionally classified as either amino- or carboxypeptidases based on their site of action at the N- or C-terminus, respectively (Theron and Divol 2014). The classification of aspartic, cysteine, metallo, threonine and serine protease is furthermore assigned on the basis of catalytic action. Further categorisation as acid (aspartic and cysteine), alkaline (serine and metallo) or neutral (threonine) proteases depends on the pH at which they are active (Theron and Divol 2014).

Proteases belonging to these various groups are widely distributed across fungi that occupy a myriad of ecological niches, each with unique properties. These range from the decaying organic matter inhabited by the aspartic protease-producing saprophytes *Aspergillus nidulans* and *Neurospora crassa*, and plants infected with *Fusarium oxysporum* with alkaline protease-secreting ability, to nematodes acting as host to the pathogenic serine protease-secreting *Clonostachys rosea*, keratin-rich human skin and nails occupied by the dermatophyte *Trichophyton rubrum*, and fermented food products populated with diverse species of *Yarrowia lipolytica*, *Debaryomyces hansenii*, *Aspergillus oryzae* and *Penicillium* spp. (Barth and Gaillardin 1997; St Leger et al. 1997; Caracuel et al. 2003; Breuer and

Harms 2006; Silveira et al. 2010; Zou et al. 2010c). The protease-producing cold adapted yeasts *Candida humicola* and *Rhodotorula mucilaginosa* have even been isolated from the Antarctic (Ray et al. 1992; Chaud et al. 2016).

Despite the genetic, proteomic and metabolic differences that inevitably occur across species and habitats, as fungi adapt and evolve to survive the unique conditions of their singular environment, there is nevertheless evidence that the impact of phylogenetic history is greater than the role that lifestyle adaptation has to play in determining the composition of the fungal secretome (Krijger et al. 2014). This finding could account for the high level of conservation observed in particular for aspartic proteases between fungal species (and even between fungi, viruses and mammals) (Cassone et al. 2016). It could also explain the great deal of homology evident between different yeast and filamentous fungi in the factors employed by the regulatory mechanisms used for extracellular protease response to environmental stimuli such as nutrient limitation and pH (Gonzalez-Lopez et al. 2002; Dabas and Morschhäuser 2008).

2.3 Regulation of fungal extracellular proteases

Knowledge regarding the regulation of protease synthesis and the signalling pathways that govern protease expression in yeasts and fungi is limited, although significant progress has been made in recent decades towards understanding those factors that play a role in the secretion of this enzyme (Gonzalez-Lopez et al. 2002). In most cases, extracellular protease production is tightly regulated and complex, all the more so in fungi to ensure efficient secretion despite the cell wall barrier, and responds to a combination of environmental stimuli (Gonzalez-Lopez et al. 2002; McCotter et al. 2016). For example, nutrient availability, pH, and temperature are all factors which influence protease production and its regulation (Figure 2.1). Presumably, this would be to avoid unnecessary protease production and energy expenditure when activity is not required, or when conditions are not suitable for optimal activity. The various regulatory stimuli may furthermore influence protease expression to different degrees in a hierarchical fashion, contributing to the complexity observed in protease regulation (Jarai and Buxton 1994).

Nevertheless, in this review, the pertinent factors will be dealt with individually, allowing an overview and comparison of the mechanisms employed by species of yeast and filamentous fungi in response to each. In most cases, the biochemical basis for protease regulation is found at the level of gene transcription, mediated by stimuli-dependent transcription factors and cell signalling pathways. Regulatory system homologues are evident between members of the Ascomycota, and therefore, filamentous fungi, dimorphic

yeasts and budding yeasts will be discussed together. Due to the considerable biotechnological interest in the secreted proteases of *Aspergillus* spp. and the prevalence of the pathogenic *C. albicans* yeast in the medical field, much of the research regarding protease production has focused on these species and their regulatory mechanisms are thus very well described. The systems employed by organisms involved in the production of food and beverage, such as *Y. lipolytica* and *A. oryzae*, are not yet as detailed and will therefore be presented in this context. A summary of the stimuli in control of extracellular protease expression in the various fungal organisms is presented in Table 2.1.

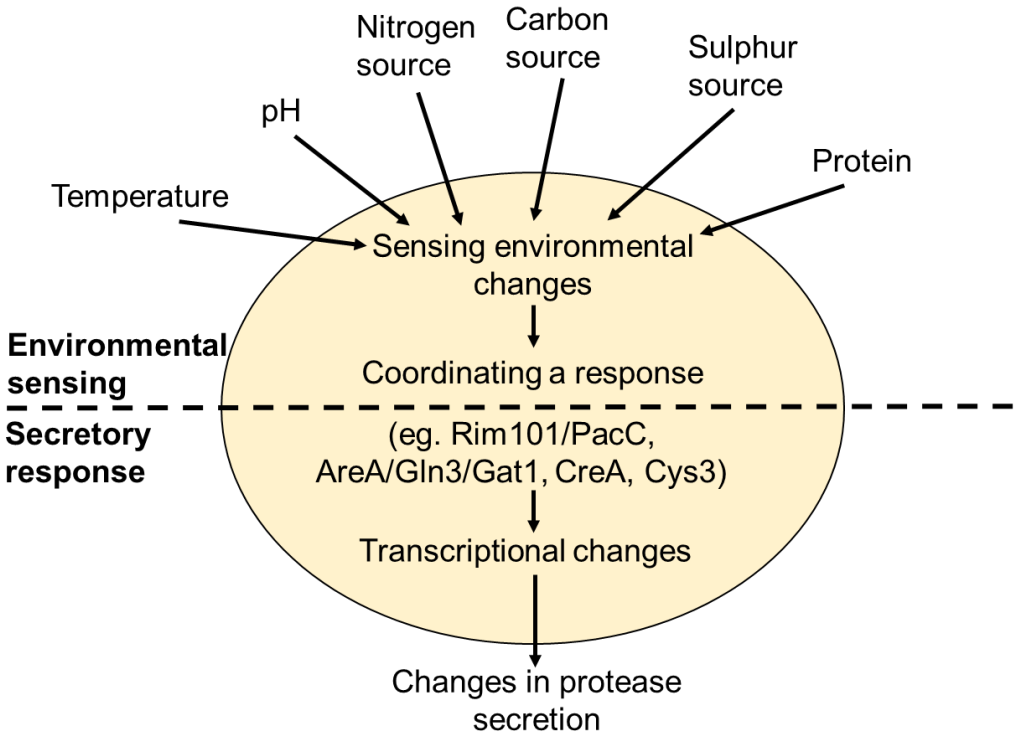

Figure 2.1 Examples of environmental factors that act as stimuli for coordinating a transcriptional response of protease expression. This response is mediated through various stimuli-dependent regulatory pathways, some of which employ elements that show homology across organisms. This regulatory response ultimately leads to changes in protease secretion. Adapted from McCotter et al. 2016.

2.3.1 Extracellular pH

Many microorganisms tailor their gene expression to the pH of their environment, particularly if they are able to grow over a wide pH range (Peñalva and Arst 2002). The pH occurring in the production of fermented foods and beverages therefore plays a significant role in the regulation of these genes, and is often a dynamic and changing environmental factor to which the organism must adapt accordingly. For example, the increase in pH observed in cheese production from the time of fermentation to the end of ripening can be substantial, especially in mould or surface-ripened cheeses (Upreti and Metzger 2007; Lee and Bae 2018). A pH regulatory system is thus useful to ensure that extracellular enzymes are

secreted under the pH conditions required for their activity (Denison 2000). For instance, *Y. lipolytica* secretes both an acid extracellular protease (Axp) and an alkaline extracellular protease (Aep), produced under acidic and neutral to alkaline environmental pH conditions, respectively (Ogrydziak 1993; Young et al. 1996; Glover et al. 1997). The type of protease secreted is therefore directly dictated by the pH of the medium. Thus, *Y. lipolytica* will continue to secrete its alkaline protease as the ambient pH approaches neutrality in ripening cheese (Watkinson et al. 2001).

Aep is produced when the *XPR2* gene is activated, which is shown to rely on the zinc finger-containing transcription factor YlRIM101p, homologous to the RIM101p and PacC factors which play a role in pH response in *C. albicans* and filamentous fungi, respectively (Tilburn et al. 1995; Ramon et al. 1999; Gonzalez-Lopez et al. 2002; Villar et al. 2007; Blanchin-Roland et al. 2008). These fungi share a conserved pH signalling pathway, called Pal in filamentous fungi and Rim in yeasts (Blanchin-Roland et al. 2008). After an initial investigation of the pathway in *A. nidulans*, the description was later extended to several other extracellular protease-producing ascomycetes, including *Y. lipolytica*, *C. albicans*, *C. rosea*, *T. rubrum* and *F. oxysporum*, as well as the basidiomycete *Ustilago maydis* (Arst et al. 1994; Glover et al. 1997; Caracuel et al. 2003; Aréchiga-Carvajal and Ruiz-Herrera 2005; Villar et al. 2007; Silveira et al. 2010; Zou et al. 2010b; Martinez-Rossi et al. 2011). Genes with homology to elements of the Pal pathway have additionally been identified in *Aspergillus niger* and *A. oryzae*, suggesting that the pathway is conserved in these fungi as well (Denison 2000). Indeed, pH regulation of the alkaline (*aplA*) and neutral (*nptB*) protease-encoding genes could be observed in *A. oryzae* as they were induced when ambient pH approached neutrality (te Biesebeke et al. 2005).

In *A. nidulans*, the products of six genes, *palA*, *palB*, *palC*, *palF*, *palH* and *palI*, transmit the pH signal to PacC under ambient alkaline conditions (Peñalva and Arst 2004). This ultimately leads to a conformational change in PacC, which is most likely the principle, and perhaps sole, form of this transcription factor to mediate expression of pH-sensitive genes (Figure 2.2) (Mingot et al. 2001; Peñalva and Arst 2004). It does so by activating alkaline-expressed genes and repressing acid-expressed genes under alkaline conditions, whereas under acidic conditions neither phenomenon occurs (Espeso and Peñalva 1996; Espeso and Arst 2000; Peñalva and Arst 2004). Indeed, expression of the alkaline protease-encoding gene *prtA* is elevated at alkaline ambient pH in *A. nidulans*, as is also the case for Aep in *Y. lipolytica* (Tilburn et al. 1995; Gonzalez-Lopez et al. 2002).

However, research suggests that in *Y. lipolytica,* the Rim pathway might still be active under acidic conditions, leading to levels of activated Rim101p too low for *XPR2* transcription, but which is required for the optimal induction of the Axp-encoding gene *AXP1* (Gonzalez-Lopez et al. 2002). In the dimorphic fungus *C. albicans* on the other hand,

environmental pH serves as a signal not only for the differential expression of its various aspartic proteinases, but also for cellular differentiation and development, particularly in terms of morphology (Chen et al. 2002; Davis 2003). Acidic conditions favour yeast growth, whereas alkaline conditions favour hyphal growth. A gene family of at least nine members (*SAP1* to *SAP9*) is responsible for the expression of secreted aspartic proteases in *C. albicans*, amongst which differences in regulation during phenotypic switching has been shown (Hube et al. 1997). The major proteases secreted by yeast cells under acidic conditions are Sap1, Sap2 and Sap3, whereas *SAP4* to *SAP6* are expressed during the yeast-to-hypha transition nearing neutral pH (White and Agabian 1995; Hube et al. 1997; Chen et al. 2002).

Environmental pH therefore plays a critical role in the regulation of many fungal proteases, and in some cases takes precedent in the hierarchy of regulatory phenomena, as observed for the aspartic protease-encoding genes *pepA* and *pepB* of *A. niger* that are not expressed under alkaline conditions, even when derepressed by the absence of preferred nitrogen and carbon sources and induced in the presence of protein (Jarai and Buxton 1994).

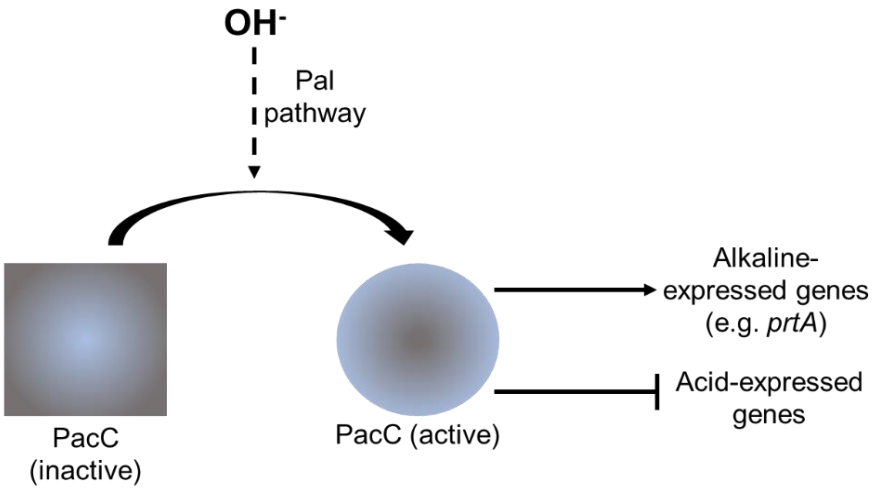

Figure 2.2 Model of protease regulation by pH in *A. nidulans* via Pal pathway signalling and PacC activation. Active PacC acts positively on alkaline-expressed genes, including the alkaline protease-encoding gene *prtA*, and negatively on acid-expressed genes. Under acidic conditions the pathway is abolished leading to a lack of alkaline gene activation and the derepression of acid-expressed genes. Adapted from Arst & Peñalva 2003.

2.3.2 Nitrogen limitation

Nitrogen forms a critical component of nearly all the macromolecules essential to the structure and function of living organisms. It is therefore unsurprising that the mechanisms in control of its uptake by most eukaryotes and prokaryotes are quite elaborate (Marzluf 1997a). Yeast and filamentous fungi are capable of utilising a diverse array of nitrogen

sources, and have well-developed regulatory mechanisms in place to do so (Wiame et al. 1985). Proteins are one such source that can be utilised to fulfil the nitrogen requirements of the organism, when degraded by extracellular proteases into oligopeptides and amino acids which are then taken up into the cell by dedicated oligopeptide transporters (Ramachandra et al. 2014).

However, certain sources of nitrogen, such as ammonia and amino acids, are preferentially used by fungi and so the utilisation of any secondary sources, such as proteins, is highly regulated (Marzluf 1997a). In fact, the genes encoding elements of the pathway, such as proteases, required for the utilisation of alternative nitrogen sources are nearly always otherwise under nitrogen catabolite repression (NCR), which prevents expression of these genes in the presence of preferred nitrogen (Dabas and Morschhäuser 2008). It is only when the absence of such sources permits a global signal indicating nitrogen derepression that genes required for the utilisation of alternative sources are activated to ensure continued nutrient uptake (Marzluf 1997a).

The global regulatory genes that play a role in this pathway and mediate nitrogen catabolite derepression have been shown to specify GATA-type zinc finger-containing transcription factors, and include *areA* in *A. nidulans*, *A. fumigatus* and *A. oryzae*, *GLN3* and *GAT1* in *C. albicans*, *nit-2* in *N. crassa*, *nmc* in *Penicillium roqueforti* and *nre* in *Penicillium chrysogenum*, which control extracellular protease expression in these organisms (Arst and Cove 1973; Fu and Marzluf 1987; Haas et al. 1995; Christensen et al. 1998; Gente et al. 1999; Dabas and Morschhäuser 2008). A bulk of the research performed on the mechanism behind the nitrogen-controlled regulation of extracellular proteases has focused on *C. albicans* and its Sap2 isoenzyme, which is the major *in vitro* secreted protease (Lerner and Goldman 1993; Hube et al. 1994; White and Agabian 1995; Martínez and Ljungdahl 2005; Reuß and Morschhäuser 2006). The general transcription factors Gln3p and Gat1p mediate the NCR response of *SAP2* in *C. albicans* under nitrogen limiting conditions by increasing expression of *STP1*, a gene encoding a specific transcription factor which is targeted to the nucleus and where it increases the expression of *SAP2* (Figure 2.3) (Dabas and Morschhäuser 2008). However, when preferred nitrogen sources such as ammonia and amino acids are available, Gln3p and Gat1p are retained in the cytoplasm and cannot activate their target genes, thus protease expression is repressed (Dabas and Morschhäuser 2008).

A similar response to nitrogen sources in terms of extracellular protease expression is also observed in numerous fungi related to food production. Thus the regulation of nitrogen metabolism affects the proteolytic chain during food fermentation and ripening, which may play a significant role in sensory perception as well as microbial succession and the fermentation process as a whole (Bolumar et al. 2006). In *Y. lipolytica*, availability of

preferred nitrogen sources led to the repression of its extracellular alkaline protease, a similar observation to that made for the aspartic (PrA) and serine (PrB) proteases of *D. hansenii* (Ogrydziak et al. 1977; Bolumar et al. 2006; Akpınar et al. 2011). Furthermore, Boutrou et al. (2006) showed that the hydrolysis of casein on the surface of cheese occurred earlier in the growth stages of *Geotrichum candidum* when simpler peptides were not initially available. A similar nitrogen catabolite repression mechanism has been described for the expression of the aspartic protease-encoding gene *aspA* in *P. roqueforti*, the extracellular carboxyl protease gene in *Rhizopus oligosporus*, and for *A. oryzae* (Farley and Ikasari 1992; Christensen et al. 1998; Gente et al. 1999).

The response of fungal protease expression to compounds in their environment that can act either as repressors (sufficient amounts of ammonium and amino acids) or inducers (protein), of which both are in abundance in food fermentations, could have a substantial impact on fungi physiology and adaptation to nutrient availability (Bolumar et al. 2006). Food fermentation processes being highly competitive microenvironments, such adaptation strategies could significantly impact the outcome of these industrial fermentations.

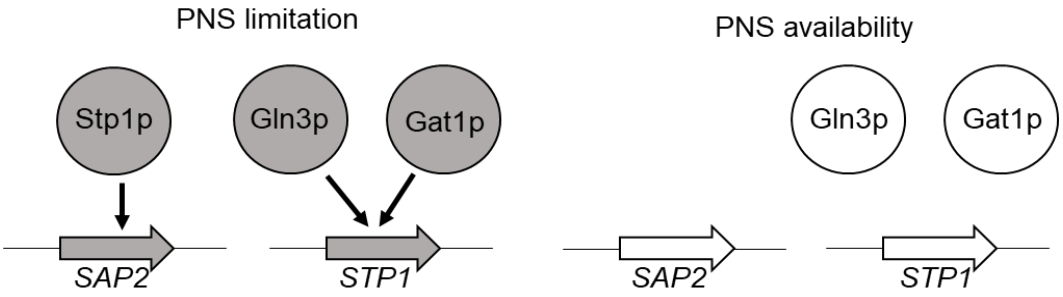

Figure 2.3 Regulation of *SAP2* expression by *C. albicans* with preferred nitrogen source (PNS) limitation or availability. In the presence of preferred nitrogen, the GATA transcription factors Gln3p and Gat1p are retained in the cytoplasm and do not activate *STP1*. However, when preferred nitrogen is limiting, *STP1* is activated and its product is targeted to the nucleus where it induces *SAP2* expression.

2.3.3 Carbon limitation

Additionally to nitrogen sources, the availability, quality and complexity of the carbon sources influence the secretome composition of fungi, including proteases (McCotter et al. 2016). This is because fungi are capable of utilising proteins not only as sources of nitrogen, but also of carbon, and do so via protein degradation by proteases (Hensel et al. 1995).

In *A. nidulans*, extracellular proteases are produced in response to carbon starvation (Cohen 1973). The transcription factor XprG has been implicated in mediating this response, as demonstrated by mutation studies in which a disruption mutation in the *xprG* gene led to abolished protease production in response to carbon starvation (Katz et al. 2006). Furthermore, wide-domain catabolic repression by preferred carbon sources such as

glucose has been shown to require the zinc finger-containing transcription factor CreA, and in *A. nidulans* the activation of this gene leads to the repression of protease expression (Hensel et al. 1995; Katz et al. 2008). However, the fact that extracellular protease levels increased in response to carbon starvation in *creA* mutants indicate that this transcription factor may play a role in the activation of protease expression in response to carbon starvation as well as their repression in the presence of preferred carbon sources as part of the carbon catabolite repression (CCR) pathway, much the same way that AreA mediates the NCR pathway in *A. nidulans* (Cohen 1972; Katz et al. 2008). Furthermore, an XprG-dependent increase in protease activity in a *creA* mutant was observed, which could indicate that an interaction between these genes or their products is involved in the regulation of extracellular proteases in response to carbon, perhaps in a manner synonymous with that of *GLN3* and *GAT1* with *STP1* in *C. albicans* in response to nitrogen (Dabas and Morschhäuser 2008; Katz et al. 2008).

Homologues of the CreA regulator have been found in *A. niger* and *A. oryzae* (Drysdale et al. 1993; Tanaka et al. 2018). Indeed, in *A. niger* carbon source depletion similarly led to the derepression of extracellular proteases, although surprisingly in *A. oryzae*, the protease-encoding genes *alpA* and *nptB* continued to be transcribed despite the presence of high concentrations of sugar, which demonstrates the differences in regulation that may occur between species (Jarai and Buxton 1994; te Biesebeke et al. 2005; Braaksma et al. 2009). Furthermore, protease production in the absence of a carbon source has previously been observed for *N. crassa*, and indeed an *xprG* homolog, *vib-1*, was found in this organism and shown to be required for the production of extracellular proteases upon carbon starvation (Cohen et al. 1975; Dementhon et al. 2006).

In *Y. lipolytica*, the addition of glucose served to reduce production of its alkaline protease whereas derepression of protease production was evident upon transfer to a carbon-free medium, confirming the regulation of protease production by a CCR pathway (Ogrydziak et al. 1977; Akpınar et al. 2011). Similarly, growth of *D. hansenii* on acetate, a poor carbon source, resulted in increased protease synthesis, and protease levels were 3-fold higher when *R. oligosporus* was inoculated into carbon-free medium than in glucose-containing minimal medium (Farley and Ikasari 1992; Bolumar et al. 2006). *Chrysosporium keratinophilim* responded in a similar fashion to glucose as a source of easily available carbon, which inhibited protease yield (Singh 2002). Presumably, the appearance of this response, characterised by the repression of protease activity in the presence of preferred carbon sources and derepression when these sources are limiting, is due to regulation by CCR mediated through a CreA homologue in these organisms.

2.3.4 Sulphur limitation

Various sulphur-containing compounds, especially cysteine, methionine and S-adenosylmethionine, are required for cell growth and activity (Marzluf 1997b). Because of the pivotal role that these compounds play in the initiation of protein synthesis, protein structure, stability and catalytic function as well as methyl group transfer and polyamine biosynthesis, fungi employ a complex regulatory circuit to ensure a steady supply of sulphur (Marzluf 1997b).

Much like the repression mechanisms in place for nitrogen and carbon regulation, elements under sulphur regulation include a diverse set of permeases and enzymes, some of which are involved in the utilisation of alternative sulphur sources, such as aromatic sulfate esters or proteins. Similarly to NCR and CCR, the sulphur catabolite repression (SCR) mechanism regulates these elements at the transcriptional level in response to the availability of preferred sources of sulphur (Marzluf 1997b). Under conditions of sulphur limitation, many fungi synthesise and secrete extracellular proteases in order to assimilate the cysteine and methionine residues from peptide degradation products released as a result of protease activity. However, the presence of preferred sulphur sources such as inorganic sulfates serves to repress protease expression (Paietta 2016).

This SCR mechanism is evidently at work and in control of extracellular protease production in *Y. lipolytica*, *R. oligosporus*, *A. niger* and *Mucor miehei,* as well as the zygomycete *Rhizopus oryzae* (Tomonaga et al. 1964; Ogrydziak et al. 1977; Lasure 1980; Farley and Ikasari 1992; Young et al. 1996; Farley and Sullivan 1998). However, the molecular basis behind protease regulation by SCR has been studied most extensively in *A. nidulans* and *N. crassa* (Cohen 1973; Hanson and Marzluf 1973; Paietta 2016).

