Investigating the secretome of non-*Saccharomyces* yeast in model wine

by

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Declaration

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Summary

Proteins from various sources, including grape berry cells, yeast, bacteria and fining agents e.g. albumin and casein, have previously been identified in wine. These proteins play various critical roles in the functioning and survival of the organisms that produced them but also exhibit oenological properties, once secreted in the juice/wine. Some of them can indeed be beneficial to winemaking, by releasing aroma compounds from grape-derived precursors, or detrimental to wine quality, by causing protein haze. Yeasts contribute significantly to the protein pool during and after alcoholic fermentation. However, while the extracellular proteins of *Saccharomyces cerevisiae*, the main wine yeast species, have been characterised, those of non-*Saccharomyces* yeasts remain largely unknown, especially under winemaking conditions. Although specific extracellular enzymes released by non-*Saccharomyces* yeasts have been the focus of many studies in recent years, the targeted approaches used have restricted our knowledge to these specific enzymes and excluded the other secreted proteins. A more comprehensive insight into entire secretomes could improve our understanding of how yeasts survive in wine and interact with other species in mixed culture fermentations.

This study aims to characterise the exo-proteome of *Saccharomyces* and selected non-*Saccharomyces* yeasts in pure and mixed cultures in a wine-like medium.

Fermentation kinetics were monitored and the extracellular proteins isolated at the end of fermentation. *M. pulcherrima* hardly fermented whereas *L. thermotolerans* fermented slowly but steadily. As expected *S. cerevisiae* completed the fermentation rapidly. In sequential fermentations, the kinetics resembled those of the non-*Saccharomyces* yeasts for a period before switching to that of *S. cerevisiae*. This period varied from 4 to 15 days for *M. pulcherrima* and *L. thermotolerans* respectively.

Visual observations of the protein content of the medium at the end of fermentation using 1D and 2D SDS-PAGE gels as well as identification of these proteins using mass fingerprinting revealed the large variety of proteins secreted and the influence of yeast interactions on each other's secretome. The fermentation kinetics observed could partially be explained by the extent of the contribution of the different yeast to the protein content.

Proteins secreted by non-*Saccharomyces* yeasts lowered the potential of wine to form protein haze, with both *M. pulcherrima* and *L. thermotolerans* in pure and mixed culture fermentations showing lower haze formation than *S. cerevisiae*.

As far as we know, this is the first report on the secretome of non-*Saccharomyces* under winemaking condition and the influence non-*Saccharomyces* proteins have on the protein haze potential of wine, providing the basis for future investigations.

Opsomming

Proteïene vanaf verskeie bronne (insluitend druiwe korrels, gis, bakterieë en verhelderings agente bv. albumien en kaseïen) is reeds in wyn identifiseer. Hierdie proteïene speel verskeie rolle in die funksionering en oorlewing van die organismes wat dit produseer, maar beskik ook oor wynkundige eienskappe sodra dit in die sap of wyn uitgeskei word. Hoewel sommige proteïene in wyn wel voordelig mag wees as gevolg van die vrystelling van aroma komponente vanuit druif-voorlopers, kan dit ook nadelig wees vir wyn kwaliteit deur die troebelheid wat dit kan veroorsaak Gis dra aansienlik by tot die totale proteïen inhoud van wyn, beide gedurende asook na alkoholiese fermentasie. Alhoewel die ekstrasellulêre proteïene van *Saccharomyces cerevisiae* (die mees algemeen gebruikte gis vir wynmaak) reeds goed gekarakteriseer is, is die proteïene van nie-*Saccharomyces* giste grootliks onbekend, veral die wat tydens wynmaak vrygestel word. Gedurende die laaste paar jaar het verskeie studies gefokus op spesifieke ekstrasellulêre ensieme wat deur nie-*Saccharomyces* giste produseer word, maar geteikende benaderings het ons kennis beperk tot net hierdie spesifieke ensieme, en enige ander afgeskeide proteïene uitgesluit. 'n Meer omvattende insig oor die algehele afgeskeide proteoom kan ons begrip van hoe gis in wyn oorleef en interaksies tussen gis spesies in gemengde kultuur fermentasies verbeter

Hierdie studie streef om die sekretoom van *Saccharomyces* en geselekteerde nie-*Saccharomyces* giste in suiwer en gemengde kultuur fermentasies van sintetiese wyn medium te karakteriseer.