The product of a putative *sulphur controller-1* (*scon-1*) gene in *N. crassa* is thought to play the role of a sulphur sensor, whereas *cys-3*$^{+}$ encodes a basic region-leucine zipper (bZIP) DNA-binding regulator protein (Burton and Metzenberg 1972; Marzluf 1997b). The level of Cys3 protein is subject to autoregulation – the control of which is the key factor by which the sulphur regulatory system exerts its ultimate effect (Figure 2.4) (Paietta 2016). Whereas Cys3 acts as a transcriptional activator of sulphur-related genes, *scon-1* and two more genes that have subsequently been given ‘sulphur controller’ designation, *scon-2* and *scon-3*, are defined as negative regulators of the sulphur regulatory system (Paietta 2016). Presumably, the sulphur-sensing function of the *scon-1* gene product leads to a change in the phosphorylation of Cys3, depending on whether sulphur conditions are limiting or sufficient. For example, the availability of sulphur may lead to the phosphorylation of Cys3, rendering its binding to the Scon2 F-box protein possible. This interaction sequesters the Cys3 protein, thus blocking transcriptional activation of sulphur-related genes (Paietta 2016).

On the other hand, should sulphur conditions become limiting as sensed by Scon1, Cys3 may exist in an alternate state (dephosphorylated), thus not favouring the binding to Scon2 and allowing for sufficient levels of Cys3 to induce sulphur-related gene expression (Paietta 2016). In this way, protease synthesis also is regulated by sulphur availability as mediated by the *cys-3*$^{+}$ control gene in *N. crassa* (Hanson and Marzluf 1973).

Homologues of the *scon* negative regulator are found in *A. nidulans* and termed *sconA*, *sconB*, *sconC* and *sconD*, whereas MetR is the transcriptional activator that corresponds to Cys3 (Natorff et al. 1993; Natorff et al. 2003). However, in contrast to *cys-3*$^{+}$, *metR* is not regulated by sulphur source or subject to autoregulation (Natorff et al. 2003). Furthermore, unlike *N. crassa*, extracellular protease production is not subject to control by the *scon* gene in *A. nidulans*, thus the regulation of extracellular proteases that is observed in response to sulphur limitation may be independent of sulphur-containing amino acid biosynthetic pathway control (Katz and Flynn 1996). Thus, although similarities exist between the sulphur regulatory mechanisms of *A. nidulans* and *N. crassa*, and indeed also between these and the pathway utilised by *S. cerevisiae* in response to sulphur utilisation, there are also many apparent differences (Paietta 2016). Further investigation into the molecular mechanisms behind these differences, and into the specific control of extracellular protease expression in response to sulphur availability, will help to gain a deeper understanding of the impact that sulphur or its limitation has on fungal extracellular protease production.

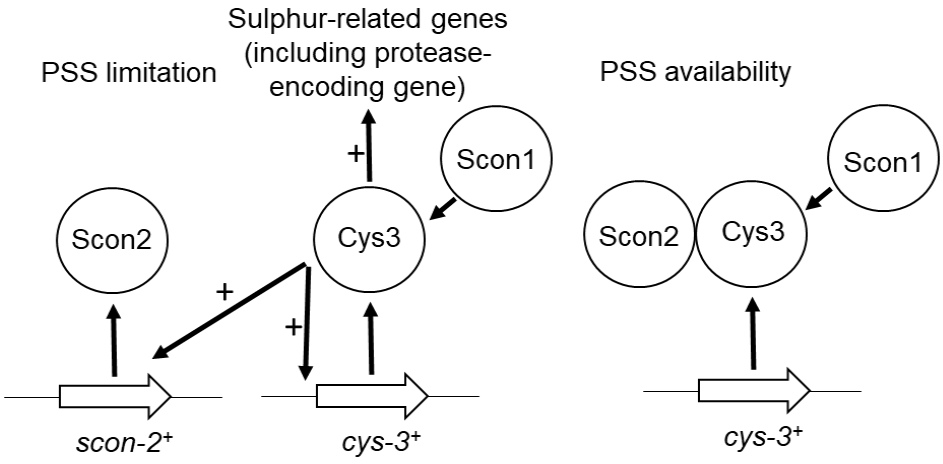

Figure 2.4 Sulphur regulation of protease expression in *N. crassa*. Under limiting conditions of preferred sulphur sources (PSS), the sulphur sensor Scon1 leads to a certain conformational state of the transcriptional activator Cys3, which does not favour binding with the F-box protein Scon2, thus allowing binding activation of *cys-3*$^{+}$ (autoregulation), *scon-2*$^{+}$ and sulphur-related genes such as the one encoding an extracellular protease. However, when preferred sulphur sources are available, Scon2 binds to the particular conformation of Cys3 which sequesters this transcriptional activator and prevents transcription of sulphur-related genes. Adapted from Paietta 2016.

2.3.5 Exogenous protein

Protease production is frequently influenced by an additional, positive signal that may accompany the derepression response elicited by either nitrogen, carbon or sulphur limitation (or any combination of the three nutrients) (Katz et al. 2008). Such a signal is provided by exogenous protein substrate or degradation products thereof, and its regulatory action on protease secretion varies among yeast and filamentous fungi (Katz et al. 2008).

N. crassa, *M. miehei* and *C. albicans* require this signal in addition to the derepression response for protease production, whereas the presence of exogenous protein merely increases protease expression in nutrient-starved *A. niger* (Cohen et al. 1975; Lasure 1980; Jarai and Buxton 1994; Dabas and Morschhäuser 2008). On the other hand, Katz et al. (2008) showed that protein induction of extracellular proteases did not occur in *A. nidulans* even under conditions of nutrient limitation, and hypothesised this to be due to the lack of a transcription factor, PrT, required for protease production in *A. niger* and found in the genomes of numerous other *Aspergillus* species (Punt et al. 2008). *R. oligosporus* showed a similar lack of protease induction by protein in medium deficient in nitrogen, carbon or sulphur (Farley and Ikasari 1992). Like *A. niger*, however, the presence of protein further increased protease expression in *P. roqueforti*, *R. oryzae* and *D. hansenii* in the absence of preferred nitrogen sources (Gente et al. 1997; Farley and Sullivan 1998; Bolumar et al. 2006). *Y. lipolytica* did not require exogenous protein for protease production although its presence enhanced production, and did so even in the presence of the preferred nitrogen source ammonia (Ogrydziak et al. 1977). However, this seems to be the exception to the rule that catabolite repression by nitrogen, carbon and sulphur is predominant over induction by exogenous protein (Gente et al. 1997). Furthermore, different protein substrates induced significantly different levels of protease secretion, as well as differential secretion of three proteases in *A. fumigatus*, demonstrating the complexity of protease regulation by protein substrate (Farnell et al. 2012)

Very little is known about the regulatory mechanism behind protease induction in fungi which has been postulated to involve a specific pathway like that found in nitrogen, carbon and sulphur repression, controlling protease expression on a transcript level (Gente et al. 1997). Another hypothesis put forward by Hanson and Marzluf (1973) states that exogenous protein may influence protease secretion rather than its synthesis through the interaction of protein with membrane-bound protease at the cell surface (Figure 2.5). A more general metabolic effect is also possible, in which exogenous protein might indirectly influence protease-encoding gene transcripts through the provision of metabolic intermediates serving as substrates for energy production or precursors for nucleotide synthesis (Farley and Sullivan 1998). It has also been suggested that it is the peptide

degradation products of exogenous proteins that occur as a result of a basal level of proteolytic activity that serve to increase protease expression via a positive feedback mechanism (Hube et al. 1994; Bolumar et al. 2006).

It has subsequently been shown that *SAP2* expression in *C. albicans* requires the presence of micromolar concentrations of amino acids, produced during the degradation of proteins by basal levels of extracellular protease activity, thus serving as an indication of the presence of extracellular proteins (Martínez and Ljungdahl 2005). Extracellular amino acids are sensed at the cell surface by the SPS sensor, a plasma membrane-localised sensor complex, which then leads to the proteolytic activation of the transcription factor Stp1p (Martínez and Ljungdahl 2005). However, even in the presence of proteins as an alternative nitrogen source, *SAP2* expression is repressed when preferred nitrogen sources are available in amounts sufficient for the nutrient requirements of the cell, because *STP1* is downregulated to levels that may not allow *SAP2* expression (Dabas and Morschhäuser 2008). Thus, both the absence of preferred nitrogen sources and the presence of an alternative source is required for the sufficient induction of *SAP2* beyond basal levels in *C. albicans*.

Considering the complexity and hierarchical nature of the various regulatory factors with unique pathways that sometimes share common elements and play a role in protease production, the effects of which are furthermore often distinct between species, it is necessary to investigate the mechanism specific to an organism and its environment for an accurate description of its regulation by nutrient availability. This could be of particular importance to the bioprocessing industry, as it is often the protein substrate that determines the level and type of protease produced, although the presence of preferred nitrogen, carbon and sulphur sources depicts whether and when protease will be produced at all.

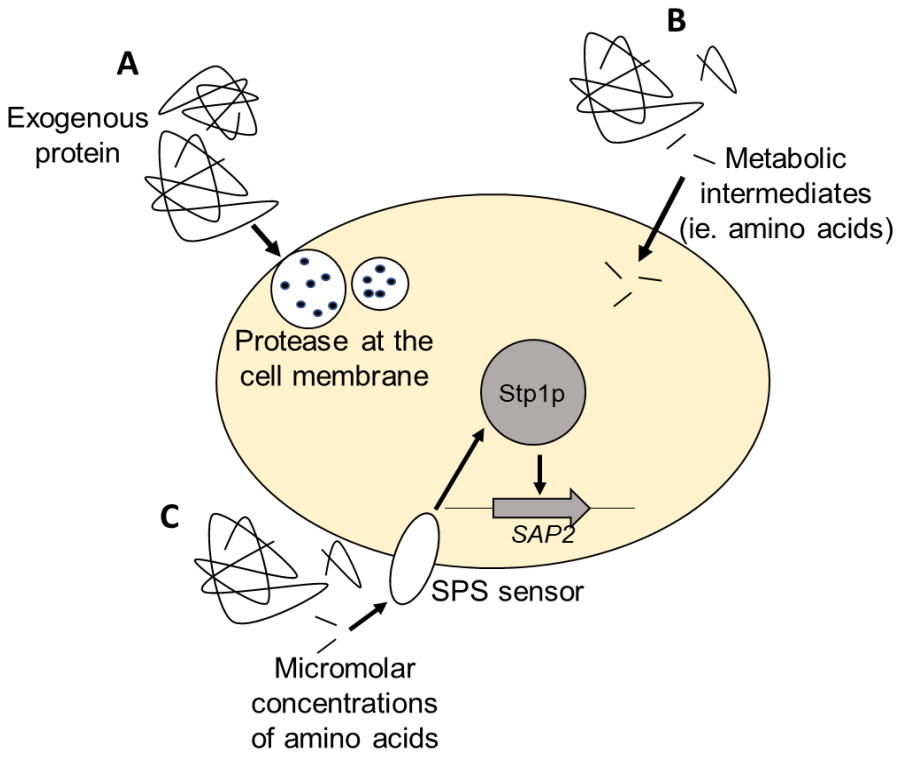

Figure 2.5 Summary of the different mechanisms proposed for protease regulation by exogenous protein. **A** The interaction of external protein with proteases at the cell surface. **B** Indirect influence on protease transcripts through the provision of metabolic intermediates such as aspartate, glycine and glutamine. **C** The regulatory pathway accepted for *C. albicans*, in which the SPS sensor at the cell surface senses micromolar concentrations of amino acids released as a result of basal levels of protease activity, thus leading to the activation of Stp1 which, in turn, increases *SAP2* expression.

2.3.6 Temperature

Despite the importance of temperature to protease activity such that optimum activity levels occur within a defined and often limited temperature range, few studies have undertaken the investigation of fungal extracellular protease regulation in response to changes in temperature beyond the optimisation of production parameters for industrial purposes. Nevertheless, temperature has been found to play an important role in the production of proteases by microorganisms (Ikram-Ul-Haq and Umber 2006).

Higher temperatures often adversely affect the metabolic activities of fungi with protease-producing abilities, which corresponds to the fact that fungal proteases are usually thermolabile with reduced activities at high temperatures (Ikram-Ul-Haq and Umber 2006). For example, protease production was tenfold higher when *C. albicans* was grown at 27°C as opposed to 37°C, and in *P. chrysogenum* protease production showed an increase from 25°C to 30°C which declined as temperature reached 45°C (Ogrydziak 1993; Ikram-Ul-Haq and Umber 2006). Similarly, the acid protease-encoding gene *pepA* was induced in a temperature-dependent fashion in *A. oryzae*, with increased expression at 30°C that decreased as temperature reached 42°C (Kitano et al. 2002).

Kitano et al. (2002) postulated that transcriptional regulation of the *pepA* promoter is responsible for temperature-dependent expression through a regulatory pathway similar to that employed for nutrient limitation signalling, as opposed to *pepA* mRNA lability, or its tendency to undergo conformational changes, at high temperatures being the cause for decreased expression. From the applied technology viewpoint, this temperature-dependent regulation is of great interest to control extracellular protease expression, for instance of *pepA* by *A. oryzae* during the fermentation of rice-koji, using temperature (Kitano et al. 2002).

On the other hand, some organisms, including psychrophiles and psychrotolerant fungi such as *C. humicola* and *R. mucilaginosa*, have developed the ability to produce optimum levels of proteases at temperatures as low as 4°C (Ray et al. 1992; Chaud et al. 2016). Such an adaptive strategy could have been employed by the fungus to survive under extreme conditions, and may be exploited in industrial processes requiring high levels of protease production at low temperatures (Alcaíno et al. 2015; Chaud et al. 2016). The optimal production of the alkaline extracellular protease of *Y. lipolytica* similarly occurred at colder temperatures, specifically at 15°C which was about 10% higher than at 25°C (and 90% higher in production per cell mass) (Ogrydziak 1993).

Then again, some thermotolerant fungi display a peak of protease release at temperatures higher than those observed for mesophiles, and *C. keratinophilim* for example produces optimum protease levels at 40°C (Singh 2002). Furthermore, a heat shock response involving the rapid induction of protease expression was observed in *C. rosea* after a sudden temperature shift from 28°C to a higher temperature (33°C-37°C) (Zou et al. 2010c). Heat shock is known to induce oxidative stress in fungi, and it is possible that the degradation products released as a result of protease activity initiate a signal transduction pathway to detoxify reactive oxygen species (ROS) after entering the cell (Zou et al. 2010c). An increase in protease activity was also observed in *A. niger* after a sudden temperature increase, suggesting a similar heat shock response (Li et al. 2008). Temperature fluctuations can occur in industrial fermentation with fungal cultures, and heat shock may therefore play a role in protease productivity under these conditions (Li et al. 2008).

Table 2.1 Fungal organisms in which extracellular protease regulation by environmental factors has been under investigation for various fundamental or biotechnological research purposes. The stimuli in control of protease expression are indicated as pH, nitrogen limitation (N), carbon limitation (C), sulphur limitation (S), exogenous protein (P) and temperature (T).

Organism	Context for interest	Stimulus	Reference
Y. lipolytica	Inoculant for cheese and meat maturation	pH	(Gonzalez-Lopez et al. 2002)
		N	(Ogrydziak et al. 1977)
		C	(Ogrydziak et al. 1977)

		S	(Ogrydziak et al. 1977)
		P	(Ogrydziak et al. 1977)
D. hansenii	Inoculant for cheese and meat maturation	N	(Bolumar et al. 2006)
		C	(Bolumar et al. 2006)
		P	(Bolumar et al. 2006)
G. candidum	Inoculant for cheese maturation	N	(Boutrou et al. 2006a)
P. roqueforti	Inoculant for cheese maturation	N	(Gente et al. 1999)
		P	(Gente et al. 1997)
R. oligosporus	Inoculant for tempe production	N	(Farley and Ikasari 1992)
		C	(Farley and Ikasari 1992)
		S	(Farley and Ikasari 1992)
		P	(Farley and Ikasari 1992)
R. oryzae	Inoculant for tempe production	S	(Farley and Sullivan 1998)
		P	(Farley and Sullivan 1998)
A. oryzae	Inoculant in tempe, miso, shoyu, koji and sake fermentation, and bioremediation of potato pulp powder	pH	(te Biesebeke et al. 2005)
		N	(Christensen et al. 1998)
		T	(Kitano et al. 2002)
C. keratinophilim	Inoculant for bioremediation of keratin waste	C	(Singh 2002)
		T	(Singh 2002)
C. rosea	Biological control agent of parasitic nematodes	pH	(Zou et al. 2010a)
		T	(Zou et al. 2010a)
M. miehei	Production of aspartic protease for cheese-making	S	(Lasure 1980)
		P	(Lasure 1980)
A. niger	Production of protease preparation and host for heterologous protein production	pH	(Jarai and Buxton 1994)
		C	(Drysdale et al. 1993)
		S	(Tomonaga et al. 1964)
		P	(Jarai and Buxton 1994)
		T	(Li et al. 2008)
P. chrysogenum	Production of protease preparation	T	(Ikram-Ul-Haq and Umber 2006)
C. humicola	Production of cold-active	T	(Ray et al. 1992)

	protease for potential use in low-temperature industrial processes		
R. mucilaginosa	Production of cold-active protease for potential use in low-temperature industrial processes	T	(Chaud et al. 2016)
A. nidulans	Filamentous fungi model organism	pH	(Peñalva and Arst 2004)
		C	(Cohen 1973)
		S	(Cohen 1973)
		P	(Katz et al. 2008)
C. albicans	Pathogenic yeast model organism	pH	(Villar et al. 2007)
		N	(Dabas and Morschhäuser 2008)
		P	(Dabas and Morschhäuser 2008)
		T	(Ogrydziak 1993)
N. crassa	Model lower eukaryote for genetic regulation studies	C	(Cohen et al. 1975)
		S	(Hanson and Marzluf 1973)
		P	(Cohen et al. 1975)
T. rubrum	Dermatophytic human pathogen	pH	(Silveira et al. 2010)
A. fumigatus	Human pathogenesis	P	(Farnell et al. 2012)
F. oxysporum	Vascular wilt disease in plants	pH	(Caracuel et al. 2003)
U. maydis	Corn smut disease	pH	(Aréchiga-Carvajal and Ruiz-Herrera 2005)

2.4 Biotechnological aspects of protease-producing fungi

Extracellular proteases are not only of metabolic and physiological importance, but also hold great commercial value (Rao et al. 1998). Fungi in particular secrete a large variety of proteases that are active over a wide pH range, exhibit broad substrate specificity, and are conveniently produced in submerged and solid-state fermentations (Rao et al. 1998). The extensive specificity of proteolytic action allows application of protease preparation to a variety of processes employed by the food, beverage, leather, pharmaceutical, medical and detergent industries (Theron and Divol 2014).

Production of these preparations is achieved relatively quickly and reliably using established fermentation methods, in which this extracellular enzyme is secreted by the fungus into the cultivation media (de Souza et al. 2015). Appropriate cultivation conditions

are essential for efficient protease production, and should therefore be optimised by taking fungal protease regulation into account. As described above, parameters such as temperature, pH and media composition in terms of nitrogen, carbon, sulphur and protein content, may all influence the production of proteases by fungi (Table 2.1). However, the efficacy of a specific protein substrate to induce protease production differs between organisms, as well as the repressive action of different preferred sources of nitrogen, carbon and sulphur (Kumar and Takagi 1999; de Souza et al. 2015). Optimisation of fermentation conditions should therefore be performed for specific organisms and secreted proteases, and could be addressed through a statistical approach, such as response surface methodology (Gupta et al. 2002).

However, protease-producing fungi can also be used as inoculants to fermentation processes for the secretion of this enzyme directly into the matrix that would benefit from its activity. Such inoculation strategies do not require the additional enzyme recovery steps that are necessary to attain protease preparations for exogenous applications, and are therefore more time- and cost-effective (Kumar and Takagi 1999). Yeasts and filamentous fungi with protease-secreting ability find application in various food, beverage and bioremediation processes as starter cultures, as summarised in Figure 2.6.

2.4.1 Food industry

The importance of proteases to the food industry can largely be attributed to their ability to coagulate proteins (Theron and Divol 2014). This activity is crucial to the manufacturing of cheese in particular in which milk proteins are coagulated, leading to the formation of curds from which cheese is prepared. Exogenous enzyme preparations such as animal rennets, microbial milk coagulate, chymosin and vegetable rennet are typically added to achieve this transformation (Theron and Divol 2014).

However, the proteolytic activities of fungi indigenous to cheese have been shown to significantly contribute to its ripening and maturation, leading to the intentional use of some strains as inoculants (Larsen et al. 1998). Strains with strong proteolytic activity have led to strong tasting and fast ripening cheese, as well as different textures and water binding capacities that can significantly affect the structure and mouthfeel of the cheese (Larsen et al. 1998). The production of different aroma and flavour compounds is furthermore frequently associated with fungal protease activity, likely due to the release of amino acid precursors from casein degradation subsequently consumed by yeast and metabolised to yield volatile aroma compounds (Larsen et al. 1998).

D. hansenii is common to many types of cheese, including soft cheeses and the brines of semi-hard and hard cheeses (Breuer and Harms 2006). While this yeast plays many diverse roles in cheese making, its proteolytic activity favours its use as a starter

culture, and has been shown to contribute to the accelerated development of a strong flavour in Cheddar cheese (Ferreira and Viljoen 2003). Similarly, *Y. lipolytica* significantly alters texture and aroma development during cheese ripening due to its secreted proteases (Wyder and Puhan 1999; Zinjarde 2014). The breakdown of casein in blue-veined cheeses is particularly intense and primarily due to the proteolytic activity of *P. roqueforti*, which has been successfully used as a starter culture in the production of this style of cheese (Larsen et al. 1998). *Penicillium camemberti* is another protease-secreting strain with application as a starter culture, with promise as a co-inoculant with *G. candidum* to soft cheese (Chrzanowska et al. 1995; Boutrou et al. 2006a). *G. candidum* is responsible for the white, velvety coat on the surface of some soft cheeses such as Camembert, where it also contributes to cheese ripening via protease production (Boutrou et al. 2006b).

The use of protease-producing fungal starter cultures in the manufacture of dry cured meats similarly contributes to the flavour and texture of products such as fermented sausages and ham (Zapelena et al. 1999). The volatile profile, and consequently the taste and flavour, of dry-cured sausages are generally altered by inoculated cultures of *D. hansenii* and *Y. lipolytica* during ripening (Breuer and Harms 2006; Patrignani et al. 2007; Zinjarde 2014). A starter culture of *P. chrysogenum* similarly demonstrated high protease activity in dry-cured ham (Rodríguez et al. 1998; Ockerman et al. 2001).

Tempe is a traditional soy product from Indonesia produced through the fermentation of soya beans, and during fermentation the secretion of proteases by the inoculated fungi *R. oryzae* and *R. oligosporus* is critical for tempe quality (Farley and Ikasari 1992; Farley and Sullivan 1998). The protease-producing fungus *A. oryzae* is furthermore used in the fermentation of several traditional Japanese foods, including miso, shoyu and koji (Kitano et al. 2002).

2.4.2 Beverage industry

Proteases are also used in the beverage industry, mainly for clarification purposes in order to prevent haze and turbidity caused by protein heat instability (Theron and Divol 2014; Van Sluyter et al. 2015). In the wine industry especially, haze formation poses a problem to consumer perception of wine quality, particularly of white wine. Several exogenous protease treatments of the aspartic type have been tested for their efficacy against grape proteins and haze formation, as they exhibit activity at the low pH of grape juice and wine (Van Sluyter et al. 2015).

The addition of aspergilloglutamic peptidase from *A. niger* prior to flash pasteurisation of grape juice and subsequent fermentation produced heat stable wines that were almost completely free from haze-forming grape proteins, whereas a protease from *Botrytis cinerea* proved effective against chitinases, the grape protein shown to play the

predominate role in causing wine haze (Marangon et al. 2012; Van Sluyter et al. 2013; Van Sluyter et al. 2015). However, these enzymes are from filamentous fungi that are not indigenous to grape juice, and the search for aspartic proteases therefore extended to organisms that occur naturally in the winemaking environment.