Fermentasie kinetika is gemonitor en die ekstrasellulêre proteïene is teen die einde van fermentasie geïsoleer. *Metschnikowia pulcherrima* het swak fermenteer terwyl *Lachancea thermotolerans* stadig tog reëlmatig fermenteer het. Soos verwag, het *S. cerevisiae* vinnig tot droog fermenteer. In agtereenvolgend geïnokuleerde fermentasies is die kinetika vir 'n tydperk soortgelyk aan die van die nie-*Saccharomyces* giste voordat dit oorskakel na die van *S. cerevisiae*. Hierdie tydperk wissel respektiewelik vanaf 4 tot 15 dae vir *M. pulcherrima* en *L. thermotolerans*.

Visuele waarnemings van die proteïen-inhoud van die medium aan die einde van die gisting met behulp van 1D en 2D SDS-PAGE gels asook identifisering van hierdie proteïene met behulp van massa vingerafdrukke onthul die groot verskeidenheid proteïene wat afgeskei word, asook die invloed van die giste se interaksies op mekaar se sekretoom. Die fermentasie kinetika waargeneem kan gedeeltelik verklaar word deur die omvang van die bydrae van die verskillende gis tot die proteïen-inhoud. Proteïene wat afgeskei word deur nie-*Saccharomyces* giste verlaag die potensiaal van wyn om proteïen troebelheid te vorm,

met beide *M. pulcherrima* en *L. thermotolerans* (in suiwer en gemengde kultuur fermentasies) wat minder troebelheid vorm as fermentasies met *S. cerevisiae.*

Sover ons kennis strek, is hierdie die eerste verslag oor die sekretoom van nie-*Saccharomyces* onder wynmaak toestande en ook oor die invloed wat nie-*Saccharomyces* proteïene op die proteïen troebelheid van wyn het, en vorm die basis vir toekomstige navorsing.

Biographical sketch

Talitha Tanya Mostert (née Greyling) was born in Johannesburg on 27 August 1983. She attended Drakensberg Primary School and matriculated from Ferrum High School in 2001. She enrolled at the University of Stellenbosch, and obtained a BSc in Animal Biotechnology in 2005. In 2006, she obtained the degree HonsBSc (Wine Biotechnology) at the Institute for Wine Biotechnology, Stellenbosch University.

Talitha has since worked as a technical officer before enrolling at Stellenbosch University for a MSc in Wine Biotechnology.

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Preface

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately and is written according to the style of *Journal of Proteomics* to which Chapter 3 will be submitted for publication

Chapter 1   **General introduction and project aims**

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CHAPTER 1

General introduction and project aims

Chapter 1 – General introduction and project aims

1.1 Introduction

The wine proteome is defined as all the proteins present in wine at a specific time point. The wine protein content is dynamic and various sources throughout the wine making process contribute to the proteome. Proteins from grape berry cells are the greatest constituent of the proteome at the beginning of fermentation. During alcoholic fermentation some of these proteins are hydrolysed by yeasts and more proteins are released into the wine by the yeasts and bacteria as wine fermentation progresses [1-3]. Other sources of proteins in wine are additives (e.g. clarifying enzyme preparations) and fining agents (e.g. albumin and casein) added during the wine making process. Many proteins are removed from the wine during general winemaking practices, such as bentonite fining in white wine, or racking during ageing in all wines. However, some proteins survive these processes and end up contributing to wine quality [4].

The contribution of yeasts to the protein pool of wine has in recent years gained more interest, especially as the need for new and improved yeast strains gained momentum. The focus has, in recent years, moved to non-*Saccharomyces* yeasts that have the ability to release proteins and enzymes of oenological interest that are active in winemaking conditions [5-10] as the "traditional" wine yeast, *Saccharomyces cerevisiae* has been reported not to secrete enzymes of oenological relevance that are active under winemaking conditions [11-13]. Even though, mannoproteins from inactivated yeasts have been shown to impact on protein haze and foam formation [14-17]. Although yeasts present in grape juice and involved in wine fermentation have been well studied over the years, very little is known about the yeast proteome and its evolution during alcoholic fermentation [18-20].

Understanding the influence these yeasts can have on the final product and how we can improve the strains used for commercial wine production [20] is dependent on knowledge of the proteins secreted by these yeasts and their activity under wine making conditions.

Extracellular proteins that have been identified for yeasts (*S. cerevisiae* and *S. paradoxus*) under fermentation conditions include a long list of mostly proteins from the cell wall but surprisingly also a few cytosolic proteins [21]. Glycolytic proteins are amongst the most abundant proteins identified in fermentation studies, along with proteins involved in ethanol production and to a lesser extent proteins from stress response and amino acid metabolism [22-25]. These proteins have functions in various cell compartments (nucleus, vacuole, cytosol and cell wall) and do not usually possess the N-terminal secretion signal that is traditionally used to identify secreted proteins. This led a few researchers to hypothesize that other pathways for protein secretion exist [26,27].

Opposed to what was believed in earlier years, yeasts present in co-inoculations or spontaneous fermentations interact with each other instead of co-existing passively in the same media [12;28]. The scope of these interactions is not well characterised, with studies mostly reporting the influence that mixed culture fermentations have on the aroma profile of wine [7;8]. More in depth investigations of these interactions will require a more holistic approach with regards to proteins and their influences on the yeasts present in mixed culture fermentations.

The limited knowledge on the secretome of yeasts, during the process of fermentation, hinders the understanding of the influence yeasts and their proteins can have on the quality of wine. Therefore, the study of the yeast secretome in fermentations has in recent years gained interest [29;30].

1.2 Rationale and scope of the study

Using targeted approaches, as was done until recently, to identify and characterise enzymes of oenological relevance that are produced by yeast does not take into consideration the interactions and influence of different yeasts and their proteins on one another when present in co-inoculations and spontaneous fermentations. Over the years, many studies have described the influence of various yeasts, present in mixed culture fermentations, on aroma (Reviewed by Ciani *et al.* [5]). Nevertheless, the contribution of the individual yeasts, either to aroma or protein content, has not been studied holistically. Therefore, to understand the contribution of individual yeasts to the wine enzyme and protein pool, a more encompassing/ inclusive approach is needed. In order to assess the influence multiple yeasts has on the secretome (nature and amount of proteins) of one another, the impact that yeasts have on each other in fermentation conditions needs to be investigated.

Specific objectives for this study:

1. To screen and identify previously isolated strains that secrete enzymes active under winemaking conditions
2. To use untargeted approaches to isolate and characterise proteins released from two non-*Saccharomyces* yeasts during alcoholic fermentation
   - By visualizing the protein profile on SDS-PAGE
   - By comparing protein profiles of pure and mixed culture fermentations on 2D SDS-PAGE
   - By identifying the proteins with mass fingerprint analyses.

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CHAPTER 2

Literature Review

Wine proteins: origin, characteristics and influence on wine quality

Chapter 2 - Wine proteins: origin, characteristics and influence on wine quality

2.1 Introduction

Greenbaum *et al.* [1] define the proteome as "the protein-coding regions of the genome" and the secretome as "the population of gene products that are secreted from the cell". In recent years, the proteomes of different micro-organisms have received a lot of attention, and especially their dynamics under different conditions in a specific organism (e.g. that of *Saccharomyces cerevisiae* during the fermentation process). Most of these studies have focused on the intracellular proteome and its evolution upon environmental changes [2-4].