In contrast to the classical wine yeast *S. cerevisiae*, non-*Saccharomyces* yeasts of oenological origin often harbour extracellular activity, including proteases (Schlander et al. 2016). Several such strains with proteolytic activity have been isolated and a handful even secrete a protease under winemaking conditions with activity against grape protein when inoculated as co-starter cultures, including *Kloeckera*, *Hanseniaspora* and even a strain of *S. cerevisiae* (Dizy and Bisson 2000; Younes et al. 2011). Considering the substantial role that *Y. lipolytica* and *D. hansenii* play in the food industry as protease-producing inoculants, there may be grape juice starter culture potential for oenological strains of these species. However, thus far no protease-secreting yeasts have been found to reduce haze-forming potential when applied as co-inoculants to wine fermentation.

The exogenous application of the purified aspartic protease MpAPr1 from one non-*Saccharomyces* yeast, *Metschnikowia pulcherrima* IWBT Y1123, to grape juice fermentation has, however, led to the partial degradation of chitinases as well as the significant reduction of wine haze after 48 h of incubation (Theron et al. 2018). This treatment furthermore resulted in a change to the composition of specific volatile compounds by the end of fermentation, some of which can be linked to amino acid metabolism by yeast, suggesting that the presence of protease activity in wine fermentation could have the additional influence of altering volatile aroma profile (Theron et al. 2018). However, it remains to be tested whether or not the inoculation of this protease-producing yeast into grape juice fermentation will result in the secretion of MpAPr1 under winemaking conditions to such an extent as to have an impact on wine haze and aroma profile.

Another beverage in which aroma profile is affected by fungal protease activity is sake, in which the inoculation of *A. oryzae* leads to protease production and degradation of proteins in the steamed rice, and subsequent liberation of amino acids and/or peptides (Kitano et al. 2002).

2.4.3 Bioprocessing and bioremediation

Protease-producing fungi are furthermore involved in several bioprocessing and bioremediation applications that require inoculation into the medium that will benefit from protease secretion and activity.

Penicillium spp. are capable of secreting acid proteases under solid substrate fermentation (SSF) and submerged fermentation (SmF), one application for which is the hydrolysis of soy protein, a by-product of soybean industries (Agrawal et al. 2004). Soybean

is rich in plant protein, but soy protein requires proteolytic modification to improve its functional properties before use as a protein enrichment additive to juices and beverages, or the fortification of food.

The protease-producing fungus *Chrysosporium keratinophilim* was isolated from a waste site containing organopollulants, and showed the ability to decompose two different keratin substrates when grown in the presence of buffalo skin and tail hair (Singh 2002). Keratin arises as a waste product through animal shedding and moulding, the death of animals and as a by-product of slaughterhouses. Its improper disposal poses a threat to human health when uncontrolled keratin degradation leads to the release of large quantities of toxic substances such as hydrogen sulphide and ammonia, thus controlled fungal degradation thereof offers a cost-effective solution.

Yet another example of the bioremedial potential for protease-producing fungi is that of potato pulp powder biotransformation by *A. oryzae* (Murthy and Kusumoto 2015). Potato pulp is produced in large quantities and its build-up poses an environmental pollution problem for the potato starch industry.

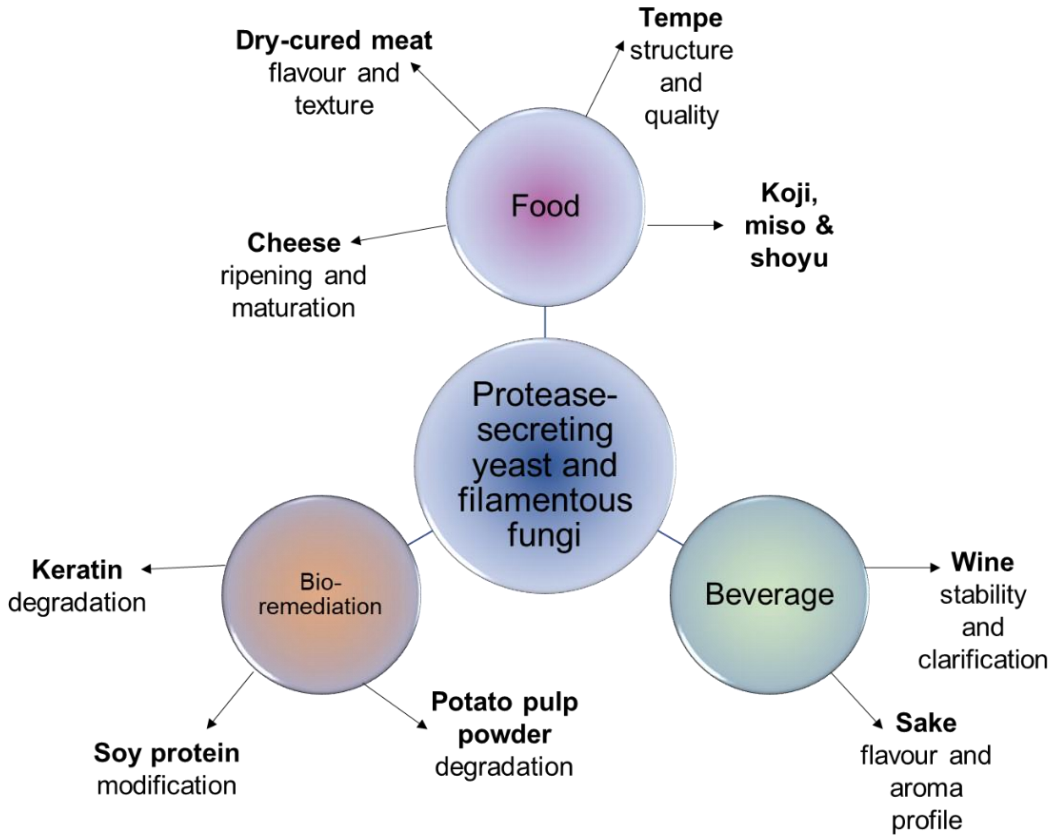

Figure 2.6 Summary of the current and potential uses of protease-secreting fungi in industrial applications.

2.5 Conclusions and future outlooks

A determining factor of where and how a fungus lives in nature, and how it can be grown by man, is whether or not the fungus is capable of secreting a protease(s), how protease expression is regulated, and the properties of the protease(s) (Ogrydziak 1993). Fungal extracellular proteases are of singular importance to the adaptive survival and physiology of the organism. Furthermore, fungal secretome changes, and specifically changes in protease production, are intrinsically linked to factors in the external environment of the cell, which include nutrient availability, pH and temperature. However, much of the molecular machinery underlying the regulatory pathways which guide protease response to the extracellular environment of the cell remains to be elucidated and requires further investigation.

Nevertheless, the biotechnological and cost-effective benefits presented by the use of protease-producing fungi as starter cultures are recognised and exploited in the food, beverage, and bioprocessing industries. However, the potential for using protease-producing fungi in new applications that could benefit from their inoculation, such as winemaking, should be explored. Although the impact of exogenous aspartic protease preparations on wine quality has been investigated, the inoculation of protease-producing yeast to grape juice fermentation has yet to be examined in terms of its ability to reduce wine haze formation.

An understanding of the changes in fungal protease secretion could benefit biotechnological applications in terms of anticipating and monitoring the environmental conditions that play a role in protease regulation, and should therefore form part of future investigations regarding protease-producing inoculants (McCotter et al. 2016). Further work should consider relating protease expression and production with its impact on properties of the designated matrix. With regard to applications that require protease secretion and recovery for exogenous use or for bioremediation purposes using SSF or SmF, the information regarding regulatory conditions for optimal production could prove immensely valuable in determining and applying parameters such as media composition, pH and temperature.

Furthermore, as the levels of protease produced by cultured fungi are often not adequate for affecting the desired biotechnological impact, methods for strain improvement other than merely optimising the medium, where possible, should be considered (Rao et al. 1998). Approaches might include screening for hyper-producing strains, random mutagenesis, cloning and over-expression using recombinant techniques, and whole-cell directed evolution through continuous cultivation using fed-batch and chemostat fermentations (Gupta et al. 2002). Knowledge regarding the transcriptional regulation of fungal extracellular proteases could provide targets for the genetic manipulation of protease

expression, however the commercial viability of a genetically modified organism (GMO) in the food and beverage industry is low (Rao et al. 1998). Directed, or adaptive, evolution is a non-GMO method based on the basic principles of genetic variation and natural selection (Pérez-Torrado et al. 2015). It involves subjecting cells to continuous cultivation with selective pressures, such as nitrogen starvation and protein induction, for a desired trait, such as enhanced protease production. The potential for applying this strategy should therefore be explored, as it may prove to be the more widely accepted method for yeast and fungal strain improvement of extracellular protease production in the food and beverage industry.

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Chapter 3

Research results

Investigating the expression, secretion and activity of the aspartic protease MpAPr1 by *Metschnikowia pulcherrima* IWBT Y1123

Chapter 3 - Investigating the expression, secretion and activity of the aspartic protease MpAPr1 by *Metschnikowia pulcherrima* IWBT Y1123

3.1 Introduction

Yeast species in the winemaking environment other than *Saccharomyces cerevisiae* have, in recent years, been considered for mixed fermentations in winemaking due to their positive contributions to wine quality and processing (Escribano et al. 2017). Species from the genera *Metschnikowia, Pichia, Torulaspora* and *Lachancea*, amongst others, have come to be used commercially as co-fermentative starter cultures due to their release of aromatic volatile compounds and enhancement of the overall aromatic profile of the wine (Oro et al. 2014). Investigations into the impact of non-*Saccharomyces* yeast species, which are mostly present during the initial stages of wine fermentation, on wine technological and oenological properties have come a long way in elucidating the metabolic attributes responsible for improving various aspects of wine quality.

The production and secretion of hydrolytic enzymes in particular present the potential of positively influencing wine clarification and filtration, juice yield, colour and aroma extraction, and wine stability (Maturano et al. 2012). However, enzymes are commonly applied exogenously to wine fermentations for these purposes, after purification from media grown with various bacteria or filamentous fungi. For example, the impact of adding the acid-tolerant aspartic proteases produced by *Aspergillus niger* and *Botrytis cinerea* to grape juice fermentations have been investigated for their ability to reduce protein haze in wine (Marangon et al. 2012; Van Sluyter et al. 2013). Although other factors play a substantial role, the incidence of haze in wine can be positively correlated to the concentration of heat-unstable grape proteins in the juice, specifically of chitinases and thaumatin-like proteins (TLPs) (McRae et al. 2018). Protein degradation through protease activity thus presents a potential preventative strategy to wine haze formation, and those produced by *A. niger* and *B. cinerea* were proven effective in reducing haze with and without additional heat treatment, respectively.

Reid et al. (2012) isolated and sequenced a gene encoding an extracellular aspartic protease from the wine yeast strain *Metschnikowia pulcherrima* IWBT Y1123 based on sequence similarities with other known aspartic proteases. This enzyme, named MpAPr1, is therefore the only known extracellular aspartic protease secreted by *M. pulcherrima* IWBT Y1123. The activity of this enzyme, MpAPr1, was further characterised and its exogenous application to grape juice fermentations showed a partial degradation of grape proteins, particularly of chitinases, as well as a decrease in haze-forming potential after 48 h of

incubation (Theron et al. 2017; Theron et al. 2018). However, an alternative could be the direct use of *M. pulcherrima* IWBT Y1123 itself as a starter culture in mixed fermentation with *S. cerevisiae*, providing that MpAPr1 be secreted into the fermentation matrix under winemaking conditions. An investigation into the regulation of this enzyme is therefore critical to elucidate the role that some environmental factors may play in its production, secretion and activity under winemaking conditions. Different factors relevant to the oenological environment such as pH, temperature, sugars and ethanol have been reported to influence enzymatic activity in yeasts (Maturano et al. 2012). Nitrogen availability, and complexity of the available sources, have been shown to influence the regulation of extracellular proteases in yeast in particular, which is known to play a role in yeast nutrition through the degradation of proteins into low molecular weight, assimilable nitrogen sources (Alexandre et al. 2001; Braaksma et al. 2009; Dunkel et al. 2014).

Nutrition is an essential prerequisite for the growth and development of microorganisms (Zaman et al. 2008). The ability of an organism to optimally utilise the nutrients available to it, and adapt to the nutritional limitations in its environment, confers an important advantage for its survival. The vital role of nitrogen in particular is manifested in the ability of free-living unicellular organisms to employ a vast array of metabolic resources for the transport and catabolism of many different nitrogenous compounds (Godard et al. 2007; Mendes-Ferreira et al. 2011). However, not all nitrogen sources support growth equally well (Beltran et al. 2004). Yeast species such as *S. cerevisiae* and *Candida albicans* have developed molecular mechanisms to ensure the prioritised utilisation of preferred nitrogen sources like ammonium and amino acids. These processes form part of the regulatory mechanism called nitrogen catabolite repression (NCR), which is also involved in the regulation of mechanisms required for the utilisation of alternative nitrogen sources when preferred sources are unavailable (Dabas and Morschhäuser 2008). Proteins can be considered as an alternative source of nitrogen in *C. albicans*, for example, as the expression of genes required for its utilisation is under the control of NCR (Dabas and Morschhäuser 2008). This includes *SAP2*, a gene encoding an extracellular aspartic protease, which is induced in the presence of proteins but repressed when preferred sources are also available (Dabas and Morschhäuser 2008). The grape juice environment includes the presence of both complex nitrogen-containing compounds such as grape proteins, and simpler, preferred nitrogen sources like ammonium ions and amino acids (Mendes-Ferreira et al. 2011).

In this study, the influence of various available nitrogen sources on MpAPr1's secretion and activity was assessed by growing *M. pulcherrima* IWBT Y1123 in a minimal yeast nitrogen base (YNB) or synthetic grape juice-like (SGJ) medium supplemented with complex (protein) or simpler (ammonium and amino acids) nitrogen-containing compounds,

or a combination thereof. Furthermore, MpAPr1's secretion and activity, and expression of *MpAPr1*, was subsequently monitored in Sauvignon blanc as a preliminary investigation into the potential of using *M. pulcherrima* IWBT Y1123 as a co-starter culture in grape juice fermentations.

3.2 Materials and methods

3.2.1 Strains and pre-culture conditions

The main strain used in this study was *Metschnikowia pulcherrima* IWBT Y1123, isolated from grape juice which was pressed from Chardonnay grapes during the 2009 harvest season in Stellenbosch, South Africa (Reid et al. 2012). For sequential inoculations in grape juice the commercial wine yeast strain *Saccharomyces cerevisiae* Lalvin® EC1118 (Lallemand, Blagnac, France) was used. Cultures were maintained in 30% glycerol at -80°C, and cultivated at 30°C on yeast peptone dextrose (YPD) agar (Biolab diagnostics, Wadenville, South Africa).

A single colony was randomly selected and grown in 10 mL YPD broth (Biolab diagnostics) for 24 h at 30°C, and inoculated into 400 mL fresh YPD broth at an optical density of 0.1 at 600 nm in a 1-L Erlenmeyer flask. Cultures were incubated at 30°C with shaking at 120 rpm for 11 h (mid-exponential growth phase) before centrifugation at 5000 x *g* for 5 min. The supernatant was discarded and cells resuspended in saline before centrifugation at 5000 x *g* for 5 min. Cells were then resuspended with the minimal medium, synthetic grape juice (SGJ) or grape juice in which experiments were performed.

3.2.2 Growth and sampling in synthetic media with different nitrogen sources

Two different media were supplemented with different nitrogen sources in this study: Yeast Nitrogen Base (YNB) medium (20 g/L glucose, 1.7 g/L YNB base without amino acids and ammonium (Difco Laboratories, MI, USA)), prepared in 0.1 M McIlvaine's buffer adjusted to pH 3.5; and SGJ without nitrogen sources as outlined in Table 3.1 (adapted from Henschke and Jiranek (1993)), and adjusted to pH 3.2 with HCl.

Table 3.1 Components of synthetic grape juice, excluding nitrogen sources

		Per litre
Carbon sources	Glucose	115 g
	Fructose	115 g
Acids	Potassium L-tartrate	2.5 g
	L-malic acid	3.0 g
	Citric acid	0.2 g
Salts	Potassium hydrogen phosphate	1.14 g

	Magnesium sulphate heptahydrate	0.44 g
	Calcium chloride dehydrate	1.23 g
Trace elements	Manganese(III) chloride tetrahydrate	200 µg
	Zinc chloride	135 µg
	Iron(III) chloride	30 µg
	Copper(III) chloride	15 µg
	Boric acid	5 µg
	Cobalt(II) nitrate hexahydrate	30 µg
	Sodium molybdate dehydrate	25 µg
	Potassium iodate	10 µg
Vitamins	Myo-inositol	100 mg
	Pyridoxine hydrochloride	2 mg
	Nicotinic acid	2 mg
	Calcium pentothenate	1 mg
	Thiamin hydrochloride	0.5 mg
	PABA K	0.2 mg
	Riboflavin	0.2 mg
	Biotin	0.125 mg
	Folic acid	0.2 mg
Anaerobic factors	Ergosterol	10 mg
	Tween 80	0.5 mL

An easily assimilable nitrogen stock solution of amino acids and ammonium chloride (NH_4^+-AA) corresponding to 20 g/L yeast assimilable nitrogen (YAN) (adapted from Henschke and Jiranek (1993)) was prepared in a 2% $NaHCO_3$ buffer solution (Table 3.2). A 2% stock solution of bovine haemoglobin (Sigma-Aldrich, MO, USA) was prepared in MilliQ water prior to use in experiments as a protein source. Nitrogen source supplementation of YNB and SGJ was performed with haemoglobin (Hb), haemoglobin and amino acids and ammonium (Hb-NH_4^+-AA), only amino acids and ammonium (NH_4^+-AA), or only ammonium (Table 3.3). For each treatment the nitrogen sources were adjusted to a final concentration of 0.2 g/L total YAN in YNB or SGJ. In the case of haemoglobin, the concentration required to reach 0.2 g/L free amino nitrogen if all peptide bonds were cleaved was considered. The final concentrations of each source in the various treatments are outlined in Table 3.3.

Table 3.2 Composition of 20 g/L amino acids and ammonium stock solution

	g/L
Alanine	9.69
Arginine	24.9

Asparagine	3.54
Aspartic acid	2.97
Cysteine	0.87
Glutamine	33.7
Glutamic acid	8.03
Glycine	1.22
Histidine	2.18
Isoleucine	2.18
Leucine	3.23
Lysine	1.14
Methionine	2.10
Phenylalanine	2.53
Proline	40.8
Serine	5.24
Threonine	5.06
Tryptophane	11.9
Tyrosine	1.22
Valine	2.97
Ammonium chloride	30.7

Table 3.3 Final concentrations of individual nitrogen source supplements to YNB and SGJ to achieve a total YAN concentration of 0.2 g/L in the different nitrogen treatments which included haemoglobin (Hb), ammonium (NH_4^+), amino acids (AA) or combinations thereof.

		Treatment			
		Hb	**Hb + NH_4^+ + AA**	**NH_4^+ + AA**	**NH_4^+ (YNB)**
Nitrogen source	**Hb**	0.16 %	0.08 %	-	-
	NH_4^+ + AA	-	100 mg/L	200 mg/L	-
	NH_4Cl	-	-	-	763.4 mg/L

Suspensions of *M. pulcherrima* IWBT Y1123 cells were inoculated at an optical density of 2.4 at 600 nm into 250-mL Erlenmeyer flasks containing 150 mL medium in triplicate, and incubated at 25°C with shaking at 110 rpm. Samples were taken 0 h, 1 h, 3 h, 6 h, 8 h, 11 h, 24 h and 48 h after inoculation into YNB, and 0 h, 1 h, 3 h, 6 h, 9 h, 12 h, 24 h and 48 h after inoculation into SGJ, for monitoring yeast growth using optical density measurements at 600 nm and protease activity against azocasein in liquid assays (as described below). Remaining samples were stored at -20°C prior to SDS-PAGE analysis (as described below).

3.2.3 Growth and sampling in Sauvignon blanc grape juice

The Sauvignon blanc grape juice used in this study was previously crushed, destemmed and pressed at the Stellenbosch University experimental cellar from grapes harvested at the Welgevallen experimental vineyard (Stellenbosch University) in 2016. No enzyme additions were made, and juice was stored at -20°C. After thawing and prior to experimental use, grape juice was centrifuged at 8000 x *g* for 15 min and subsequently filtered through 11 µM and 2.5 µM (Whatman™ filter paper, GE Healthcare, IL, USA); 1.2 µM, 0.45 µM and 0.2 µM (cellulose acetate filters, Sartorius Stedim, Göttingen, Germany) filters using a Nalgene® vacuum filtration system (Sigma-Aldrich). The appropriate enzyme kits were used to determine the grape juice, of pH 3.3, glucose (Enzytec™ Liquid D-Glucose, R-Biopharm, Darmstadt, Germany) and fructose (D-Fructose, Thermo Scientific, MA, USA) concentrations at 116 g/L each, and ammonia (Enzytec™ Fluid Ammonia, R-Biopharm) and primary amino nitrogen (PAN) (PANOPA Assay Kit, Megazyme, Bray, Ireland) concentrations at 48.3 mg/L and 116 mg/L respectively, in conjunction with photometric determination (Arena 20XT Photometric Analyzer, Thermo Scientific). Suspensions of *M. pulcherrima* IWBT Y1123 cells were inoculated at an optical density of 2.4 at 600 nm into 250-mL Erlenmeyer flasks containing 100 mL grape juice, sealed with rubber bungs and S-bend airlocks, and incubated at 25°C with shaking at 110 rpm. After 48 h incubation *S. cerevisiae* was inoculated at a concentration of 10^6 cells/mL. Grape juice with no prior inoculation of *M. pulcherrima* IWBT Y1123 was inoculated separately with *S. cerevisiae* as a control. Fermentations were carried out in triplicate and fermentation kinetics were monitored by measuring flask weight loss daily (as an estimation of CO_2 released). Samples were taken at 0 h, 3 h, 6 h, 9 h, 12 h, 24 h, 48 h and 120 h after *M. pulcherrima* IWBT Y1123 inoculation for monitoring protease activity against azocasein in liquid assays (as described below), and the remaining sample was stored at -20°C prior to SDS-PAGE analysis (as described below). Additional 1 mL samples were resuspended in 20% glycerol and flash-frozen in liquid nitrogen, and subsequently stored at -80°C before RNA extractions and qPCR analyses (as described below).

3.2.4 Endo-protease activity assay

Protease activity in sample supernatant was measured against azocasein (Megazyme), a chromogenic substrate associated with the release of acid-soluble material in the presence of endopeptidase activity. Assay parameters were adapted from Theron et al. (2017). Cultures were centrifuged at 6000 x *g* for 5 min, and 160 µL supernatant was added to 160 µL 2% azocasein substrate (dissolved in McIlvaine's buffer pH 4.5). A sample of 160 µL was taken before and after incubation at 40°C for 24 h, and added to 160 µL 20% TCA solution

to stop the reaction. The mixture was briefly vortexed and centrifuged at 16 000 x *g* for 5 min, before transferring supernatant in 100 µL aliquots to a 96-well plate. Absorbance was measured at 440 nm using a Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer with SkanIt™ software. All experiments were performed in triplicate. One arbitrary unit (AU) of protease activity was defined as the amount of protease causing an increase in absorbance at 440 nm of 0.001 through a path length of 1 cm, per millilitre supernatant-substrate sample incubated at 40°C for an hour.

3.2.5 Protein extraction and quantification

Culture samples from YNB and SGJ containing only haemoglobin, and haemoglobin with ammonium and amino acids, as well as from grape juice, were thawed prior to protein extraction, and centrifuged at 6000 x *g* for 5 min. An aliquot of 100 µL supernatant was diluted in 400 µL MilliQ and 1500 µL 100% acetone, and left at -20°C overnight before centrifugation at 21 380 x *g* for 30 min. The pellet was resuspended in MilliQ and protein concentrations were determined using the Pierce® BCA Protein Assay kit (Thermo Scientific) according to the manufacturer's instructions. Colorimetric detection was performed by measuring absorbance at 540 nm using a PowerWave™ Microplate Scanning Spectrophotometer (BioTek Instruments Inc, VT, USA).

3.2.6 Protein visualisation

For each treatment, protein samples were diluted to the same extent required for the non-inoculated control sample to reach 1 mg/mL total protein concentration. Thus, the Hb-only samples from YNB and SGJ were diluted 2X more than samples from the Hb-NH_4^+-AA combination treatment.

Proteins were visualised through the use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Laemmli 1970). Gels containing 15% bis-acrylamide were loaded with 40 µL of the suspended protein samples, and run on a Bio-Rad Mini-Protean® Tetra Cell System (Bio-Rad Laboratories, CA, USA). Electrode chambers were filled with Tris-Glycine buffer (50 mM Tris, 200 mM glycine, 0.2% SDS). Gels were stained overnight in staining solution (1 g Coomassie blue R250 (Merck, Darmstadt, Germany) in 50 % (v/v) ethanol, 10% (v/v) acetic acid), and destained with 12.5% isopropanol and 10% (v/v) acetic acid. Gels were captured using a Molecular Imager® Gel Doc™ System (Bio-Rad Laboratories) using Image Lab™ Software v6.0 (Bio-Rad Laboratories).

3.2.7 RNA extraction

RNA extractions were performed on biological triplicates of grape juice cultures which were thawed and centrifuged at 21 380 x *g* for 30 min before resuspending the pellet in saline. After centrifugation at 6000 x *g* for 5 min, the saline wash step was repeated. The resultant pellet was resuspended in 600 µL high salt buffer (0.5 M NaCl, 20 mM Tris/HCl, 10 mM EDTA, 2% SDS), to which 200 µL acid phenol and 200 µL chloroform was added before vortexing the mixture vigorously for 3 min in the presence of glass beads. The mixture was centrifuged at 15 000 x *g* for 10 min, and the acqueous layer added to 400 µL chloroform. Another centrifugation step was performed at 15 000 x *g* for 10 min before the acqueous layer was suspended in 1 mL 100% isopropanol and placed at -20°C overnight. After centrifugation at 21 380 x *g* for 10 min the pellet was resuspended in 100 µL MilliQ.

3.2.8 Reverse transcription and qPCR

Genomic DNA and RNA concentrations were quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Products, DE, USA). Contaminating DNA was removed with DNAse I treatments performed according to the manufacturer's instructions (Roche, Mannheim, Germany). Reverse transcription of 760 ng RNA was performed using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche) according to the manufacturer's instructions. The oligo$(dT)_{15}$ primer of the kit was used to initiate reverse transcription of poly$(A)^+$ mRNA molecules.

Quantitative PCR (qPCR) was performed in triplicate on the cDNA obtained from 3 biological repeats on a 7500 real-time PCR system (Applied Biosystems, Johannesburg, South Africa). Relative quantification was determined using the 2^{ddCt} method, and the *ACT1* gene was used for normalisation purposes. Additionally, the linear regression of efficiency (LRE) method was used for absolute quantification of *MpAPr1* transcripts present at the start of qPCR amplification, using the *CAL1* amplicon from lambda DNA (Roche) as optical calibrator (Rutledge and Stewart 2010). The KAPA SYBR Fast qPCR kit (Kapa Biosystems, Cape Town, South Africa) was used for the reaction mix according to the manufacturer's instructions. The primer sets MpAPr1-qPCR-fw/rev (ACACCCAAGGCGTCATACTC/ ACAGGTCAATCGGGTACAGC) and MpACT1-qPCR-fw/rev (CTCCATGCCTCACGGTATTT/ CTCCTGCTCAAAGTCCAAGG) were used to amplify the *MpAPr1* and the *ACT1* genes of *Metschnikowia pulcherrima* IWBT Y1123, respectively (Reid et al. 2012). The *CAL1* forward (AGACGAATGCCAGGTCATCTGAAACAG) and reverse (CTTTTGCTCTGCGATGCTGATACCG) primers were used to amplify this gene in lambda DNA. The qPCR program consisted of an initial denaturation at 95°C for 3 min followed by

40 cycles comprising of a denaturation step at 95°C for 5 s and an annealing step at 60°C for 1 min.

The concentration of starting cDNA was subsequently determined using the Qubit® 2.0 Fluorometer (Thermo Scientific), and used to normalise the Ct values obtained from *MpAPr1* and *ACT1* amplification before application of the 2^{ddCt} method. The LRE Analyzer program (Rutledge 2011) was used to calculate the initial number of *MpAPr1* transcripts, which were then normalised with starting cDNA concentrations.

3.2.9 Statistical analysis

Statistical analyses were performed using the computer software GraphPad Prism 6 (GraphPad, CA, USA) to determine significant differences between treatments and time-points.

3.3 Results

3.3.1 YNB with haemoglobin, ammonium and amino acids

3.3.1.1 Growth kinetics

Growth of *M. pulcherrima* IWBT Y1123 occurred in all treatments, including when haemoglobin was provided as the sole source of nitrogen (Figure 3.1). Cell density in the Hb-only treatment was less pronounced than in treatments containing simple nitrogen sources during the first 8 h of growth. However, from 24 h after inoculation, growth had exceeded that of the other nitrogen conditions - a trend that was amplified by 48 h when an optical density of 20 at 600 nm was reached compared to an average of 14 for those treatments containing simple nitrogen sources. An opposite trend was observed when the yeast was grown with ammonium and amino acids, and ammonium only. With only simple nitrogen sources available, *M. pulcherrima* IWBT Y1123 showed less pronounced growth by 24 h after inoculation. The Hb-NH_4^+-AA combination treatment displayed optical density values at 600 nm intermediate between the Hb-only and simple nitrogen source-only conditions (13 and 15 at 24 h and 72 h, respectively).

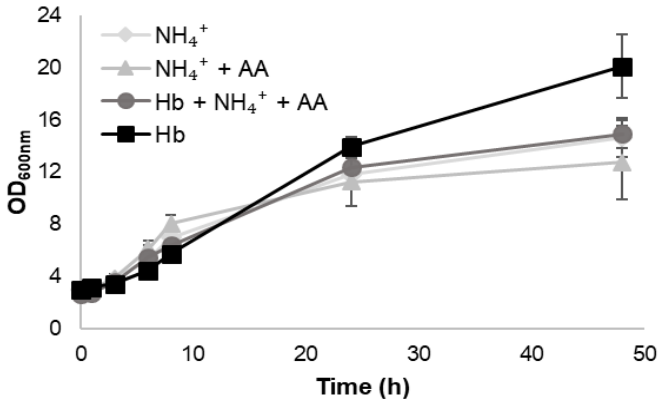

Figure 3.1 Growth of *M. pulcherrima* IWBT Y1123 over time in in YNB media containing ammonium (NH_4^+), ammonium and amino acids (NH_4^+ + AA), ammonium and amino acids and haemoglobin (Hb + NH_4^+ + AA), or haemoglobin (Hb) only. The data points shown are means for three independent experiments and the error bars indicate standard deviation between triplicates.

3.3.1.2 Protease activity

Protease assays revealed a basal level of activity for all treatments throughout the duration of sampling (Figure 3.2). A significant increase in activity was recorded for Hb-only samples from 3 h as compared to simple nitrogen source-containing samples. In the Hb-NH_4^+-AA combination treatment, a significant increase was observed from 6 h. Activity increased to a maximum of 230 AU and 256 AU at 8 h for Hb-only and Hb-NH_4^+-AA treatments, respectively. A decrease in activity of 81 AU and 93 AU for Hb-only and Hb-NH_4^+-AA treatments was observed from 8 h to 11 h. Thereafter, from 11 h to 24 h, Hb-only and Hb-NH_4^+-AA treatment activity levels increased again by 63 AU and 26 AU, respectively. Data obtained from statistical comparison between means can be found in supplementary material (Tables S3.1 and S3.2).

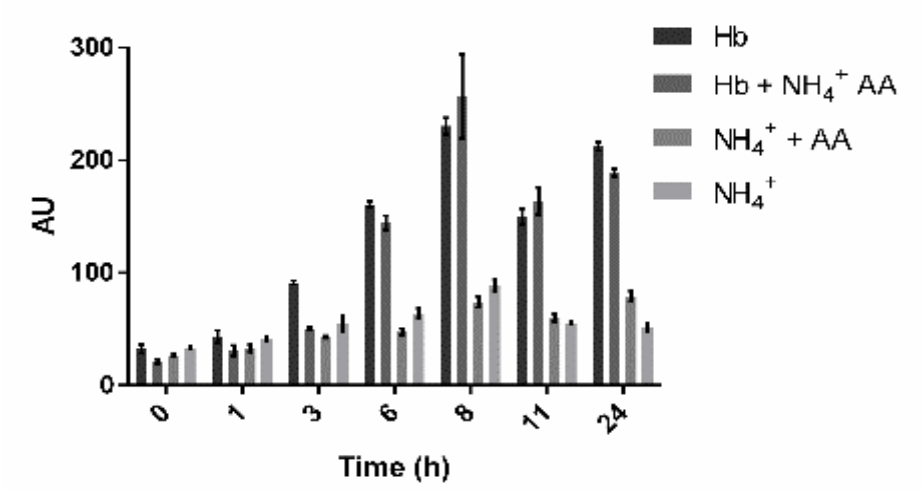

Figure 3.2 Protease activity expressed in AU calculated for supernatant samples of *M. pulcherrima* IWBT Y1123 taken at different time points from YNB media containing ammonium (NH_4^+), ammonium and amino acids (NH_4^+ + AA), ammonium and amino acids and haemoglobin (Hb + NH_4^+ + AA), or haemoglobin only (Hb). The data points shown are means for three independent experiments and the error bars indicate standard deviation between triplicates.

3.3.1.3 Protein visualisation

Haemoglobin degradation and the presence of MpAPr1 was also monitored in samples taken over 48 h after *M. pulcherrima* IWBT Y1123 inoculation into the Hb-only and Hb-NH_4^+-AA combination media. Precipitated proteins were diluted to reach what would have been 1 mg/mL haemoglobin in non-inoculated media according to the initial haemoglobin concentration. Thus, the haemoglobin-only samples were diluted 2X more than samples from the combination treatment. Proteins were subsequently visualised using SDS-PAGE (Figure 3.3). In the 0 h and control (incubated but non-inoculated medium) samples for both treatments, the different subunit associations of haemoglobin are clearly visible as bands at ~64 kDa, ~32 kDa and ~16 kDa, indicative of haemoglobin tetramers, dimers and monomers, respectively (Huang et al. 2013). These bands are absent from subsequent samples taken 3 h to 48 h after inoculation, for both treatments. Additionally, a low molecular weight (<5 kDa) smear appears in the 3 h lanes, absent from subsequent samples. The appearance of a ~42 kDa band is evident from 6 h in both treatments, becoming more prominent towards 48 h. This band can be attributed to the production of MpAPr1, through comparison of molecular weight (Reid et al. 2012; Theron et al. 2017). Although this band seems more intense in Hb-NH_4^+-AA combination samples, the difference in dilution of protein precipitate must be taken into account.

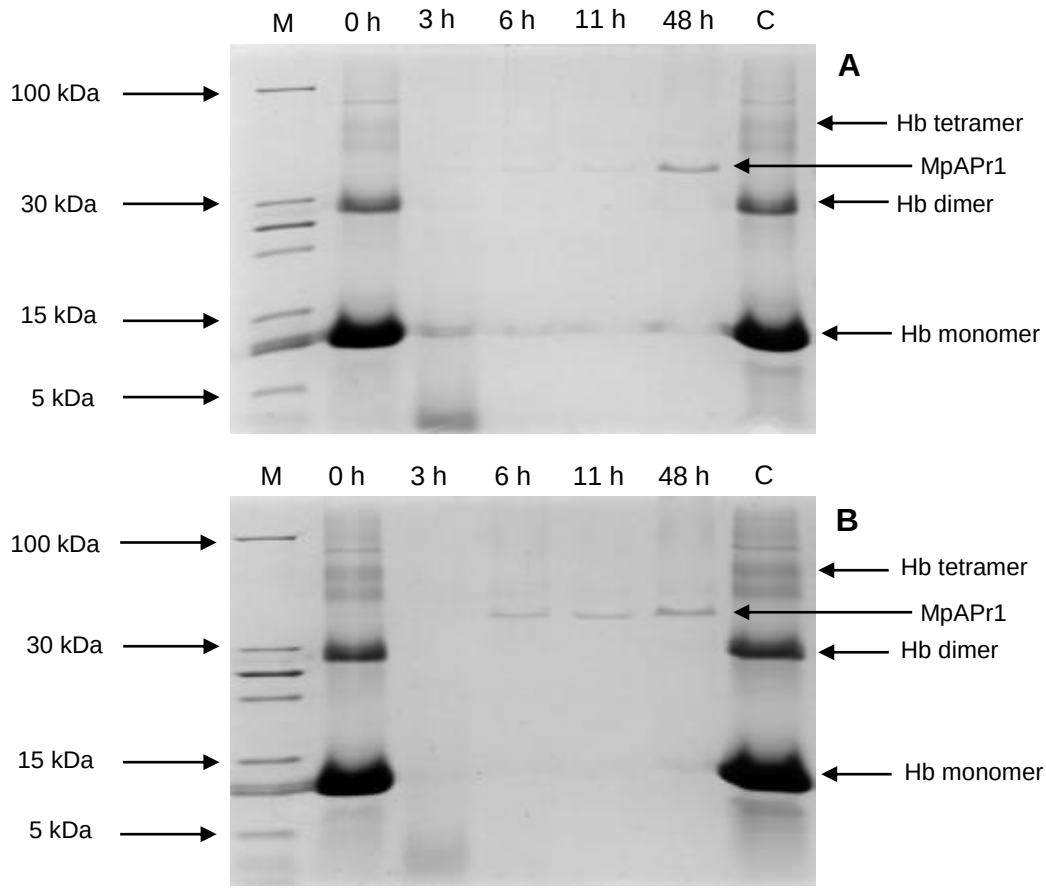

Figure 3.3 Visualisation of proteins precipitated from samples of *M. pulcherrima* IWBT Y1123 grown in YNB media taken at 0 h, 3 h, 6 h, 11 h and 48 h post-inoculation, and of a negative control obtained from incubated but non-inoculated medium (C). **A** Proteins obtained from haemoglobin-only (Hb) samples. **B** Proteins obtained from Hb-NH_4^+-AA samples. M: molecular weight marker (PageRuler™ Unstained Low Range Protein Ladder, Thermo Scientific). Arrows to the right of the gel indicate protein bands identified as MpAPr1 or various structural conformations of haemoglobin through comparison of molecular weight (Huang et al. 2013, Theron et al. 2017).

3.3.2 Synthetic grape juice with haemoglobin, ammonium and amino acids

3.3.2.1 Growth kinetics

The previous experiment was repeated in synthetic grape juice (230 g/L glucose and fructose, pH 3.2) with haemoglobin and/or ammonium and amino acids.

From 6 h after inoculation with *M. pulcherrima* IWBT Y1123, growth in the presence of ammonium and amino acids once again exceeded that of the Hb-only treatment, with an optical density of 3.3 at 600 nm as opposed to 2 for Hb-only (Figure 3.4). This trend was amplified with the progress of time to 12 h after inoculation, although by 48 h the difference in cell density between treatments became less, with the optical density of Hb-only samples reaching 10 as opposed to 11 in NH_4^+-AA -only samples. Yeast grown with a combination of haemoglobin and NH_4^+-AA performed slightly better than in NH_4^+-AA -only conditions from mid to late exponential phase of growth.

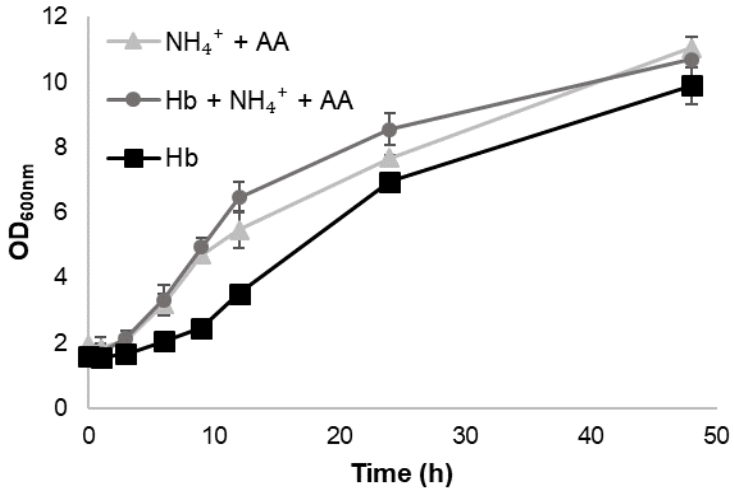

Figure 3.4 Growth of *M. pulcherrima* IWBT Y1123 over time in SGJ media containing ammonium and amino acids (NH_4^+ + AA), ammonium and amino acids and haemoglobin (NH_4^+ + AA + Hb), or haemoglobin only (Hb). The data points shown are means for three independent experiments and the error bars indicate standard deviation between triplicates.

3.3.2.2 Protease activity

Protease activity of samples against azocasein is shown in Figure 3.5. Hb-only samples showed significantly higher levels of activity than samples from NH_4^+-AA -containing media, increasing from 3 h to 48 h after inoculation with *M. pulcherrima* IWBT Y1123. Maximum activity levels at 274 AU was reached at 48 h. A similar trend was observed in Hb-NH_4^+-AA combination samples, although activity increased significantly only from 9 h as opposed to 3 h, and a maximum of 223 AU was reached at 48 h. The basal level of activity observed in NH_4^+-AA -only samples remained 3-fold to 9-fold less than Hb-only samples for this duration. Data obtained from statistical comparison between means can be found in supplementary material (Tables S3.3 and S3.4).

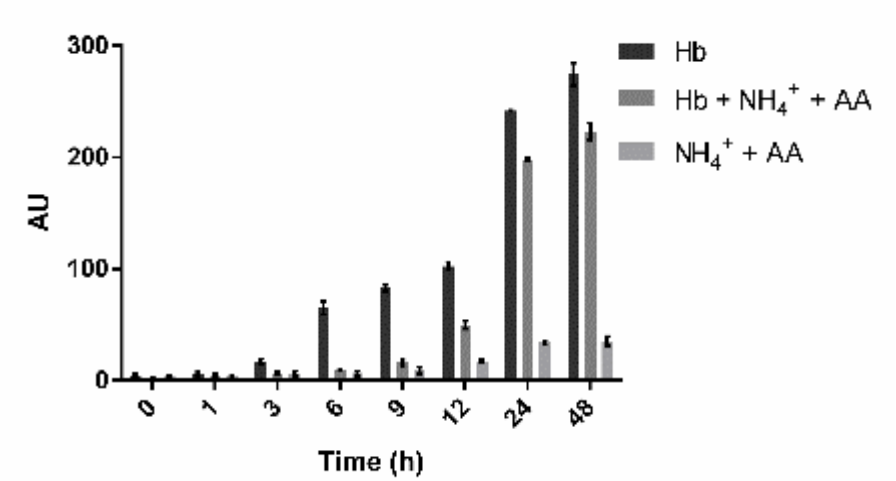

Figure 3.5 Protease activity expressed in AU calculated for supernatant samples of *M. pulcherrima* IWBT Y1123 taken at different time points from SGJ media containing ammonium and amino acids (NH_{4+} + AA), ammonium and amino acids and haemoglobin (NH_4^+ + AA + Hb), or haemoglobin only (Hb). The data points shown are means for three independent experiments and the error bars indicate standard deviation between triplicates.

3.3.2.3 Protein visualisation

Proteins were extracted from samples taken from Hb-only and Hb-NH_4^+-AA treatments and visualised using SDS-PAGE (Figure 3.6). Bands corresponding to haemoglobin tetramers, dimers and monomers were drastically reduced in Hb-only samples by 6 h after inoculation and had disappeared from 12 h onwards, and in Hb-NH_4^+-AA samples these bands were absent from 24 h onwards. Low molecular weight (< 10 kDa) smears were evident in 6 h, 12 h and 24 h lanes of haemoglobin-only samples, and in 12 h and 24 h lanes of Hb-NH_4^+-AA combination samples. A band corresponding to MpAPr1 is apparent from 12 h after inoculation for the Hb-only treatment, and 24 h from the Hb-NH_4^+-AA condition.

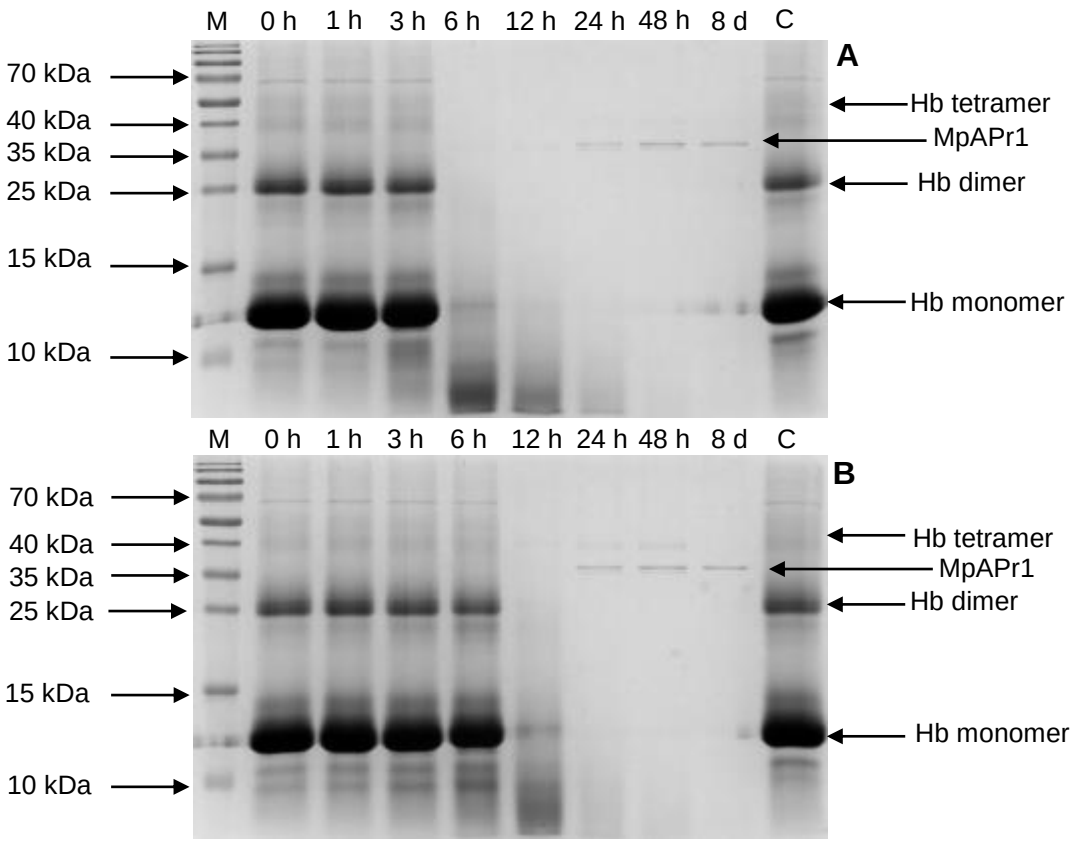

Figure 3.6 Visualisation of proteins precipitated from supernatant samples of SGJ inoculated with *M. pulcherrima* IWBT Y1123, taken at 0 h, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h and 8 days post inoculation, and of a negative control obtained from incubated but non-inoculated medium (C). **A** Proteins obtained from haemoglobin-only (Hb) samples. **B** Proteins obtained from Hb-NH_4^+-AA samples. M: molecular weight marker (PageRuler™ Prestained Protein Ladder, Thermo Scientific). Arrows to the right of the gel indicate protein bands tentatively identified as MpAPr1 or various structural conformations of haemoglobin through comparison of molecular weight (Huang et al. 2013, Theron et al. 2017).