In fermentation studies, the most abundant proteins identified are unsurprisingly those involved in glycolysis and ethanol production [4,5]. Nevertheless, other proteins involved in stress response, amino acid metabolism and carbohydrate metabolism are also commonly identified [4,5]. The latter proteins are mostly intracellular or connected to the cell wall [2,5,6] and very little is known about the extracellular proteins as the secretome of yeast during the process of fermentation has not yet been fully described and has gained some interest only in the last few years [7-10].

In the case of wine, the proteome is dynamic as wine is the product of the fermentation process and therefore the proteome of wine refers to all proteins present in wine at a specific time point and includes those from grape berry cells, yeast, bacteria and external origins (i.e. wine additives such as clarifying and fining agents). Wine proteins are crucial to quality as they affect taste, clarity, and stability among other wine quality parameters [11].

The "traditional" wine yeast, *S. cerevisiae* has been extensively studied as a model organism [2,6]. As a result, a great deal is known about its transcriptome and genome [4]. Yet there is still very little known about the proteome and its evolution during alcoholic fermentation [5,6,12]. This information is crucial to our understanding of the influence these yeasts can have on the final product and how we can improve strains used for commercial wine production [12]. Furthermore, Rossouw *et al.* [6] showed that proteomic analyses can be a powerful means to interpret omics-related data and also to understand metabolic and physiological changes during the fermentation process. Goméz-Pastor *et al.* [12] focused on the proteomic analyses of yeast strains during biomass production to understand the adaptation mechanisms for the metabolic transitions the yeast have to pass. They report that the combination of transcript, protein and enzymatic analyses gives a better understanding of the mechanisms involved in adaptation to the biomass propagation process.

The scope of the present review is to provide an overview of the proteins present in wine and their evolution during the fermentation process. The contribution of yeasts,

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including non-*Saccharomyces* yeasts will be discussed and examples of oenologically relevant secreted enzymes will also be mentioned.

2.2 Protein content of Wine

2.2.1 Techniques used to identify wine proteins

Various techniques have been implemented to characterise the wine proteome. The purpose of this review is not to describe all the methods available, but a brief overview is detailed below.

Obtaining concentrated protein extracts is generally achieved by precipitation or dialysis and ultrafiltration. Precipitation is normally simple and effective but should be used cautiously as many of the precipitating agents can cause irreversible denaturation [11]. Dialysis is a non-denaturing method for removing the low molecular mass compounds and isolating proteins from complex media while ultrafiltration allows for sample concentration while also removing low molecular mass compounds [11].

Techniques such as ion exchange chromatography and gel filtration are used to fractionate and analyse proteins [11,13] and in recent years, various methods have been tested and improved to collect, identify and quantify low abundance proteins present in wine [3,14-17]. Techniques that have been successfully used recently include Combinatorial Peptide Ligand library (CPLL), ELISA, nano-LC-MS as well as combinations of these techniques [15,18]. For visualization of proteomic profiles two-dimensional (2D) SDS-PAGE electrophoresis is still the most used method [2,3]. SDS-PAGE has inherent limitations with regards to which proteins can be visualized [19,20]. Rabilloud *et al.* [20] reviewed the development of 2D gel electrophoresis and summarized these shortcomings of the method very well. In short, the low efficiency in analysing hydrophobic proteins and the high sensitivity to dynamic range of the sample make the use of 2D SDS-PAGE a highly debated issue. These challenges have not stopped researchers from investigating the wine proteome, with an increasing number of studies reporting on this subject in recent years [16,17,21-24].