3.3.3 Sauvignon blanc sequential inoculation with *S. cerevisiae*

3.3.3.1 Fermentation kinetics

M. pulcherrima IWBT Y1123 was subsequently inoculated into Sauvignon blanc grape juice to monitor the expression, secretion and activity of MpAPr1 in a winemaking environment. A sequential inoculation of *S. cerevisiae* EC1118 was performed 48 h later, to observe the fermentation kinetics when *M. pulcherrima* IWBT Y1123 is applied as a co-starter culture (Figure 3.7). Both fermentations with and without the use of *M. pulcherrima* IWBT Y1123 as a co-starter culture had finished fermenting by the 9th day after *S. cerevisiae* was inoculated, reaching 99 g/L cumulated weight loss.

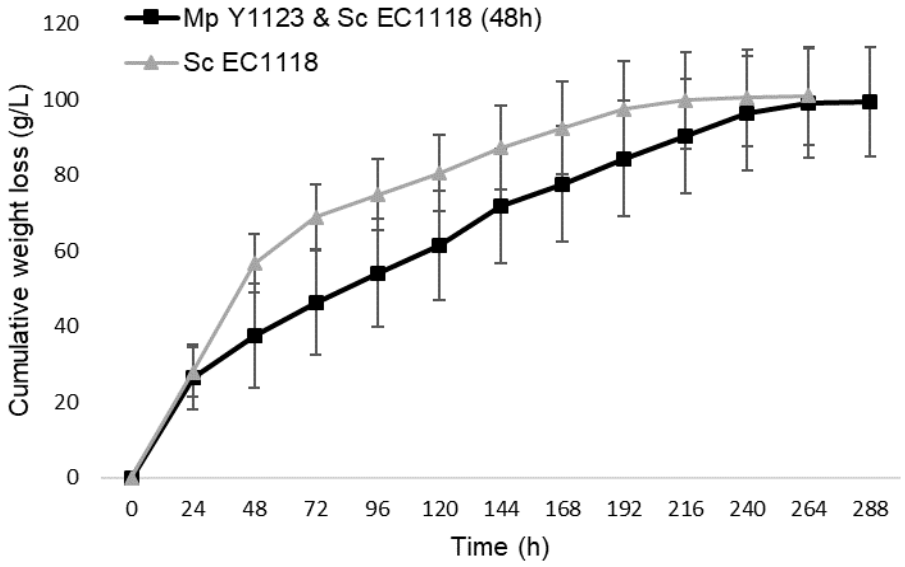

Figure 3.7 Loss of CO_2 during fermentation monitored by accumulated weight loss of flasks containing *M. pulcherrima* IWBT Y1123 in 48h sequential inoculation with *S. cerevisiae* EC1118, and pure *S. cerevisiae* EC1118, in Sauvignon blanc grape juice. The data points shown are means for three independent experiments and the error bars indicate standard deviation between triplicates.

3.3.3.2 Protease activity

Protease activity was followed in samples from the *M. pulcherrima* IWBT Y1123 treatment prior to *S. cerevisiae* inoculation (Figure 3.8). Activity increased significantly throughout time from 0 h to 24 h at which point a maximum of 80 AU was reached. Thereafter, activity decreased significantly by 48 h.

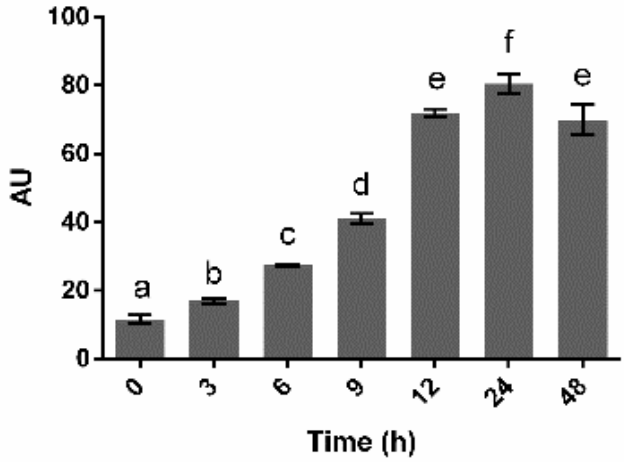

Figure 3.8 Protease activity calculated for supernatant samples of *M. pulcherrima* IWBT Y1123 and *S. cerevisiae* EC1118 taken at different time points throughout the first 48 h of growth in Sauvignon blanc grape juice. The data points shown are means for three independent experiments and the error bars indicate standard deviation between triplicates. Different letters indicate significant differences between samples ($P<0.05$) as analysed by one-way ANOVA and the Fisher's LSD test.

3.3.3.3 Protein visualisation

The presence of MpAPr1 and degradation of grape proteins was monitored in the sequentially inoculated fermentation using SDS-PAGE (Figure 3.9). Bands corresponding to the known molecular weight of MpAPr1 could be observed at 12 h, 24 h, 48 h and 120 h after inoculation with *M. pulcherrima* IWBT Y1123. Grape proteins with known molecular weights corresponding to the bands on the gel are also indicated. The degradation of these bands are evident in the fading intensity from 48 h to the end of fermentation – particularly of chitinases.

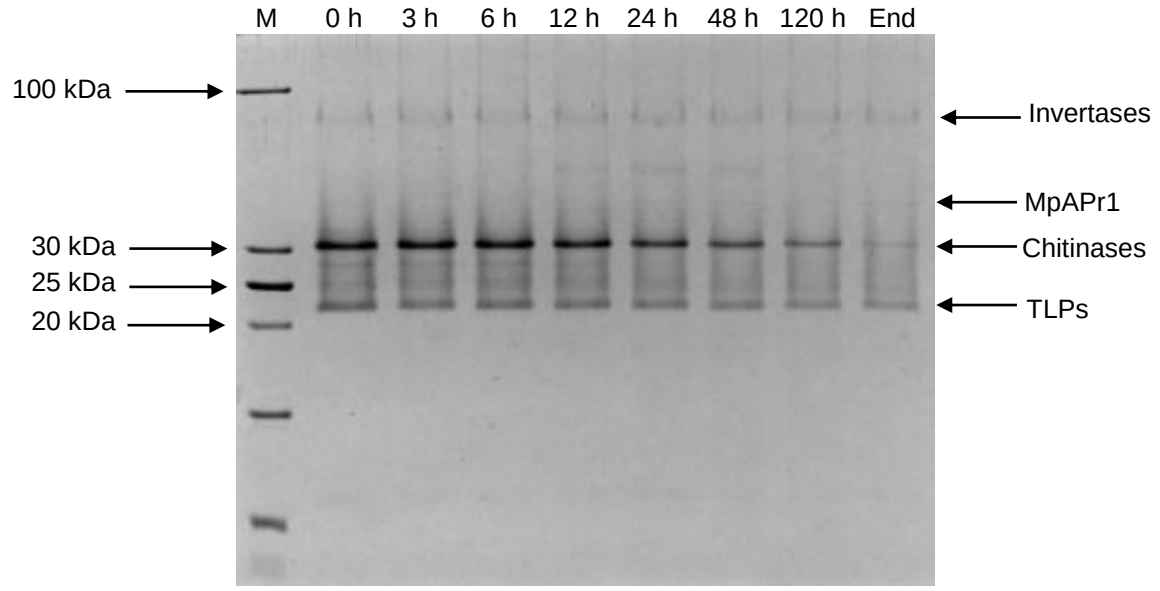

Figure 3.9 Visualisation of proteins precipitated from samples taken during and after fermentation by *M. pulcherrima* IWBT Y1123 with 48 h sequential inoculation of *S. cerevisiae* EC1118 (0 h, 3 h, 6 h, 12 h, 24 h, 48 h and 120 h after *M. pulcherrima* IWBT Y1123 inoculation, and at the end of fermentation). M: molecular weight marker (PageRuler™ Unstained Low Range Protein Ladder, Thermo Scientific). Arrows to the right of the gel indicate protein bands identified as MpAPr1 or grape proteins through comparison of molecular weight (Van Sluyter et al. 2015, Theron et al. 2017).

3.3.3.4 Protease gene expression

The expression of the *MpAPr1* gene was furthermore monitored in *M. pulcherrima* IWBT Y1123 using quantitative PCR (qPCR) analysis (Figure 3.10). The Ct values obtained were normalised by the concentration of the initial cDNA template of the appropriate sample. Subsequently, two different normalisation techniques were compared: the use of *ACT1* as a housekeeping gene (Figures 3.10A and 3.10B) and LRE (linear regression of efficiency) (Figure 3.10C). In Figure 3.10A, despite an apparent initial decrease in expression from 0 h to 3 h, expression increased significantly from 3 h to 9 h after inoculation. Expression levels reached a maximum at 12 h and plateaued until 24 h, before decreasing to 48 h. Figure 3.10B shows an increasing fold change in expression relative to 0 h from 3 h to 24 h, for samples already normalised to *ACT1*. The change becomes less pronounced by 48 h. The

number of *MpAPr1* transcripts (N0) reverse transcribed into cDNA before qPCR amplification was calculated using the LRE method, and normalised to cDNA concentration of the initial cDNA template (Figure 3.10C). This method demonstrated a significant increase in relative transcript levels from 0 h to 3 h, and again from 9 h to 12 h. The maximum level of transcripts was reached at 12 h and subsequently decreased by 24 h. Transcripts were almost absent from 48 h samples.

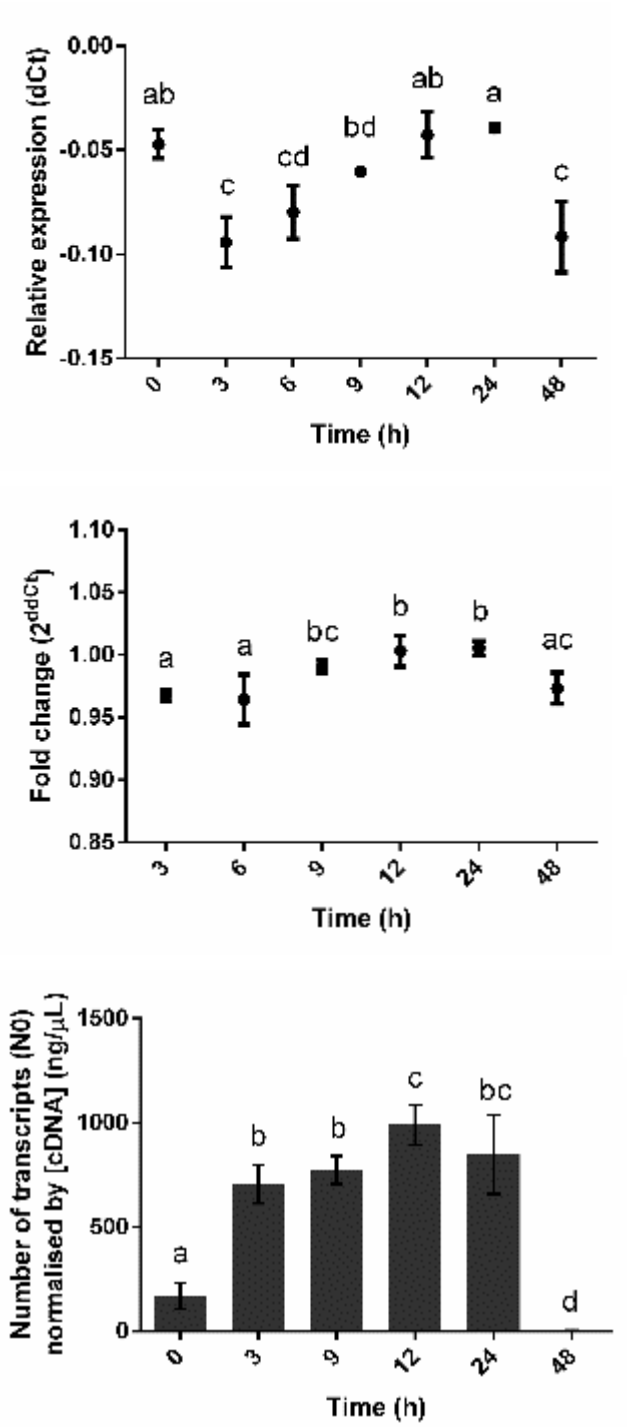

Figure 3.10 Gene expression analysis of *MpAPr1* in samples of *M. pulcherrima* IWBT Y1123 taken throughout the first 48 h of growth in Sauvignon blanc juice. **A** *MpAPr1* expression relative to *ACT1*. Ct values were normalised with cDNA concentration obtained through Qubit analysis before calculating dCt (Ct(*ACT1*)-Ct(*MpAPr1*)). **B** Fold change of *MpAPr1* expression over time obtained through normalisation of dCt values to T0 (2^{ddCt}). **C** Number of *MpAPr1* transcripts present at the start of qPCR amplification quantified using LRE with the CAL1 amplicon from lambda DNA as optical calibrator, normalised with cDNA concentration. The data points shown are means for three independent experiments and the error bars indicate standard deviation between triplicates. Different letters indicate significant differences between samples ($P<0.05$) for similar protein bands as analysed independently by one-way ANOVA and the Fisher's LSD test.

3.4 Discussion and partial conclusion

In this study, protease activity and production of MpAPr1, as well as the degradation of protein substrate, was measured in media supplemented with different nitrogen sources inoculated with *M. pulcherrima* IWBT Y1123. Protease activity assays revealed a distinct and reproducible response to Hb-only and NH_4^+-AA-only conditions. In the absence of complex nitrogen sources in NH_4^+-AA-only treatments, only basal activity levels were evident. Similar observations have been reported for the protease-producing yeasts *Candida humicola* and *Debaryomyces hansenii*, in which only a basal level of activity was observed when yeasts were grown with ammonium as the sole nitrogen source (Ray et al. 1992; Bolumar et al. 2006). This suggests that the presence of more complex nitrogen sources, such as proteins, are required for the induction of protease activity, a hypothesis accepted for the extracellular protease-producing fungi *Neurospora crassa* and *C. albicans* (Cohen et al. 1975; Dabas and Morschhäuser 2008). Additionally, work with *C. albicans* showed that a basal level of protease activity produced micromolar concentrations of amino acids from protein degradation, which acted as end-product inducers for expression of the protease-encoding gene *SAP2* (Dabas and Morschhäuser 2008). In *C. albicans* this basal level of activity even in the presence of assimilable nitrogen sources may be explained by the basal expression of *STP1*, a transcription factor responsible for activating *SAP2*, even in the absence of the NCR-controlled general transcription factors Gln3p and Gat1p which increase *STP1* expression under nitrogen limitation (Dabas and Morschhäuser 2008). The results described in this study show that *M. pulcherrima* IWBT Y1123 grown in the presence of haemoglobin led to significantly increased protease production when compared to NH_4^+-AA-only treatments. Haemoglobin is a tetrameric, acid-soluble protein and has been used extensively as a substrate for the detection of protease activity, and for this reason was used as the complex nitrogen source in the acidic conditions employed in this study (Sternberg 1970; Kalisz 1988).

Treatments containing a combination of haemoglobin and simple nitrogen sources showed a similar response to that of Hb-only, although delayed in timing of onset. This observation suggests that another factor is playing a role in protease regulation regarding the nature and availability of nitrogen sources: that protease production and activity is influenced not only by the presence of complex nitrogen sources, but also by that of simple nitrogen sources such as ammonium and amino acids. This corresponds well with NCR regulation, and this hypothesis has been explored extensively for numerous protease-producing yeast and fungal species including *C. albicans*, *Yarrowia lipolytica*, *D. hansenii*, *Geotrichum candidum*, *Penicillium rocqueforti*, *Rhizopus oligosporus* and *Aspergillus oryzae* (Ogrydziak et al. 1977; Farley and Ikasari 1992; Christensen et al. 1998; Gente et al. 1999;

Boutrou et al. 2006; Bolumar et al. 2008; Dabas and Morschhäuser 2008). Growth of these yeasts in the presence of proteins with and without low molecular weight nitrogen sources such as ammonium revealed that protease secretion and activity did not occur when easily assimilable and preferred nitrogen sources were available. For example, Sap2 secretion by *C. albicans* and the subsequent degradation and utilisation of BSA as a nitrogen source was blocked when low molecular weight nitrogen sources were also available in the medium (Dunkel et al. 2014). Thus, in the context of media containing both simple and complex nitrogen sources, a consequence of NCR regulation is the preferential consumption of low molecular weight compounds before mechanisms required for the utilisation of alternative sources are activated. Indeed, in the presence of proteins, protease-producing *Saccharomyces* strains showed increased protease activity only after easily assimilable nitrogen sources had been mostly consumed (Lekkas et al. 2009; Szopinska et al. 2016). It is therefore possible that this is the phenomenon being observed for *M. pulcherrima* IWBT Y1123 under conditions including both haemoglobin and NH_4^+-AA. As assimilable nitrogen sources in combination treatments are consumed protease repression is alleviated, leading to an increase in protease activity. Activity in haemoglobin-containing treatments as detected through endo-protease assays could furthermore be correlated to the complete degradation of haemoglobin, as well as the detection of MpAPr1 using SDS-PAGE techniques.

Differences in the timing and magnitude of protease activity were evident between YNB and SGJ media. Activity in YNB peaked at 8 h as opposed to 48 h in SGJ. This could be due to a major difference in the composition between the two media, which besides the presence of various vitamins, salts and trace elements in SGJ, was sugar concentration. The YNB medium was made up of 20 g/L glucose, whereas SGJ consists of 230 g/L total sugars (115 g/L glucose and 115 g/L fructose), thus making it 11.5X more concentrated. Glucose (and fructose) is the preferred carbon source for many yeast including *S. cerevisiae*, and its utilisation is under the strict regulation of a carbon catabolite repressor system (Mendes-Ferreira et al. 2011). Amino acids, one of the degradation products of protease activity, are also carbon-containing compounds, although not the preferred source of carbon for yeast. Mechanisms leading to increased amino acid concentration in the environment, such as extracellular protease production, may thus also be subject to carbon catabolite repression when abundant preferred sources such as glucose are available. This carbon repression mechanism, and subsequent de-repression in the event of carbon starvation, has been observed for several protease-producing yeast and fungal strains (Cohen et al. 1975; Ogrydziak et al. 1977; Geisseler and Horwath 2008; Katz et al. 2008; Braaksma et al. 2009; Chaud et al. 2016). It can therefore be hypothesised that protease production by *M. pulcherrima* IWBT Y1123 too may be subject to carbon catabolite repression, such that under the same nitrogen conditions but in media which differed largely in sugar

concentration, protease activity reached a maximum under high sugar conditions almost 40 h after that of low sugar. However, it also possible that the complex SGJ medium introduced factors in addition to elevated sugar levels that interfered with the protease activity assay conditions, possibly leading to competition for the azocasein substrate or inhibition of protease activity, a phenomenon previously observed for MpAPr1 (Theron et al. 2017).

Yeast growth performance also differed between nitrogen treatments and the media used. In YNB there was an initial lag in growth during early exponential phase for Hb-only treatments, which could indicate the time during which protease expression, production and secretion occurred as a result of induction by proteins or peptides and de-repression by the absence of NH_4^+-AA. During this time, advanced growth in NH_4^+-AA-supplemented treatments would be due to the immediately accessible sources of nitrogen. Thereafter, during late exponential phase, growth with only haemoglobin exceeded that of NH_4^+-AA-supplemented treatments in minimal medium and remained so for the remainder of sampling time-points. A correlation could thus be made between protease activity and enhanced cell growth, presumably due to the release of an additional pool of assimilable nitrogen sources. A similar out-performance by Hb-only grown cells did not occur in SGJ. Instead, cell densities for this treatment for SGJ were lower than in NH_4^+-AA-containing treatments for the duration measured. This could be as a direct result of the effect that carbon catabolite repression had on protease activity, leading to the delayed de-repression of protease production and subsequent release of assimilable nitrogen sources for growth. In fact, the presence of MpAPr1 is detected in YNB at least 6 h, and the complete degradation of haemoglobin at least 3 h, before SGJ. Taken together, these results suggest that although the presence of NH_4^+-AA and high levels of glucose and fructose played a role in the repression of MpAPr1, this effect only served to delay the onset of protease activity and protein degradation. However, it is possible that the delay in MpAPr1 production could be attributed to a delayed adaptation of *M. pulcherrima* IWBT Y1123 to the high sugar concentrations of SGJ as opposed to YNB. Furthermore, it is important to note that normalisation of cell density to protease activity revealed a similar increased protease activity in the Hb-only treatments compared to those containing low molecular weight nitrogen sources, confirming that this observation was not due to differences in growth (data not shown).

Having thus established the secretion and activity of MpAPr1 under conditions mimicking that of grape juice, *M. pulcherrima* IWBT Y1123 was subsequently grown in Sauvignon blanc grape juice. As observed previously when grown in the presence of both simple and complex nitrogen sources, protease activity and the presence of MpAPr1 could be detected. However, the maximum activity detected was 80 AU – almost 3-fold less than in SGJ with haemoglobin as protein substrate, which may be attributed to limitations

experienced by the activity assay due to grape juice composition in terms of potential protease inhibitors or competitors for protein substrate. Nevertheless, grape proteins, especially chitinases and TLPs, are known to be highly resistant to proteases in their native state due to their rigid peptide backbone structure (Van Sluyter et al. 2015). If, as mentioned earlier, protease regulation involves induction by degradation products produced from basal protease activity, it is possible that lower activity levels were the result of lower concentrations of these products due to inefficient degradation of grape proteins by MpAPr1. Indeed, protein degradation as visualised using SDS-PAGE appeared to be minimal within the first 48 h of growth, with a visible decrease in intensity occurring only after 5 days and at the end of fermentation. Protease presence and activity as detected by SDS-PAGE techniques and endo-protease assays could furthermore be correlated to the expression of *MpAPr1*. Relative to *ACT1* expression, *MpAPr1* was up-regulated from 3 h to 24 h, the same period during which protease activity increased. However, although *ACT1* has previously been used as a reference gene to normalise the expression of *MpAPr1*, and is commonly used for gene expression analysis in *S. cerevisiae*, its stable expression in *M. pulcherrima* IWBT Y1123 has not been validated (Teste et al. 2009; Reid et al. 2012). Moreover, it is becoming evident that even commonly used housekeeping genes can vary considerably in expression, and the normalisation with the mean of at least three selected reference genes is therefore recommended to smooth individual variations of single genes (Vandesompele et al. 2002). The genome of *M. pulcherrima* IWBT Y1123 has not been sequenced or annotated at this time to assist the search for additional candidate reference genes, thus an alternative normalisation method was utilised. The LRE method makes use of the fact that amplification efficiency is linearly coupled to amplicon quantity, and derives a target quantity value (F0) based on reaction fluorescence during amplification (Rutledge and Stewart 2010). When used in conjunction with an optical calibrator of defined amplicon size from DNA of known concentration – in this case, *CAL1* from lambda DNA – an optical calibration factor (OCF) can be derived and used to convert F0 into an absolute number of transcripts (N0) of the gene of interest, taking into account the amplicon size of the amplified transcript. Despite the difference in normalisation techniques, a similar trend of *MpAPr1* up-regulation was observed after LRE analysis where a significant increase in expression is evident from 3 h to 12 h. Taken together, these results show that *MpAPr1* expression was up-regulated in *M. pulcherrima* IWBT Y1123 upon inoculation into grape juice, reaching maximum expression levels around 12 h after inoculation and leading to the production and secretion of MpAPr1 and its subsequent activity which peaked at 24 h.

The results obtained in this study aid in elucidating the regulatory role that some environmental factors play in the production and activity of MpAPr1 by *M. pulcherrima* IWBT Y1123. Although nitrogen catabolite repression leads to the repression of protease activity

when easily assimilable nitrogen sources are present, this mechanism is balanced by protease induction in the presence of proteins. Furthermore, high sugar levels present in the medium led to a delayed increase in protease activity, presumably as a result of the carbon catabolite repression mechanism. When *M. pulcherrima* IWBT Y1123 was inoculated into grape juice, which includes a combination of simple and complex nitrogen sources as well as high sugar levels, *MpAPr1* expression was up-regulated and protease secretion and activity could be observed. Furthermore, the application of *M. pulcherrima* IWBT Y1123 as a starter culture did not influence the overall fermentation kinetics upon inoculation of *S. cerevisiae*. These results show promise for the use of *M. pulcherrima* IWBT Y1123 as a protease-producing co-starter culture to grape juice fermentations. However, the impact thereof on wine properties such as protein haze formation remains to be explored in further depth.

3.5 References

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3.6 Supplementary material

3.6.1 Protease activity in minimal medium: comparison of means

Table S3.1 Protease activity (AU) in samples of *M. pulcherrima* IWBT Y1123 grown in YNB with ammonium (NH_4^+), ammonium and amino acids (NH_4^+ + AA), ammonium and amino acids and haemoglobin (Hb + NH_4^+ + AA), or haemoglobin (Hb). Means were compared between treatments, within time-points.

					P-value					
Time (h)	**Hb**	**Hb + NH_4^+ + AA**	**NH_4^+ + AA**	**NH_4^+**	**Hb vs Hb + NH_4^++ AA**	**Hb vs NH_4^+ + AA**	**Hb vs NH_4^+**	**Hb + NH_4^+ + AA vs NH_4^+ + AA**	**Hb + NH_4^+ + AA vs NH_4^+**	**NH_4^+ + AA vs NH_4^+**
0	32.2 ± 3.65^{ab}	21.5 ± 1.75^{a}	26.3 ± 1.06^{ab}	33.1 ± 0.867^{b}	0.0647	0.2985	0.8745	0.4067	0.0458	0.2321
1	43.2 ± 5.89^{a}	30.8 ± 4.86^{b}	33.2 ± 3.06^{ab}	41.2 ± 2.27^{ab}	0.0335	0.0856	0.7215	0.668	0.0737	0.1693
3	91.4 ± 1.63^{a}	50.7 ± 1.03^{b}	43.1 ± 0.898^{b}	55.1 ± 6.88^{b}	<0.0001	<0.0001	<0.0001	0.188	0.4905	0.0646
6	160 ± 2.57^{a}	144 ± 6.56^{b}	47.7 ± 2.92^{c}	64.1 ± 4.61^{d}	0.0061	<0.0001	<0.0001	<0.0001	<0.0001	0.0056
8	230 ± 7.14^{a}	256 ± 37.7^{b}	74.3 ± 4.96^{c}	89.1 ± 5.60^{d}	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0242
11	149 ± 7.34^{a}	163 ± 12.4^{b}	60.2 ± 3.62^{c}	55.5 ± 1.46^{c}	0.0189	<0.0001	<0.0001	<0.0001	<0.0001	0.4149
24	212 ± 4.01^{a}	189 ± 3.19^{b}	79.2 ± 4.55^{c}	51.1 ± 3.67^{d}	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Values are means of protease activity in AU ± SD, n=3 per treatment group. $^{a-c}$ Means in a row with different superscript letters differ significantly (P<0.05) as analysed by two-way ANOVA and the Fisher's LSD test.

Table S3.2 Comparison of protease activity (AU) means in samples of *M. pulcherrima* IWBT Y1123 grown in YNB with ammonium (NH_4^+), ammonium and amino acids (NH_4^+ + AA), ammonium and amino acids and haemoglobin (Hb + NH_4^+ + AA), or haemoglobin (Hb). Means were compared between time-points, within treatments.

NH_4^+

Time (h)	**0**	**1**	**3**	**6**	**8**	**11**	**24**
0	-	ns	**	****	****	***	**
1	-	-	*	***	****	*	ns
3	-	-	-	ns	****	ns	ns
6	-	-	-	-	***	ns	*
8	-	-	-	-	-	****	****
11	-	-	-	-	-	-	ns
24	-	-	-	-	-	-	-

NH_4^+ + AA

Time (h)	**0**	**1**	**3**	**6**	**8**	**11**	**24**

0	-	ns	**	***	****	****	****
1	-	-	ns	*	****	****	****
3	-	-	-	ns	****	**	****
6	-	-	-	-	****	*	****
8	-	-	-	-	-	*	ns
11	-	-	-	-	-	-	**
24	-	-	-	-	-	-	-

NH_4^+ + AA + Hb

Time (h)	**0**	**1**	**3**	**6**	**8**	**11**	**24**
0	-	ns	****	****	****	****	****
1	-	-	***	****	****	****	****
3	-	-	-	****	****	****	****
6	-	-	-	-	****	**	****
8	-	-	-	-	-	****	****
11	-	-	-	-	-	-	****
24	-	-	-	-	-	-	-

Hb

Time (h)	**0**	**1**	**3**	**6**	**8**	**11**	**24**
0	-	ns	****	****	****	****	****
1	-	-	****	****	****	****	****
3	-	-	-	****	****	****	****
6	-	-	-	-	****	ns	****
8	-	-	-	-	-	****	**
11	-	-	-	-	-	-	****
24	-	-	-	-	-	-	-

Means in AU (n=3 per treatment group) were analysed by two-way ANOVA and the Fisher's LSD test.
ns: P>0.05
*: P≤0.05
**: P≤0.01
***: P≤0.001
****: P≤0.0001

3.6.2 Protease activity in synthetic grape juice: comparison of means

Table S3.3 Protease activity (AU) in samples of *M. pulcherrima* IWBT Y1123 grown in SGJ with ammonium and amino acids (NH_{4+} + AA), ammonium and amino acids and haemoglobin (NH_4^+ + AA + Hb), or haemoglobin only (Hb). Means were compared between treatments, within time-points.

				P-value		
Time (h)	**Hb**	**Hb + NH_4^+ + AA**	**NH_4^+ + AA**	**Hb vs Hb + NH_4^+ + AA**	**Hb vs NH_4^+ + AA**	**Hb + NH_4^+ + AA vs NH_4^+ + AA**
0	4.77 ± 1.64	1.95 ± 0.31	4.28 ± 0.84	0.3911	0.8815	0.5165
1	5.82 ± 1.67	4.23 ± 1.64	4.41 ± 0.82	0.5875	0.6305	0.9508
3	17.3 ± 1.89^a	6.13 ± 1.15^b	6.32 ± 2.29^b	0.0013	0.0005	0.9516
6	64.8 ± 5.70^a	9.25 ± 0.33^b	6.79 ± 2.14^b	< 0.0001	< 0.0001	0.403
9	83.1 ± 3.07^a	16.0 ± 3.04^b	8.96 ± 3.38^c	< 0.0001	< 0.0001	0.0194
12	102 ± 3.76^a	49.9 ± 3.55^b	17.1 ± 1.32^c	< 0.0001	< 0.0001	< 0.0001
24	241 ± 0.15^a	197 ± 1.04^b	34.0 ± 1.76^c	< 0.0001	< 0.0001	< 0.0001

48	274 ± 10.2[a]	223 ± 7.52[b]	35.4 ± 4.14[c]	< 0.0001	< 0.0001	< 0.0001

Values are means of protease activity in AU ± SD, n=3 per treatment group. [a-c] Means in a row with different superscript letters differ significantly (P<0.05) as analysed by two-way ANOVA and the Fisher's LSD test.

Table S3.4 Comparison of protease activity (AU) means in samples of *M. pulcherrima* IWBT Y1123 grown in SGJ with ammonium and amino acids (NH_4^+ + AA), ammonium and amino acids and haemoglobin (Hb + NH_4^+ + AA), or haemoglobin only (Hb). Means were compared between time-points, within treatments.

NH_4^+ + AA

Time (h)	**0**	**1**	**3**	**6**	**9**	**12**	**24**	**48**
0	-	ns	ns	ns	ns	***	****	****
1	-	-	ns	ns	ns	****	****	****
3	-	-	-	ns	ns	***	****	****
6	-	-	-	-	ns	***	****	****
9	-	-	-	-	-	**	****	****
12	-	-	-	-	-	-	****	****
24	-	-	-	-	-	-	-	ns
48	-	-	-	-	-	-	-	-

NH_4^+ + AA + Hb

Time (h)	**0**	**1**	**3**	**6**	**9**	**12**	**24**	**48**
0	-	ns	ns	*	****	****	****	****
1	-	-	ns	ns	***	****	****	****
3	-	-	-	ns	**	****	****	****
6	-	-	-	-	*	****	****	****
9	-	-	-	-	-	****	****	****
12	-	-	-	-	-	-	****	****
24	-	-	-	-	-	-	-	****
48	-	-	-	-	-	-	-	-

Hb

Time (h)	**0**	**1**	**3**	**6**	**9**	**12**	**24**	**48**
0	-	ns	****	****	****	****	****	****
1	-	-	***	****	****	****	****	****
3	-	-	-	****	****	****	****	****
6	-	-	-	-	****	****	****	****
9	-	-	-	-	-	****	****	****
12	-	-	-	-	-	-	****	****
24	-	-	-	-	-	-	-	****
48	-	-	-	-	-	-	-	-

Means in AU (n=3 per treatment group) were analysed by two-way ANOVA and the Fisher's LSD test.
ns: P>0.05
*: P≤0.05
**: P≤0.01
***: P≤0.001
****: P≤0.0001

Chapter 4

Research results

Investigating the impact of the protease-secreting yeast *Metschnikowia pulcherrima* IWBT Y1123 on wine properties

Chapter 4 - Investigating the impact of the protease-secreting yeast *Metschnikowia pulcherrima* IWBT Y1123 on wine properties

4.1 Introduction

Wine clarity plays an important role in the perception of wine quality, and any signs of turbidity are generally regarded as a fault by consumers (Waters et al. 2005). Protein heat instability is a key component in the formation of haze and is easily affected by inappropriate shipping and storage conditions (Van Sluyter et al. 2015). In response to stimuli such as elevated storage temperatures, heat-unstable grape proteins unfold and aggregate, before cross-linking with other aggregates to form increasingly larger particles that eventually become visible to the naked eye and are perceived as haze. There are many non-proteinaceous wine components that also contribute to this process, such as pH, ionic strength, phenolic substances and polysaccharides (McRae et al. 2018). However, protein concentration, specifically that of the pathogenesis-related (PR) grape proteins chitinases and thaumatin-like proteins (TLPs), is shown to correlate positively with haze formation. In fact, chitinases, which unfold and aggregate at a lower temperature than TLPs and are shown to be generally less stable, are considered to play the major role in wine haze formation (Marangon et al. 2011b). Most TLP isoforms require interaction with other wine components, such as salts and polyphenolics, before contributing to visible haze and their impact is therefore wine-dependent (Van Sluyter et al. 2015). Methods for the elimination of wine haze therefore focus primarily on the removal of grape proteins.

Bentonite is most commonly employed by wine-makers as a fining agent, due to its protein-binding and settling properties (Waters et al. 2005). However, this clay is associated with significant disadvantages such as the labour-intensive and time-consuming nature of its application, concerns regarding disposal of spent bentonite, as well as losses of wine yield and quality due to dilution of the wine and the non-specific binding of bentonite to potentially sensory-relevant compounds (Van Sluyter et al. 2015; Jaeckels et al. 2016). The quest to improve bentonite efficiency and to find alternative stabilisation strategies is therefore of great interest to the wine industry (Van Sluyter et al. 2015; Muhlack and Colby 2018). Novel fining agents that have been explored as alternatives to bentonite include zirconium dioxide, chitin and the seaweed polysaccharide carrageenan (Vincenzi et al. 2005; Marangon et al. 2011a; Marangon et al. 2013). More recently, the use of mesoporous nanomaterials and magnetic nanoparticles and of chitosan both as a single adjuvant and as a support for enzyme immobilisation, have been investigated for their efficacy in reducing wine haze

(Benucci et al. 2017; Mierczynska-Vasilev et al. 2017; Colangelo et al. 2018; Dumitriu et al. 2018).

Another promising strategy for protein removal is through the use of proteolytic enzymes – a particularly appealing avenue because it minimizes wine volume loss and aroma stripping (Van Sluyter et al. 2015). In fact, their application harbours the potential added benefit of increasing the assimilable nitrogen content of wine, thus potentially preventing stuck fermentations and enhancing the sensory profile of wine through the release of volatile compounds from yeast metabolism (Zhang et al. 2016). Aspartic proteases from various fungal origins, including *Aspergillus niger* and *Botrytis cinerea*, have been added as exogenous treatments to grape juice in testing their ability to degrade grape proteins and subsequently reduce wine hazing potential (Marangon et al. 2012; Van Sluyter et al. 2013). However, enzymes of indigenous origin to the winemaking environment are particularly sought after, and several protease-producing yeast strains of oenological origin have been isolated (Lagace and Bisson 1990; Charoenchai et al. 1997; Dizy and Bisson 2000; Strauss et al. 2001; Younes et al. 2011; Mateo et al. 2015; Schlander et al. 2016; Escribano et al. 2017).

Metschnikowia pulcherrima IWBT Y1123 is one such wine yeast that has been isolated from grape juice and shown to display extracellular proteolytic activity (Reid et al. 2012). Its secreted aspartic protease, MpAPr1, was able to degrade grape proteins, showing activity particularly against chitinases (Theron et al. 2017). Furthermore, grape juice treatment with MpAPr1 was shown to significantly reduce haze formation after 48 h incubation, and influence the volatile composition of the wine (Theron et al. 2018). However, although *MpAPr1* expression and protease activity was evident under winemaking conditions when grape juice was inoculated with *M. pulcherrima* IWBT Y1123, as described in Chapter 3, the impact of this yeast on wine properties when used as a co-starter culture remains to be elucidated. This direct addition of a starter culture to grape juice for the purpose of protease secretion and activity would eliminate the need for enzyme purification steps, presenting a time- and cost-effective strategy that does not suffer from the same administrative restrictions that should be considered for enzyme preparations (Schlander et al. 2016). However, non-*Saccharomyces* yeasts are typically weak fermenters and must therefore be used in combination with *S. cerevisiae* in order to complete fermentation (Mateo et al. 2015).

The application of enzyme-producing non-*Saccharomyces* yeasts to winemaking is of great biotechnological interest to the wine industry, for various technological and sensory reasons (Maturano et al. 2012; Maicas and Mateo 2015; Claus and Mojsov 2018). However, the endogenous enzymes of yeasts (as well as grapes and other microorganisms) are typically not secreted efficiently enough, nor show sufficient enough activity, under

winemaking conditions to catalyse the relevant biotransformation processes to such an extent as to have a significant impact on wine properties (van Rensburg and Pretorius 2000). Numerous studies have reported about the enzyme activities by non-*Saccharomyces* yeasts in winemaking and fermentations (Zamuz et al. 2004; Fia et al. 2005; Comitini et al. 2011; Maturano et al. 2012). However, few studies relate enzymatic activity in mixed cultures of *Saccharomyces* and non-*Saccharomyces* to its actual impact on wine properties, such as the aromatic profile (Maturano et al. 2015; Padilla et al. 2016). Such knowledge is crucial for developing effective inoculation strategies based on the desired properties of the final wine, taking into account the effect of secreted enzymatic activities and microbial interactions during fermentation.

The aim of this work was to assess the impact of inoculating the protease-producing yeast *M. pulcherrima* IWBT Y1123 as a grape juice starter culture in mixed fermentation with *Saccharomyces cerevisiae* on wine properties including grape protein degradation, haze formation potential and composition of volatile compounds. *M. pulcherrima* has already been shown to positively influence wine sensory properties and any further contribution in terms of haze reduction would be an advantage for wine-makers towards reducing or eliminating the need for bentonite (Barbosa et al. 2018).

4.2 Materials and methods

4.2.1 Strains and fermentation conditions

The strains of *Metschnikowia pulcherrima* used in this study were *M. pulcherrima* IWBT Y1123 and the commercial wine yeast strain *M. pulcherrima* Flavia® MP346 (Lallemand) as a protease-negative control. *S. cerevisiae* Lalvin® EC1118 was used for sequential inoculations. Cultures were maintained and pre-cultures were grown as described in Chapter 3.

The Nouvelle grape berries used in this study were crushed, destemmed and pressed at the Stellenbosch University experimental cellar from grapes harvested at the Welgevallen experimental vineyard (Stellenbosch University) in 2018. Berries from these vines were chosen due to the heat instability observed after a protein haze assay in wine produced from a preliminary fermentation with *S. cerevisiae* EC1118 (data not shown). No enzyme additions were made and juice was stored at -20°C. After thawing and prior to experimental use, grape juice was centrifuged at 8000 x *g* for 15 min. Grape juice was not filter-sterilised, in order to prevent excessive oxidation. Grape juice (pH 3.8) glucose and fructose concentrations were determined at 110 g/L and 105 g/L respectively, and ammonium and PAN concentrations at 82 mg/L and 124 mg/L respectively, as described in Chapter 3. Suspensions of *M. pulcherrima* cells were inoculated at an optical density of 2.4

at 600 nm into 250-mL Erlenmeyer flasks containing 150 mL grape juice, sealed with rubber bungs and S-bend airlocks, and incubated at 25°C with shaking at 110 rpm. After 48 h incubation *S. cerevisiae* was inoculated at a concentration of 10^6 cells/mL. Grape juice with no prior inoculation of *M. pulcherrima* was inoculated separately with *S. cerevisiae* as a control. Fermentations were carried out in triplicate and fermentation kinetics were monitored by measuring flask weight loss daily (as an estimation of CO_2 released). Samples were taken at 0 h, 9 h, 24 h, 48 h and 72 h after *M. pulcherrima* inoculation and at the end of fermentation, for monitoring protease activity against azocasein in liquid assays (as described in Chapter 3) and the haze-forming potential of the samples (as described below). The remaining sample was stored at -20°C prior to SDS-PAGE analysis and protein quantification (as described below). Samples taken at the end of fermentation were analysed for major volatile compounds (as described below).

4.2.2 Protein haze assay

Protein haze potential of grape juice and wine samples was determined as described by Pocock and Waters (2006). Samples were centrifuged at 6000 x *g* for 5 min before the supernatant absorbance was measured at 520 nm using a Lambda UV/Vis spectrophotometer (Perkin Elmer, MA, USA). Samples were then incubated at 80°C for 2 h and thereafter allowed to return to room temperature for 45 min before again measuring absorbance at 520 nm. Haze-forming potential is depicted as the change in absorbance between heated and non-heated samples. Samples were considered unstable or prone to haze when this difference was greater than an absorbance unit of 0.02 (Pocock et al. 2007).

4.2.3 Protein quantification, visualisation and identification

The protein content of sample supernatants was quantified and visualised using BCA assays and SDS-PAGE techniques, respectively, as described in Chapter 3. Relative protein quantification of bands visualised through SDS-PAGE was determined through densitometry analyses of protein band intensities using Image Lab™ Software v6.0 (Bio-Rad Laboratories) and a protein band obtained from a 2 mg/mL sample of protease from *Aspergillus saitoi* as common standard. Absolute protein quantification of bands was also determined using the Image Lab™ Software, with a standard curve derived from the band intensities of a gel ran with a serial dilution of known concentrations of protease from *A. saitoi.* Selected protein bands were excised from the gels and sequenced by LC-MS/MS after trypsin in-gel digestion at the Centre for Proteomic and Genomic Research (CPGR, Cape Town, South Africa). Database interrogation was performed with Byonic Software v2.6.46 (Protein Metrics, CA, USA) using the proteomes of *Metschnikowia bicuspidate*, *Clavispora lusitaniae*, *Debaryomyces hansenii*, *Meyerozyma guilliermondii, Lodderomyces elon*gis*porus* and

Saccharomyces cerevisiae, as well as protein databases for *Metschnikowia fructicola* and *Metschnikowia pulcherrima,* sourced from UniProt.

4.2.4 Major volatile analysis

A selection of major volatiles (alcohols, esters, and acids) were analysed using a gas chromatography – flame ionisation detection (GC-FID) method previously described by Louw (2007). Sample preparation involved a liquid-liquid extraction method in which 1 mL diethyl ether was added to 5 mL wine sample, followed by the addition of 100 µL internal standard stock solution (0.5 mg/L 4-methyl-2-pentanol). The samples were vortexed for 10 s followed by sonication for 5 min before cooling to 4°C for 10 min. After centrifugation at 4000 rpm for 3 min the ether layer was removed and dried over anhydrous sodium sulfate. Analyses were carried out on a Hewlett Packard 6890 Series II GC coupled to an HP 3396A auto-sampler and injector. An Agilent J&W DB-FFAP capillary GC column (60 m x 0.32 mm internal diameter with a 0.5 µM thick coating) was used, with hydrogen as the carrier gas for FID. The following conditions were used: injection temperature at 250°C, split ratio 15:1, flow rate 3.3 ml/min. Oven temperature was increased from 33°C to 240°C at 12°C/min. Each extract was injected and analysed in duplicate.

4.2.5 Statistical analysis

Statistical analyses were performed using the computer software GraphPad Prism 6 (GraphPad) to determine significant differences between treatments and time-points.

4.3 Results

4.3.1 Fermentation kinetics

M. pulcherrima IWBT Y1123 (Mp Y1123) and Flavia (Mp Flavia) were inoculated into non-sterile Nouvelle grape juice, followed by *S. cerevisiae* EC1118 (Sc) sequential inoculation 48 h later. The fermentation kinetics were followed, as well as that of *S. cerevisiae* inoculated into 48 h incubated grape juice not inoculated with *M. pulcherrima* (Figure 4.1). Weight loss differences between treatments were not significant for most of the duration of fermentation, although CO_2 loss was also observed in the non-inoculated grape juice during the 48 h before *S. cerevisiae* inoculation. All fermentations reached dryness by 200 h after inoculation.

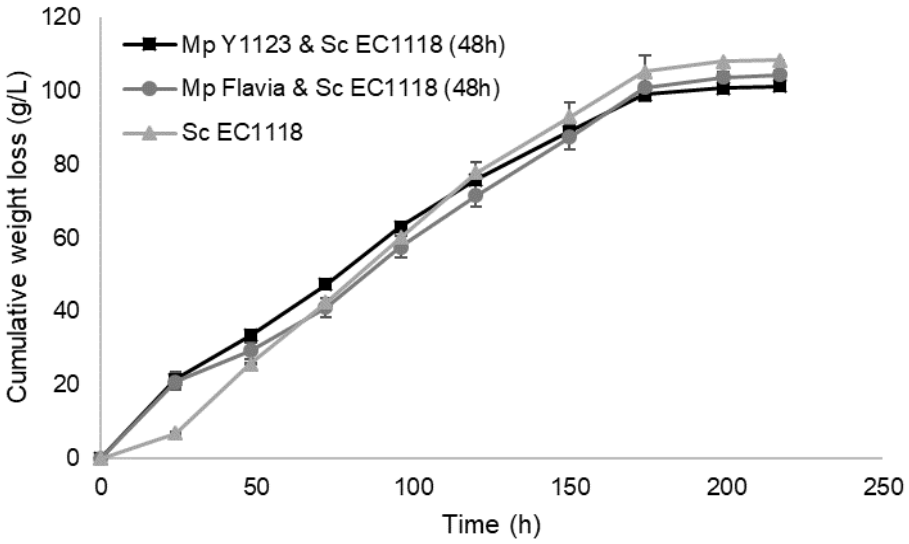

Figure 4.1 Loss of CO_2 during fermentation monitored by accumulated weight loss of flasks containing *M. pulcherrima* IWBT Y1123 (Mp Y1123) or *M. pulcherrima* Flavia (Mp Flavia) in 48 h sequential inoculation with *S. cerevisiae* EC1118, and pure *S. cerevisiae* EC1118 (Sc EC1118), in grape must. The data points shown are means for three independent experiments and the error bars indicate standard deviation between triplicates

4.3.2 Protease activity

Protease activity against azocasein was monitored in supernatant samples of *M. pulcherrima* IWBT Y1123, *M. pulcherrima* Flavia and *S. cerevisiae* taken during the first 48 h following their inoculation into grape juice (Figure 4.2). Activity in Mp Y1123 samples was significantly higher than in those of Mp Flavia or Sc at corresponding time-points, except at 0 h for which Mp Y1123 and Sc did not show a difference. Protease activity increased significantly in Mp Y1123 from 0 h to 9 h, and again to 24 h, after which activity decreased significantly to 48 h after inoculation. Differences in activity in Mp Flavia and Sc samples between time-points were mostly insignificant. Data obtained from statistical comparison of means between treatments can be found in supplementary data (Table S4.1).