2.2.2 Biological origin and evolution of the wine proteome

The protein content of wine is dynamic and diverse, as it contains proteins from different sources as represented in Fig. 2.1. At the beginning of fermentation the greatest constituents of the grape juice protein content originate from the grape berry (seed and skin) and fungi present on the berry, but during the fermentations, microbial proteins are released into the must and some of the grape proteins are hydrolysed by enzymes from the microorganisms present during fermentation [3,7,25]. Some of the proteins are very unstable and cannot

resist the changes that occur during alcoholic fermentation. The pathogen-related proteins, including chitinases and thaumatin-like proteins, together with some other proteins such as invertases are some of the most abundant proteins found in grape juice [11] and their stability ensures their survival throughout alcoholic fermentation. During crushing, commercial enzymes are sometimes added to enhance juice extraction or colour development. These enzymes are mostly not pure single enzyme extracts, but a combination of various enzymes. Yeasts secrete various compounds, such as mannoproteins, enzymes and polysaccharides, during fermentation and also upon autolysis [14,26,27]. In some wines, malolactic fermentation can also contribute to the protein pool, as this fermentation is carried out by bacteria that also release proteins either via secretion or autolysis. At the end of the fermentations, wine can be aged on lees in barrels where more proteins are released into the wine through the process of autolysis and some wood aroma compounds (tannins and polyphenols) might be released into the wine. When the wine is nearly ready to be bottled, most winemakers add fining agents such as egg albumin or bovine casein to stabilize the wine and trace residues can be found in final protein content as it is not always completely removed through filtering and bentonite treatment [18].

The proteins remaining at the end of the winemaking process are highly resistant to proteolysis and low pH. They are heterogeneous in size and range from 10 – 560 kDa [9,11]. Although the protein concentration in wine is relatively low, around 10 to 500 mg/L, some of these proteins are responsible for a number of faults that can impair or at least reduce the acceptance of the product by consumers [11]. Fukui and Yokotsuka [7] reported on the origin of proteins found in young wines and concluded that in Cabernet Sauvignon ~56% of the number of proteins are from grape juice, ~7 % from grape skin, seeds and yeast during the initial stages of fermentation and ~35% is derived from yeast and protein hydrolysis during the later stages of fermentation. During bottle maturation the protein content decreased significantly in the first 2 months after bottling (from 108 mg/L to 95 mg/L in Chardonnay) but some proteins survived up to 10 months of ageing in white wine while in red wine no protein bands could be detected on a PAGE gel after 10 months of maturation [7]. In a similar study, Wigand *et al*. [17] found that the protein content of wines from different cultivars vary in composition, although all wines had similar proteins present. They concluded that about 48% of the proteins identified in wine are derived from grape and 24% from yeast, the rest they attributed to fining agents. Although not representing the majority of proteins, the proteins secreted by the yeast during fermentation have an important role to play in the final wine product, for example releasing aroma compounds from grape derived precursors or affecting taste by reducing bitterness of polyphenols [7].
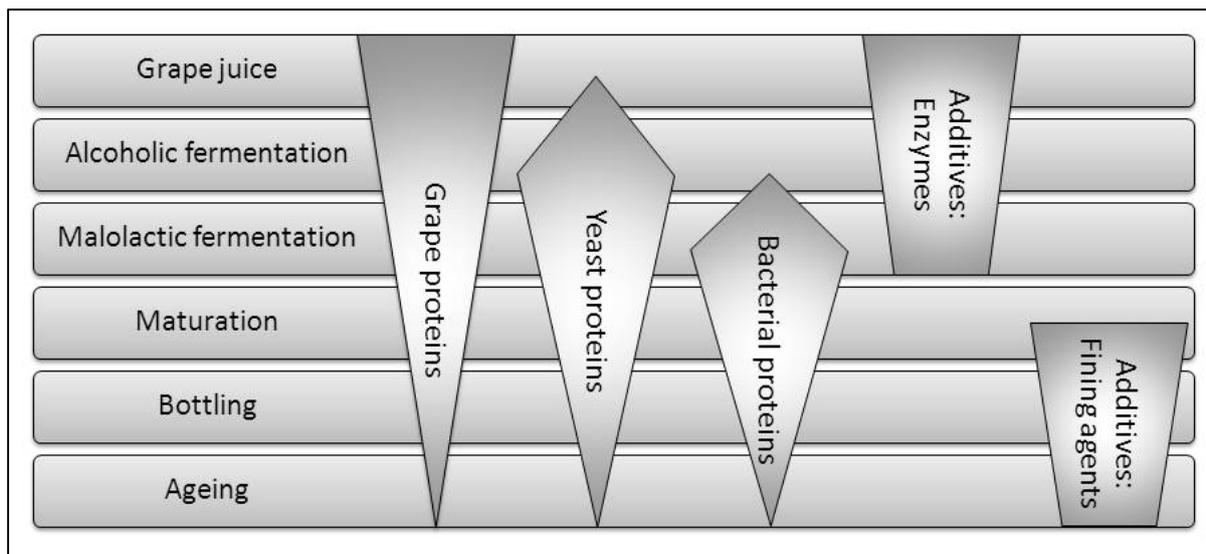
Fig. 2.1- The evolution of the wine proteome during all phases of wine production. The wine proteome consists of proteins from different sources, namely grape berries, fungi present on the grapes, yeast and bacteria. Other proteins that can contribute to the wine proteome are those that are added during different stages of wine production such as maceration enzymes and fining agents (e.g. albumin).