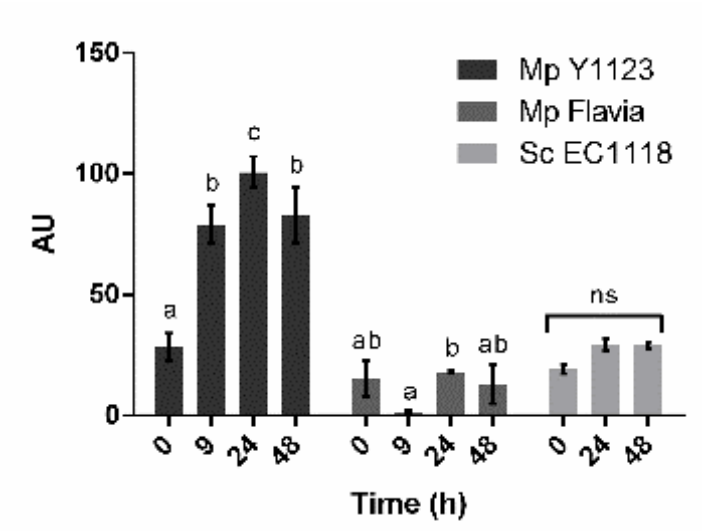

Figure 4.2 Protease activity calculated for supernatant samples of *M. pulcherrima* IWBT Y1123 (Mp Y1123), *M. pulcherrima* Flavia (Mp Flavia) and *S. cerevisiae* EC1118 (Sc EC1118) taken at different time points throughout the first 48 h of growth in grape juice. The data points shown are means for three independent experiments and the error bars indicate standard deviation between triplicates. Different letters indicate significant differences between samples ($P<0.05$) for similar treatments as analysed independently by two-way ANOVA and the Fisher's LSD test. Non-significant differences across samples for a similar treatment are indicated by ns.

4.3.3 Protein haze assay

Haze-forming potential in samples of Mp Y1123 and Mp Flavia with sequential Sc inoculation, grape juice inoculated with only Sc, and a non-inoculated control, are shown in Figure 4.3. A similar trend was observed in samples from all treatments, with an initial increase in haze-forming potential over the first 24 h after inoculation for Mp Flavia and Sc, and over the first 48 h for Mp Y1123. However, at the end of fermentation a significant decrease in haze was observed in all treatments, with both Mp samples displaying significantly lower levels of haze than in Sc, although not significantly different from each other. Data obtained from statistical comparison of means can be found in supplementary data (Tables S4.2 and S4.3).

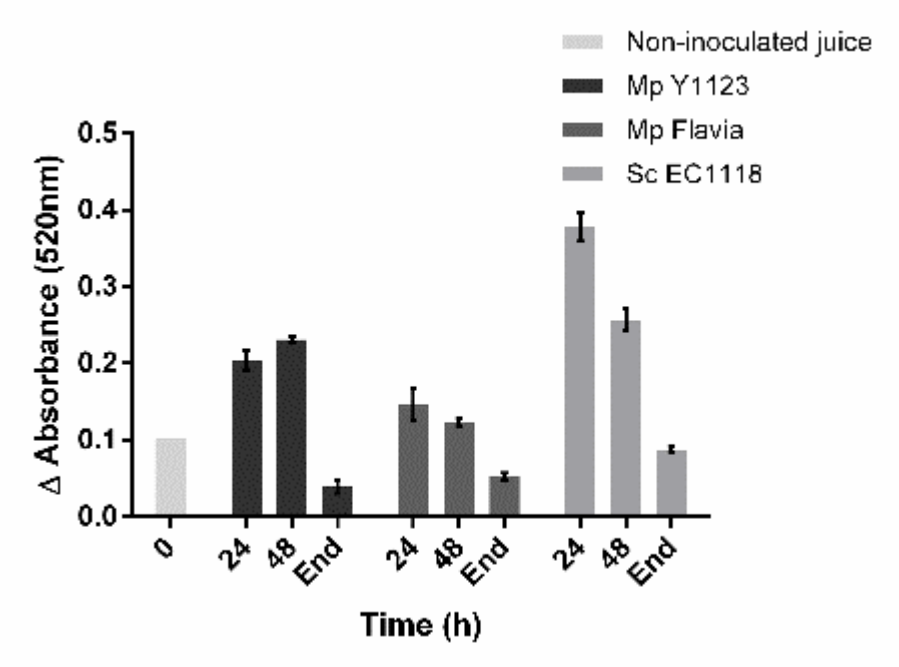

Figure 4.3 Heat stability of non-inoculated grape juice, and grape juice fermented with *S. cerevisiae* EC1118 (Sc EC1118) with or without the inoculation of *M. pulcherrima* IWBT Y1123 (Mp Y1123) or *M. pulcherrima* Flavia (Mp Flavia) 48 h prior to *S. cerevisiae* inoculation. The data points shown are means for three independent experiments and the error bars indicate standard deviation between triplicates.

4.3.4 Protein visualisation, identification and densitometry analysis

Proteins were extracted from 24 h, 48 h and end-point samples of Mp Y1123 and Mp Flavia, and from 48 h and end-point samples of Sc, and visualised with SDS-PAGE (Figure 4.4). The protein band in a sample of Mp Y1123 at ~35 kDa was identified as MpAPr1 through LC-MS/MS analysis. A | Log Prob | of 22.90, the absolute value of the log base 10 of the protein p-value, which is the likelihood of peptide-spectrum matches (PSMs) to the protein arising by random chance, was obtained in the search against MpAPr1. A total number of 11 unique peptides were matched to MpAPr1 which covered 39.68% of the MpAPr1 protein sequence. A protein band of unknown identity was furthermore observed at ~40 kDa in samples of Mp Y1123 and Mp Flavia at 24 h and 48 h. However, peptides sequenced from these samples yielded no match to the databases used in this study. Bands corresponding to grape proteins are visible in all samples and decrease in intensity by the end of fermentation for all treatments.

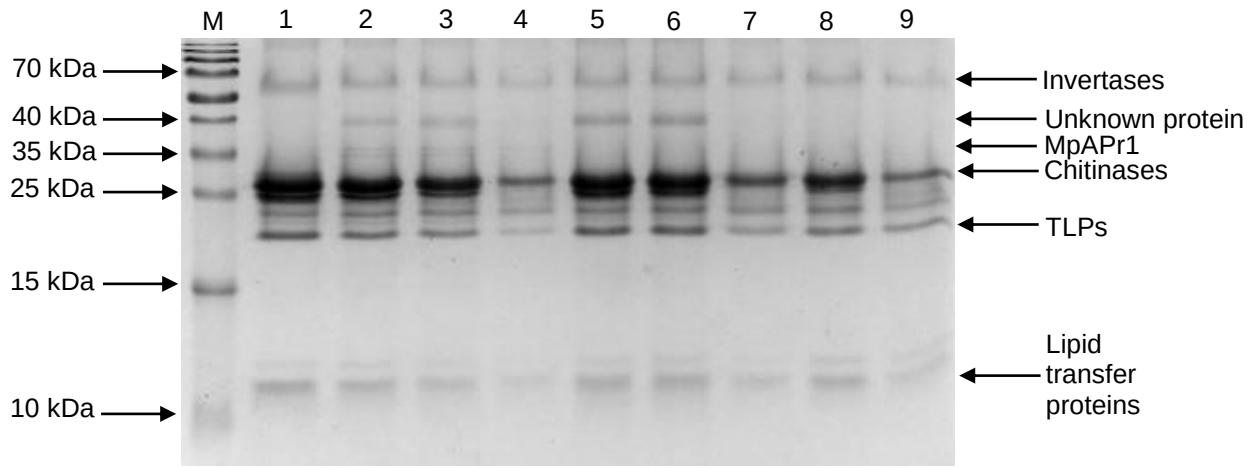

Figure 4.4 Visualisation of proteins precipitated from samples taken from non-inoculated grape juice (lane 1), throughout grape juice fermentation by *M. pulcherrima* IWBT Y1123 with 48 h sequential *S. cerevisiae* inoculation (24 h and 48 h after *M. pulcherrima* IWBT Y1123 inoculation and at the end of fermentation, lanes 2-4 respectively), by *M. pulcherrima* Flavia with 48 h sequential *S. cerevisiae* inoculation (24 h and 48 h after *M. pulcherrima* Flavia inoculation and at the end of fermentation, lanes 5-7 respectively), and by *S. cerevisiae* EC1118 (48 h after inoculation and at the end of fermentation, lanes 8 and 9 respectively). M: molecular weight marker (PageRuler™ Prestained Protein Ladder, Thermo Scientific). Arrows to the right of the gel indicate protein bands identified as MpAPr1 through LC-MS/MS analysis, or grape proteins through comparison of molecular weight (Van Sluyter et al. 2015, Theron et al. 2017).

Densitometry analyses were performed on three distinct gels loaded with proteins as in Figure 4.4, on four sets of tentatively identified protein bands: invertase, chitinase, TLPs and lipid transfer proteins (LTPs) (Figure 4.5). Changes in band intensities of invertases and LTPs remained mostly insignificant between treatments within time-points and were significantly decreased by the end of fermentation within both *M. pulcherrima* treatments. By the end of fermentation, chitinase band intensities had decreased significantly within all treatments, as had TLPs within the *M. pulcherrima* treatments. Chitinase and TLP band intensities were significantly decreased in Mp Y1123 treatments compared to Mp Flavia at the end of fermentation, although not when compared to Sc. Data obtained from statistical comparison of means can be found in supplementary data (Tables S4.4 and S4.5).

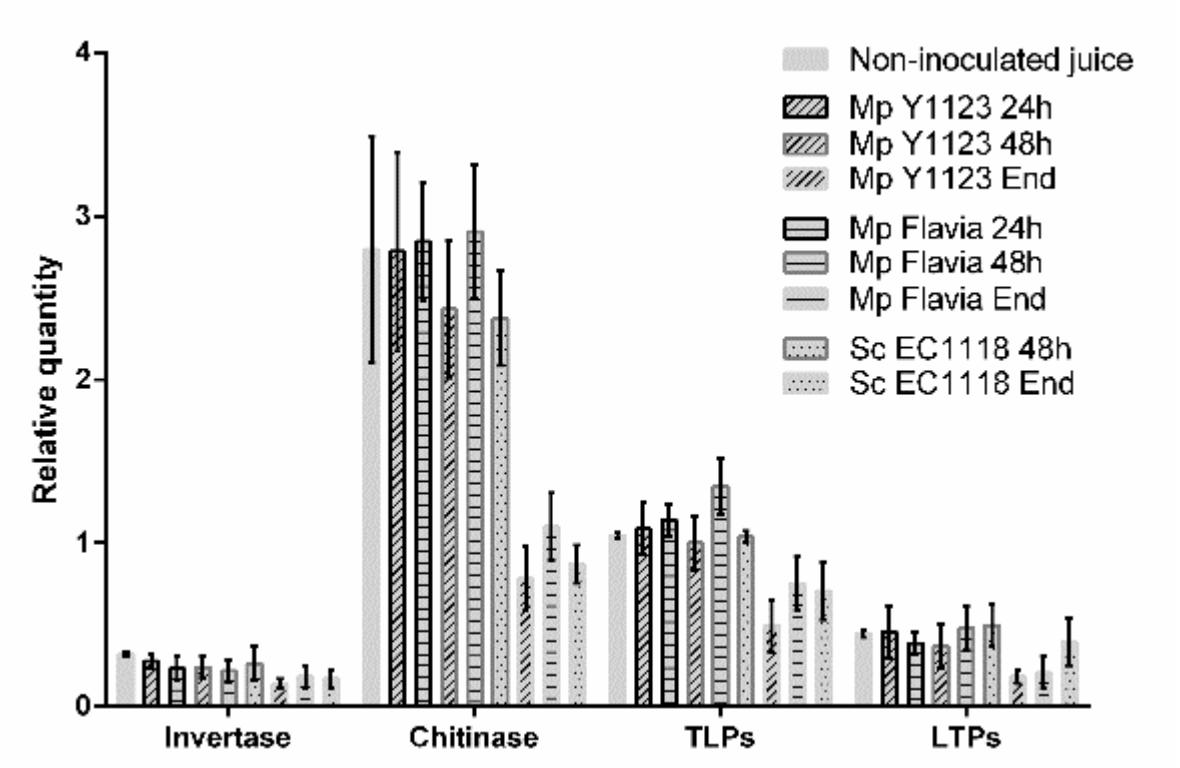

Figure 4.5 Densitometry analysis of SDS-PAGE gel of grape proteins extracted from grape juice fermented with *S. cerevisiae* EC1118 (Sc EC1118) with or without the inoculation of *M. pulcherrima* IWBT Y1123 (Mp Y1123) or *M. pulcherrima* Flavia (Mp Flavia) 48 h prior to *S. cerevisiae* inoculation (Figure 4.4). Relative quantity of identified grape proteins were determined through comparison of band intensities with a common standard (2 mg/mL protease from *A. saitoi*). The data points shown are means for three independent experiments ran on three gels and the error bars indicate standard deviation between triplicates.

Bands identified as MpAPr1 in 24 h and 48 h samples of Mp Y1123 were also subjected to densitometry analysis to determine absolute concentration calculated using a standard curve derived from the band intensities of a gel ran with a serial dilution of known concentrations of protease from *A. saitoi*. Average concentrations were estimated at 97 mg/L and 116 mg/L for 24 h and 48 h samples, respectively (data not shown).

4.3.5 Protein quantification

Total protein concentrations for samples of Mp Y1123, Mp Flavia and Sc are depicted in Figure 4.6. Protein content decreased in all treatments by the end of fermentation. The decrease from 24 h to 48 h was significant in Mp Y1123 and Sc although not so in Mp Flavia, but in all treatments the decrease in protein concentration from 48 h to the end of fermentation was significant. Protein concentration was significantly lower in 24 h and 48 h samples of Mp Y1123 than in the same time-points of Mp Flavia, whereas Sc was less than both of these. However, at the end of fermentation protein concentration was significantly lower in Mp Y1123 than both Mp Flavia and Sc, at 402 µg/mL. Data obtained from statistical comparison of means can be found in supplementary data (Tables S4.6 and S4.7).

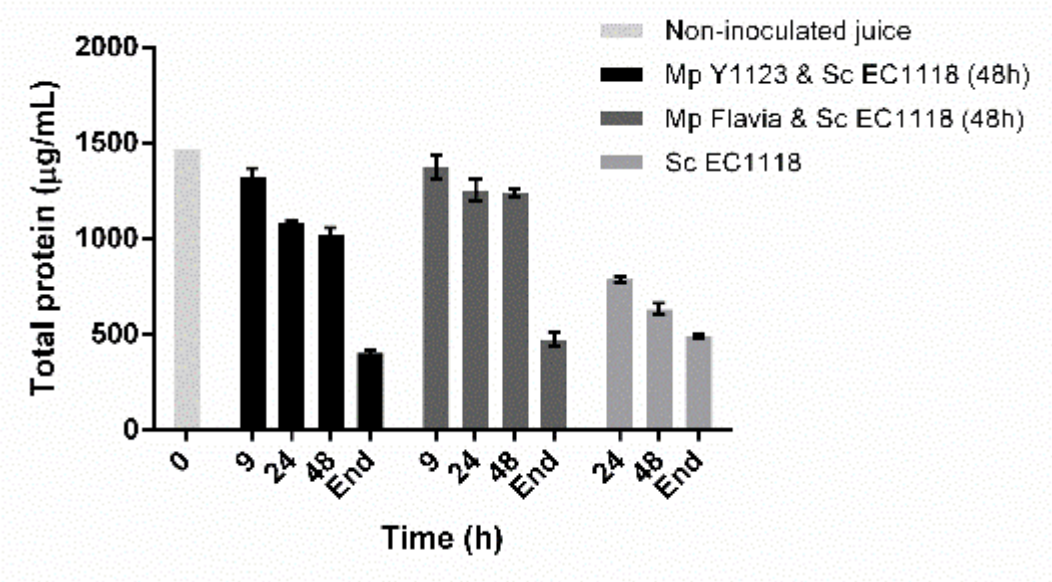

Figure 4.6 Total protein content in samples of grape juice fermented with *S. cerevisiae* EC1118 (Sc EC1118) with or without the inoculation of *M. pulcherrima* IWBT Y1123 (Mp Y1123) or *M. pulcherrima* Flavia (Mp Flavia) 48 h prior to *S. cerevisiae* inoculation, determined by BCA protein quantification. The data points shown are means for three independent experiments and the error bars indicate standard deviation between triplicates.

4.3.6 Major volatile compounds

Major volatile compounds were analysed in wine samples fermented with Sc, with or without prior inoculation of Mp Y1123 or Mp Flavia. Compounds with statistically different concentrations between treatments are depicted in Figure 4.7. Grape juice inoculated with Mp Y1123 and Mp Flavia resulted in wine with significantly lower levels of ethyl acetate and acetic acid. Acetic acid and propanol were present in lower concentrations in wine made from juice inoculated with Mp Y1123 compared to Mp Flavia. The higher alcohols 2-phenethylethanol, isobutanol and isoamyl alcohol were present in significantly higher concentrations with Mp Y1123 and Mp Flavia than only Sc. When Mp Flavia was inoculated, 2-phenethylethanol concentrations were significantly higher whereas Mp Y1123 led to higher levels of isobutanol and isoamyl alcohol than Mp Flavia. Data obtained from statistical comparison of means can be found in supplementary data (Table S4.8).

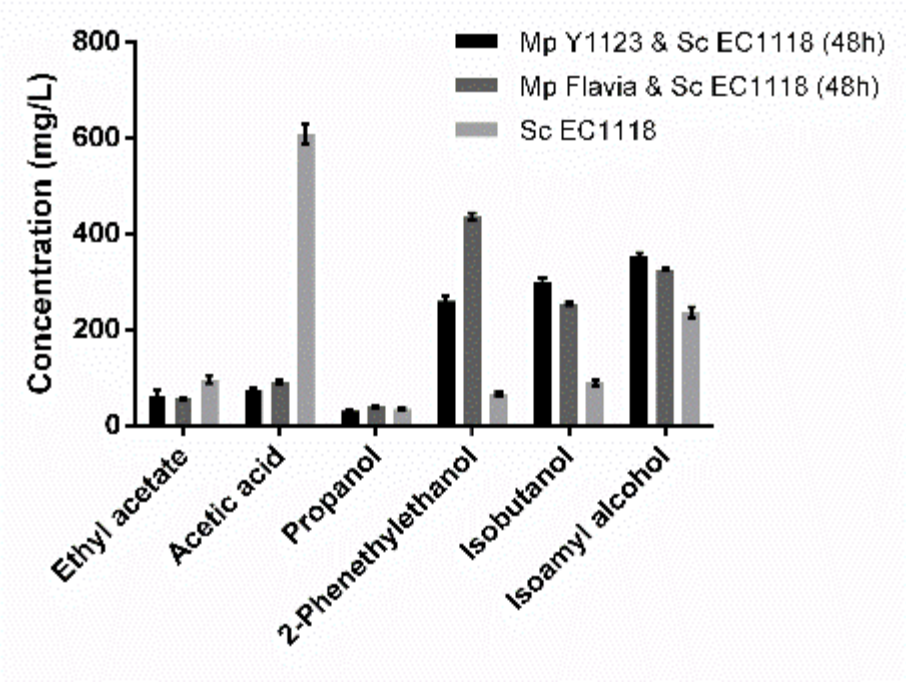

Figure 4.7 Major volatile compounds quantified by GC-FID in wine samples fermented by *S. cerevisiae* EC1118 (Sc EC1118) with or without prior inoculation of *M. pulcherrima* IWBT Y1123 (Mp Y1123) or *M. pulcherrima* Flavia (Mp Flavia). Compounds shown are significantly different in concentration ($P<0.05$) between treatments as analysed by two-way ANOVA and the Fisher's LSD test. The data points shown are means for three independent experiments and the error bars indicate standard deviation between triplicates

4.4 Discussion and partial conclusion

Since 1990, the ability of wine non-*Saccharomyces* yeasts to secrete proteases has been the topic of several investigations, because of their potential to degrade haze-forming proteins in wine and provide nutrient sources for microorganisms (Lagace and Bisson 1990). The ability of some yeast strains to secrete extracellular proteases that are active under winemaking conditions during fermentation has been demonstrated (Dizy and Bisson 2000; Younes et al. 2011; Maturano et al. 2012; Szopinska et al. 2016; Schlander et al. 2016). Dizy and Bisson (2000) furthermore reported on protease-producing strains of *Kloeckera* and *Hanseniaspora* that even reduced the protein content of the final wine, whereas Younes et al. (2011) showed that fermentation with the protease-producing *S. cerevisiae* strain PlR1 led to grape protein degradation, albeit only after incubation of the wine sample supernatants at 38°C for 48 h. However, thus far no reports have linked the application of a protease-producing yeast in grape juice fermentation to a reduction of wine haze-forming potential and a change in volatile composition.

The results obtained in this study show that the co-fermentation of Nouvelle grape juice with *M. pulcherrima* IWBT Y1123 and *S. cerevisiae* led to a reduction in wine haze-forming potential from 24 h after inoculation compared to when only *S. cerevisiae* was

inoculated and that this trend persisted until the end of fermentation. An initial increase in haze-forming potential, as observed for Mp Y1123 samples from 0 h to 48 h and for Mp Flavia and Sc samples from 0 h to 24 h, was similarly demonstrated by Lagace and Bisson (1990) in grape juice samples incubated with protease suspensions from non-*Saccharomyces* yeasts, but which eventually showed haze levels below that of control samples. The authors hypothesised that this initial increase could be contributed to by the protease protein itself, as well as other proteins or protein by-products present in the crude suspension that were secreted by the yeast, a hypothesis that can possibly be extended to the similar phenomenon observed in study. Protease activity could be detected in supernatant samples of Mp Y1123 over the 48 h following its inoculation into grape juice, as well as the presence of MpAPr1 in the fermenting juice. As similarly demonstrated with protease assays in Sauvignon blanc grape juice inoculated with Mp Y1123, described in Chapter 3, activity peaked at 24 h after inoculation. Furthermore, by the end of fermentation, the total protein content was lower in Mp Y1123 co-fermentations compared to *S. cerevisiae* alone. The degradation of specific, tentatively identified grape proteins was visualised through SDS-PAGE, and by the end of fermentation Mp Y1123 samples showed significantly lower levels of chitinase and TLPs compared to Mp Flavia.

Although total protein concentration was significantly less in Mp Y1123 co-fermentation samples at the end of fermentation compared to both the Mp Flavia co-fermentation and *S. cerevisiae* alone, a significant reduction in the latter two treatments had also been observed over time. Moreover, a decrease in haze-forming potential could be observed over time not only in Mp Y1123 co-fermentations, but also in Mp Flavia co-fermentations and *S. cerevisiae* alone. This decrease in protein content and haze-forming potential for Mp Flavia and *S. cerevisiae* cannot be attributed to protease activity, as activity detected by the protease assay remained consistently low in these samples and can be regarded as background noise. It is possible to attribute the gradual depletion of grape proteins in all treatments to natural precipitation during the fermentation process. For example, this could be due to changes in solubility of many wine components leading to protein precipitation, as well as decreased protein retention during the changes in pH throughout fermentation (Waters 1991). Another hypothesis is that these strains contain high levels of chitin in their cell wall, which could lead to the adsorption and subsequent removal of the haze-forming chitinases (Ndlovu et al. 2018). Furthermore, the release of macromolecules from the yeast cell wall, particularly mannoproteins, has been positively correlated to improved protein stability in wine and lower haze potential (Dizy and Bisson 2000; Gonzalez-Ramos et al. 2008). It is possible that this extracellular product was released during grape juice fermentation by the yeasts used in this study, thus contributing to the observed decrease in haze-forming potential. Indeed, the presence of an unidentified

protein in samples of Mp Y1123 and Mp Flavia taken 24 h and 48 h after inoculation was observed using SDS-PAGE techniques, and should be further investigated regarding its identity and potential role in haze reduction as these treatments showed significantly reduced haze-forming potential by the end of fermentation compared to Sc. These hypotheses are supported by the evidence for higher chitin levels in some non-*Saccharomyces* yeasts compared to *S. cerevisiae*, as well as the higher capacity of some non-*Saccharomyces* yeasts to release polysaccharides than *S. cerevisiae* (Xie and Lipke 2010; Domizio et al. 2014). Moreover, mannoproteins are what Dizy and Bisson (2000) hypothesised was responsible for their observation of decreased levels of haze formation in *S. cerevisiae* fermentations. It could therefore be of interest to monitor protein degradation and haze potential in grape juice inoculated with Mp Y1123, without the inoculation of *S. cerevisiae*, to determine its impact in the absence of other potentially influential factors.