Despite the studies mentioned above, accurate analysis and characterisation of wine proteins represents a number of challenges, due to the variety in size and composition of the proteins present in wine. One of the greatest challenges is the variety of low abundance proteins [3,11,18,26] which are extremely difficult to isolate and characterize. Another challenge is the presence of salts, sugars and polyphenols in wine that make selective precipitation of proteins difficult [3,11,16]. Various analytical techniques have been developed to study wine and grape proteins, from methods dedicated to isolation from grape juice and wine to techniques to study proteins and their evolution during the fermentation process [11].

2.2.3 Oenological wine derivatives

The two aspects of protein content that were the focus of attention in studies over the years are: on the one side, the detection and characterisation of residual proteins that might have negative implications on the final product, such as haze formation [28-31] and on the other side the analysis of the total proteome of untreated wine, to better understand the proteins that have a positive impact on the final product, for instance the foaming properties of sparkling wine [3,9,14,32]. Some studies have also focused on the proteins that can influence aroma and flavour [21,33,34].

Proteins present in the final wine product that might have negative implications are those that can cause allergic reactions in the consumer, including the proteins added during fining such as milk casein and egg albumin [14] and the so called haze proteins [3]. The

grape proteins that are resistant to proteolysis and low pH and can withstand the alcoholic fermentation process are involved in haze formation and foam stabilization of sparkling wines and therefore became the interest of a few studies in recent years [7,14,35]. Righetti *et al.* [32] provided a comprehensive review of the latest results in various studies on the wine proteome and suggested that proteins that survive the fermentation are small sized or components of high molecular mass proteins such as artefacts of albumin and casein added as fining agents by winemakers around the world. These fining agents are not always removed in downstream processes such as bentonite treatment and filtration [14,18] and can have some allergenic response in a small number of consumers. Some other known allergenic proteins have also been identified in wine, i.e. lipid transfer protein (LTP) that might have its origin in the yeast or grape [3,13]. The grape protein that is the most resistant to treatment seems to be thaumatin [18,26], a protein recognised as one of the main contributors to wine protein instability [31,36].

Stability of wine proteins, mostly those responsible for haze, has been tested in several studies [37,38], as haze is an recurring problem that can lead to great financial losses [39]. Fukui and Yokotsuka [7] found that wine proteins are stable even after heat-chill treatment and therefore suggested a method other than heat testing to predict wine stability. In their study, they also discovered that a significant portion of wine proteins remain in the wine even after bentonite treatment and suggest that these proteins should be characterized to understand the mechanism by which they are protected from bentonite adsorption or heat-chill instability and the influence they have on the final wine quality.