Grape proteins have been shown to be resistant to proteolytic activity and their effective degradation has in several cases only been achieved with a heating step in conjunction with enzyme treatment (Waters et al. 1995; Marangon et al. 2012). This was not the case for exogenous MpAPr1 treatment, however the secretion and subsequent activity of this protease by *M. pulcherrima* IWBT Y1123 may not have been sufficient to completely degrade grape proteins by the end of fermentation. Furthermore, although Mp Y1123 co-fermentations did lead to a significant decrease in wine haze by the end of fermentation than when grape juice was fermented with only *S. cerevisiae*, the difference between the absorbance units measured at 520 nm of the heated and non-heated control samples was 0.04, thus greater than the 0.02 threshold which has typically been regarded as the pass-fail point in protein stability tests in the past (Pocock et al. 2007). The concentration of exogenous MpAPr1 that was demonstrated to have an impact on wine haze after 48 h incubation was much higher (0.5 g/L) than detected here at 24 h and 48 h after inoculation with Mp Y1123 (ca. 0.1 g/L) (Theron et al. 2018). On the other hand, the addition of 0.1 g/L exogenous MpAPr1, a similar concentration secreted by Mp Y1123 in this study, was not effective against wine haze. The potential for improving MpAPr1 secretion by Mp Y1123, using techniques such as directed evolution, should therefore be investigated to enhance the impact of Mp Y1123 against the formation of haze. Nevertheless, the heat assay itself that was used to determine the haze-forming potential of the samples could lead to false interpretation, as the extreme temperatures to which samples were subjected are not representative of the typical conditions that wine would be exposed to (Pocock and Waters 2006).

It could furthermore be interesting to follow protease activity, protein degradation and haze potential over a longer period of time, and extending past the end of fermentation. It has been observed previously that the evolution of enzymatic activities during winemaking is

not necessarily constant throughout the process (Maturano et al. 2015). The ability of MpAPr1 to continue degrading proteins even after fermentation has started has furthermore been demonstrated, although it does lose activity by the end of fermentation as an exogenous treatment (Theron et al. 2018). Thus, monitoring protease activity by *M. pulcherrima* IWBT Y1123 over a longer period of time could provide valuable information regarding its impact on wine haze and protein content.

Grape protein degradation as a result of protease secretion and activity has been hypothesised to have the potential added benefit of releasing assimilable nitrogen compounds into the fermentation matrix which may be utilised by fermenting yeasts, ultimately leading to the production of certain aroma compounds. Volatile compound analysis revealed six compounds which differed significantly in concentration between treatments after fermentation. Reduced levels of ethyl acetate and acetic acid were observed in *M. pulcherrima* co-fermentations, which has previously been observed for sequential inoculation with this species (González-Royo et al. 2014). The other compounds investigated in this study are higher alcohols which can be linked to amino acid metabolism via the Ehrlich pathway. The production of 2-phenethyl ethanol has typically been associated with *M. pulcherrima*, lending co-fermentations with this yeast its characteristic rose/floral aroma, and indeed sequential inoculation with both Mp Y1123 and Mp Flavia led to drastically increased levels of this compound (Comitini et al. 2011). However, 2-phenethyl ethanol and propanol levels were significantly lower for Mp Y1123 compared to Mp Flavia, whereas isobutanol and isoamyl alcohol were higher in Mp Y1123. While a similar increase in isoamyl alcohol and decreased propanol was observed in wine treated with exogenous MpAPr1, isobutanol levels had decreased and 2-phenethyl ethanol remained unaffected (Theron et al. 2018). However, the matrix involved in the two experiments differed in several ways, including the grape juice used, the strain of *S. cerevisiae* and the presence of *M. pulcherrima*, all of which would impact considerably on the final wine volatile profile. Nevertheless, a different study which investigated the impact of protease treatment on wine volatile profile found that isobutanol and isoamyl alcohol levels had increased after enzyme application (Zhang et al. 2016). Changes to the wine volatile composition due to these higher alcohols, amongst other compounds, resulted in wine with greater complexity and enhanced aroma.

Enzyme-producing non-*Saccharomyces* yeasts are attractive tools for enhancing wine characteristics and improving fermentation processing. Steps taken towards understanding enzyme secretion and activity under winemaking conditions and impact thereof on yeast interactions and wine quality have led to informed decision-making regarding co-inoculation strategies, as in the case of numerous β-D-glucosidase-producing strains (Padilla et al. 2016). This study shows that a wine yeast strain, *M. pulcherrima* IWBT

Y1123, can be applied as a co-starter culture with *S. cerevisiae* to grape juice fermentation for the production of an extracellular aspartic protease to reduce haze potential and influence the wine volatile profile. The application of this protease-producing yeast did not significantly impact the fermentation kinetics of *S. cerevisiae*, however it is possible that *S. cerevisiae* EC1118 influenced the haze assays in terms of chitin in its membrane and/or release of mannoproteins. Future experiments could therefore seek to compare chitin levels in the cell walls of *M. pulcherrima* and *S. cerevisiae* species, and to perform *M. pulcherrima* IWBT Y1123 co-fermentations with a strain of *S. cerevisiae* without these properties. Furthermore, the ability of non-*Saccharomyces* yeasts to release mannoproteins, and the impact thereof on wine haze, should be a focus of future work. Prolonged monitoring of protease activity and the impact of *M. pulcherrima* growth in terms of proteolysis and haze potential could also be considered, with and without *S. cerevisiae* sequential inoculation. A sensory evaluation would furthermore elucidate whether the resulting wine is of suitable organoleptic quality. The potential to improve proteolytic activity by *M. pulcherrima* IWBT Y1123 through non-GMO techniques such as directed evolution should also be considered for the development of a potentially marketable strain with protease secretion and activity levels that are efficient and sufficient enough under winemaking conditions to significantly improve wine clarity.

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4.6 Supplementary material

4.6.1 Protease activity: comparison of means

Table S4.1 Protease activity in samples of *M. pulcherrima* IWBT Y1123 (Mp Y1123), *M. pulcherrima* Flavia (Mp Flavia) and *S. cerevisiae* EC1118 (Sc EC1118). Means were compared between treatments, within time-points.

				P-value		
	Mp Y1123	**Mp Flavia**	**Sc EC1118**	**Mp Y1123 vs. Mp Flavia**	**Mp Y1123 vs. Sc EC1118**	**Mp Flavia vs. Sc EC1118**
0	28.17 ± 5.75^{a}	15.21 ± 7.32^{b}	19.32 ± 1.96^{ab}	0.0183	0.0931	0.4216
24	100.62 ± 6.59^{a}	17.94 ± 0.52^{b}	29.17 ± 2.57^{c}	< 0.0001	< 0.0001	0.0373
48	82.96 ± 11.35^{a}	12.99 ± 8.05^{b}	29.06 ± 1.34^{c}	< 0.0001	< 0.0001	0.0047

Values are means of protease activity in AU ± SD, n=3 per treatment group. $^{a-c}$ Means in a row with different superscript letters differ significantly (P<0.05) as analysed by two-way ANOVA and the Fisher's LSD test.

4.6.2 Heat stability: comparison of means

Table S4.2 Multiple comparison of means of heat stability readings obtained for samples within similar time-points, between different treatments of grape juice inoculated with *M. pulcherrima* IWBT Y1123 (Mp Y1123) or *M. pulcherrima* Flavia (Mp Flavia) with sequential inoculation of *S. cerevisiae* EC1118 (Sc EC1118), or only *S. cerevisiae* EC1118.

				P-value		
Time	**Mp Y1123**	**Mp Flavia**	**Sc EC1118**	**Mp Y1123 vs. Mp Flavia**	**Mp Y1123 vs. Sc EC1118**	**Mp Flavia vs. Sc EC1118**
24	0.21 ± 0.013[a]	0.15 ± 0.021[b]	0.38 ± 0.019[c]	< 0.0001	< 0.0001	< 0.0001
48	0.23 ± 0.004[a]	0.12 ± 0.005[b]	0.26 ± 0.014[c]	< 0.0001	0.0191	< 0.0001
End	0.04 ± 0.009[a]	0.05 ± 0.005[a]	0.09 ± 0.004[b]	0.1322	0.0001	0.0022

Values are means of heat stability readings (change in optical density at 520 nm) ± SD, n=3 per treatment group. [a-c] Means in a row with different superscript letters differ significantly (P<0.05) as analysed by two-way ANOVA and the Fisher's LSD test.

Table S4.3 Multiple comparison of means of heat stability readings obtained for samples between different time-points, within similar treatments of grape juice inoculated with *M. pulcherrima* IWBT Y1123 (Mp Y1123) or *M. pulcherrima* Flavia (Mp Flavia) with sequential inoculation of *S. cerevisiae* EC1118 (Sc EC1118), or only *S. cerevisiae* EC1118.

				P-value		
Treatment	**24**	**48**	**End**	**24 vs. 48**	**24 vs. End**	**48 vs. End**
Mp Y1123	0.21 ± 0.013[a]	0.23 ± 0.004[b]	0.04 ± 0.009[c]	0.0181	< 0.0001	< 0.0001
Mp Flavia	0.15 ± 0.021[a]	0.12 ± 0.005[b]	0.05 ± 0.005[c]	0.0342	< 0.0001	< 0.0001
Sc EC1118	0.38 ± 0.019[a]	0.26 ± 0.014[b]	0.09 ± 0.004[c]	< 0.0001	< 0.0001	< 0.0001

Values are means of heat stability readings (change in optical density at 520 nm) ± SD, n=3 per treatment group. [a-c] Means in a row with different superscript letters differ significantly (P<0.05) as analysed by two-way ANOVA and the Fisher's LSD test.

4.6.3 Densitometry: comparison of means

Table S4.4 Multiple comparison of means of protein band intensities obtained for samples within similar time-points, between different treatments of grape juice inoculated with *M. pulcherrima* IWBT Y1123 (Mp Y1123) or *M. pulcherrima* Flavia (Mp Flavia) with sequential inoculation of *S. cerevisiae* EC1118 (Sc EC1118), or only *S. cerevisiae* EC1118.

	24 h		**48 h**		**End**		
	Mp Y1123 vs Mp Flavia	Mp Y1123 vs Mp Flavia	Mp Y1123 vs Sc EC1118	Mp Flavia vs Sc EC1118	Mp Y1123 vs Mp Flavia	Mp Y1123 vs Sc EC1118	Mp Flavia vs Sc EC1118
Invertase	ns	ns	ns	ns	ns	ns	ns
Chitinase	ns	ns	ns	ns	*	ns	ns
TLPs	ns	*	ns	ns	**	ns	ns
LTPs	ns	ns	ns	*	ns	ns	*

Means (n=3 per treatment group) were analysed by one-way ANOVA and the Fisher's LSD test.
ns: P>0.05
*: P≤0.05
**: P≤0.01

Table S4.5 Multiple comparison of means of protein band intensities obtained for samples between different time-points, within similar treatments of grape juice inoculated with *M. pulcherrima* IWBT Y1123 (Mp Y1123) or *M. pulcherrima* Flavia (Mp Flavia) with sequential inoculation of *S. cerevisiae* EC1118 (Sc EC1118), or only *S. cerevisiae* EC1118

	0 h vs	**0 h vs**	**0 h vs**	**24 h**	**24 h vs**	**48 h vs**

		24 h	48 h	End	vs 48 h	End	End
Mp Y1123	Invertase	ns	ns	*	ns	**	ns
	Chitinase	ns	ns	*	ns	*	**
	TLPs	ns	ns	*	ns	**	**
	LTPs	ns	ns	**	ns	ns	ns
Mp Flavia	Invertase	ns	ns	ns	ns	**	ns
	Chitinase	ns	ns	*	ns	**	*
	TLPs	ns	ns	ns	ns	ns	***
	LTPs	ns	ns	*	ns	*	**
Sc EC1118	Invertase		ns	ns			ns
	Chitinase		ns	*			*
	TLPs		ns	ns			ns
	LTPs		ns	ns			ns

Means (n=3 per treatment group) were analysed by one-way ANOVA and the Fisher's LSD test.
ns: P>0.05
*: P≤0.05
**: P≤0.01
***: P≤0.001

4.6.4 Total protein: comparison of means

Table S4.6 Multiple comparison of means of total protein concentrations for samples within similar time-points, between different treatments of grape juice inoculated with *M. pulcherrima* IWBT Y1123 (Mp Y1123) or *M. pulcherrima* Flavia (Mp Flavia) with sequential inoculation of *S. cerevisiae* EC1118 (Sc EC1118), or only *S. cerevisiae* EC1118.

				P-value		
	Mp Y1123	**Mp Flavia**	**Sc EC1118**	**Mp Y1123 vs. Mp Flavia**	**Mp Y1123 vs. Sc EC1118**	**Mp Flavia vs. Sc EC1118**
24	1079.94 ± 12.41^{a}	1253.73 ± 58.20^{b}	787.97 ± 14.42^{c}	< 0.0001	< 0.0001	< 0.0001
48	1023.88 ± 33.88^{a}	1238.27 ± 19.05^{b}	631.61 ± 30.43^{c}	< 0.0001	< 0.0001	< 0.0001
End	402.37 ± 16.37^{a}	473.12 ± 34.36^{b}	488.73 ± 8.49^{b}	0.0084	0.002	0.5219

Values are means of protein concentration in µg/mL ± SD, n=3 per treatment group. $^{a-c}$ Means in a row with different superscript letters differ significantly (P<0.05) as analysed by two-way ANOVA and the Fisher's LSD test.

Table S4.7 Multiple comparison of means of total protein concentrations for samples between different time-points, within similar treatments of grape juice inoculated with *M. pulcherrima* IWBT Y1123 (Mp Y1123) or *M. pulcherrima* Flavia (Mp Flavia) with sequential inoculation of *S. cerevisiae* EC1118 (Sc EC1118), or only *S. cerevisiae* EC1118.

				P-value		
	24	**48**	**End**	**24 vs. 48**	**24 vs. End**	**48 vs. End**
Mp Y1123	1079.94 ± 12.41^{a}	1023.88 ± 33.88^{b}	402.37 ± 16.37^{c}	0.0306	< 0.0001	< 0.0001
Mp Flavia	1253.73 ± 58.20^{a}	1238.27 ± 19.05^{a}	473.12 ± 34.36^{b}	0.5259	< 0.0001	< 0.0001
Sc EC1118	787.97 ± 14.42^{a}	631.61 ± 30.43^{b}	488.73 ± 8.49^{c}	< 0.0001	< 0.0001	< 0.0001

Values are means of protein concentration in µg/mL ± SD, n=3 per treatment group. $^{a-c}$ Means in a row with different superscript letters differ significantly (P<0.05) as analysed by two-way ANOVA and the Fisher's LSD test.

4.6.5 Major volatiles: comparison of means

Table S4.8 Multiple comparison of means of major volatile concentrations between wine samples fermented by *S. cerevisiae* EC1118 (Sc EC1118) with or without prior inoculation of *M. pulcherrima* IWBT Y1123 (Mp Y1123) or *M. pulcherrima* Flavia (Mp Flavia).

				P-value		
	Mp Y1123	**Mp Flavia**	**Sc EC1118**	**Mp Y1123 vs. Mp Flavia**	**Mp Y1123 vs. Sc EC1118**	**Mp Flavia vs. Sc EC1118**
Ethyl acetate	62.68 ± 9.34^{a}	55.92 ± 2.13^{a}	96.44 ± 8.41^{b}	0.6479	< 0.0001	< 0.0001
Acetic acid	73.95 ± 4.92^{a}	90.82 ± 4.29^{b}	607.39 ± 21.47^{c}	< 0.0001	< 0.0001	< 0.0001
Propanol	30.66 ± 1.29^{a}	39.24 ± 2.37^{b}	34.62 ± 1.82^{ab}	0.0034	0.1721	0.1116
2-Phenethylethanol	259.55 ± 10.89^{a}	434.85 ± 8.03^{b}	65.60 ± 3.02^{c}	< 0.0001	< 0.0001	< 0.0001
Isobutanol	299.78 ± 7.90^{a}	253.49 ± 2.74^{b}	89.19 ± 6.49^{c}	< 0.0001	< 0.0001	< 0.0001
Isoamyl alcohol	351.63 ± 7.77^{a}	325.17 ± 2.80^{b}	235.26 ± 11.77^{c}	< 0.0001	< 0.0001	< 0.0001

Values are means of major volatile concentration in mg/L ± SD, n=3 per treatment group. $^{a-c}$ Means in a row with different superscript letters differ significantly (P<0.05) as analysed by two-way ANOVA and the Fisher's LSD test.

Chapter 5

General discussion and conclusions

Chapter 5 - General discussion and conclusions

5.1 General discussion

The biotechnological interest in protease-secreting yeasts and filamentous fungi is vast, spanning from the food and beverage industries to that of bioremediation (de Souza et al. 2015). Whereas purified enzyme or crude preparations are commonly applied to these processes as exogenous treatments, inoculation of the organism itself into the matrix that would benefit from protease activity can often present a time- and cost-effective alternative (Kumar and Takagi 1999). The aspartic protease-secreting yeast *Metschnikowia pulcherrima* IWBT Y1123 is one such organism with potential for application as a starter culture to wine fermentation. Its secreted protease, MpAPr1, has already shown an effect against grape proteins and wine haze formation when added as a purified preparation to grape juice fermentation (Theron et al. 2018).

An understanding of protease regulation in inoculants such as *M. pulcherrima* IWBT Y1123 could prove valuable for predicting production, activity and ultimate effect thereof, as well as manipulating environmental parameters for optimal production. In fact, the strict regulation of protease expression by extracellular conditions attests to the great ecological significance of this enzyme in its determining role of how the organism interacts with and survives in its environment (McCotter et al. 2016). Whether or not an organism secretes a protease influences where it can grow in nature and whether it can utilise proteins as an alternative nutrient substrate under nitrogen-, carbon- and/or sulphur-limiting conditions (Ogrydziak 1993). Indeed, indicative of their importance to the biological function of extracellular proteases, certain environmental factors, including nutrient limitation and protein availability, have been shown to play a regulatory role in the control of protease gene expression in fungi.

In this study, the impact of nitrogen sources and protein availability on the production of MpAPr1 by *M. pulcherrima* IWBT Y1123 was investigated. When the easily assimilable nitrogen sources ammonium and amino acids were available, only basal protease activity levels were detected. However, in terms of protease activity the presence of the haemoglobin protein alleviated this repressive effect in both treatments with and without additional ammonium and amino acids, albeit delayed when the easily assimilable sources were also initially available. Nitrogen catabolite repression of protease activity is therefore evident in the presence of ammonium and amino acids, but is balanced by protease induction in the presence of proteins in both YNB and SGJ. Moreover, when *M. pulcherrima* IWBT Y1123 was grown in glucose-rich medium, a delayed increase in protease activity was observed, possibly due to the effect of the carbon catabolite repression mechanism.

In this way, the results of this study contribute to fundamental knowledge regarding the regulation of fungal extracellular proteases. The information generated in these experiments indicated that in terms of the nitrogen sources available in grape juice, which include preferred sources such as ammonium and amino acids, as well as alternative protein sources, protease production and activity would be favoured in a fermentation inoculated with *M. pulcherrima* IWBT Y1123, if delayed in onset. Subsequently, the biotechnological aspect of *M. pulcherrima* IWBT Y1123 application as a protease-producing starter culture to grape juice fermentation for the improvement of wine properties was also addressed.

When *M. pulcherrima* IWBT Y1123 was grown in grape juice, expression analyses of *MpAPr1* and protease activity assays revealed that protease expression and activity occurred under winemaking conditions, and that activity reached a peak at 24 h after inoculation. In order to relate enzyme production and activity as a result of *M. pulcherrima* IWBT Y1123 inoculation to its impact on final wine properties, grape protein degradation, haze formation potential and major volatiles were also measured. At the end of fermentation, total protein content was significantly lower in *M. pulcherrima* IWBT Y1123 with *S. cerevisiae* co-inoculations compared to treatments of the *M. pulcherrima* Flavia control and only *S. cerevisiae*. Furthermore, protein bands indicative of chitinases and TLPs, as visualised through SDS-PAGE and densitometry techniques, were significantly less intense in *M. pulcherrima* IWBT Y1123 samples than in *M. pulcherrima* Flavia co-inoculations. Moreover, a significant reduction in wine haze-forming potential was observed in *M. pulcherrima* IWBT Y1123 co-inoculations when compared to wine fermented by only *S. cerevisiae*. However, the haze assay yielded a value of 0.04, thus above the 0.02 threshold thought to indicate protein stability.

It should be noted that protein content and haze formation potential also decreased in the *M. pulcherrima* Flavia and pure *S. cerevisiae* controls. It may be that these organisms release macromolecules such as mannoproteins into the fermentation matrix, which have been positively correlated with improved protein stability in wine and lower haze potential. High levels of yeast cell wall chitin have moreover been shown to reduce protein haze by binding grape chitinase, and it is therefore possible that these strains contain chitin-rich cell walls (Ndlovu et al. 2018). However, natural precipitation of grape proteins during fermentation has also been shown to lead to decreased levels of protein concentration.

Volatile compound analysis furthermore revealed changes in aroma- and flavour-active compounds between *M. pulcherrima* IWBT Y1123 and *M. pulcherrima* Flavia treatments that can be linked to amino acid metabolism via the Ehrlich pathway. It is therefore possible that the co-inoculation of grape juice with *M. pulcherrima* IWBT Y1123 leads to changes in the environmental amino acid composition and subsequent volatile profile.

5.2 Future outlooks

This study was subject to a number of limitations, including the use of artificial substrates for both protein induction of protease and endo-protease assays which are not representative of protease activity against grape proteins. Future experiments should investigate the potential of using more oenologically relevant substrates for direct determination of protease regulation in response to, and activity against, these intended substrates. Furthermore, the haze assay parameters used in this study are not representative of the actual conditions under which haze may form in wine and optimisation of this assay is therefore required.

Future work should also include investigating the regulatory role of more factors relevant to the wine fermentation environment, such as sulphur sources and temperature, on MpAPr1 production by *M. pulcherrima* IWBT Y1123. Further experiments regarding the regulatory response of protease production should also involve expression analyses of *MpAPr1*, as well as an exploration of the regulatory elements and possible transcription factors in *M. pulcherrima* IWBT Y1123, to provide a more in-depth analysis of the genetic control exerted by environmental factors such as nitrogen and carbon sources.

The potential for improving protease production by *M. pulcherrima* IWBT Y1123 using non-GMO techniques, such as directed evolution through continuous cultivation, should also be investigated for the production of a commercially viable protease-producing strain with the ability to significantly reduce wine haze. Grape protein degradation, haze formation and protease activity could also be measured over a longer period of time during and after fermentation for a more holistic analysis of the impact of the protease over time, and on the final wine. Sensory evaluation of wine co-inoculated with *M. pulcherrima* IWBT Y1123 would also give an indication of its impact on organoleptic quality.

Future experiments could explore the haze-reducing potential of chitin levels in the cell walls of the organisms used in this study, as well as in other potential candidates, as an additional route by which non-*Saccharomyces* starter cultures may be used to improve wine protein stability. Similarly, the release of mannoproteins is another promising phenomenon which should be investigated and potentially exploited for reducing wine haze.

5.3 References

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