Proteins found in wine have some oenological and technological applications. However, they are poorly characterised and described in literature. In one of a limited number of studies on the characteristics of proteins in wine, Palmisano *et al.* [21] identified and characterised the glycoproteins derived from yeast as those involved in assembly and disassembly of the cell wall and specifically lipid catabolism while the grape derived proteins they identified showed high homology with known allergens. Characterisation of these proteins is an important part of understanding how they interact and influence wine quality.

2.3 Yeast secretome

2.3.1 Introduction

The secretion of proteins by yeast cells has significant use in biological and technological processes. The secretome of species that have biotechnological importance have been studied widely in recent years [40,41]. While some studies have focused on the adaptation of yeast proteome upon inoculation into various synthetic media [40,42,43], others focused on the "native" secretome as the yeast are mostly used in heterologous expression of proteins

in the biotechnology industry [23,41,44]. Some of the reported adaptations that the yeast proteome undergoes after inoculation into a high sugar medium are the activation of genes in the non-oxidative branch of the pentose pathway, genes in the fermentation pathway and genes related to protein biosynthesis [42]. Very few studies have however focused on the changes in the extracellular protein fraction of the yeast, but rather on the intracellular changes.

Various proteins are transported to the cell surface of yeast cells. These proteins are either integrated into the cell wall structure or secreted into the extracellular medium [2,8]. In literature all these proteins, together with intracellular proteins that end up in the extracellular medium, are referred to as the secretome of the yeast [1], nevertheless some of these proteins pass purposefully through the cell wall and are secreted into the environment. There is therefore a need to distinguishing between the secretome "senso lato" and secretome "senso stricto" when reading literature. The "senso lato" group (sometimes also called the exo-proteome) would include proteins that are bound to the cell wall (surface proteins), proteins that are secreted into the surrounding area but have no concrete link to the cell wall and also proteins that have an intracellular function, mostly those that are active at a neutral pH (pI between 5 and 8) and not secreted through the conventional protein secretion pathway. The "senso stricto" group would only contain the proteins that are synthesised to be secreted from the cell.

The nature and function of the proteins belonging to the secretome are highly variable between species and also dependent on the growth conditions [2,40]. Carbon and nitrogen sources in particular have the greatest influence on the proteins secreted, e.g. invertase production is induced when *Candida utilis* is grown on xylose media, whereas xylose inhibits invertase production in *S. cerevisiae* [40].

Comparing the exo-proteome of different yeast species with biotechnological and clinical importance, Buerth and colleagues [40] found a conserved core secretome between species of non-*Saccharomyces* yeasts. This core secretome consists of 10 proteins secreted by all species tested; these proteins are mostly linked to cell wall assembly or function and include glucanases (Scw4p, Scw11p, Bgl2p and Exg2p all involved in building and remodelling of cell walls), transglucosylase (Gas1p), chitinase (Cts1p), glyceraldehyde-3-phosphate dehydrogenase (Tdh3p, a cell wall protein that mediates adhesion to host cells in *C. glabrata*) and two cell wall proteins (Tos1p and Pry1p) whose functions have not yet been defined. Endoglucanase (Egt2p) is also regularly found in the secretome of yeasts and is required for proper cell separation [45].

The proteins identified as the core secretome during the Buerth *et al.* [40] study, have functions in different cellular compartments, not always as extracellular proteins; leaving the reader with questions about the secretion pathway these proteins follow through the cell.

2.3.2 The yeast secretion system

The secretome contains proteins from various cell compartments and these can be grouped into three distinct groups: the actively secreted proteins, membrane bound (cell wall and membrane) proteins and intracellular proteins. The actively secreted proteins are those that generally contain an N-terminal secretion signal peptide and are transported via the classical endoplasmic reticulum-Golgi pathway [8,40]. Evidence from past studies however shows that many proteins lacking the N-terminal signal peptide also reach the cell surface [8]. These were previously thought to be intracellular proteins, but Nombela and colleagues [8] suggested that there might be a non-conventional pathway for protein secretion in yeast. The membrane-bound proteins are those that are anchored in the cell membrane by an anchor peptide or by binding to other cell wall structures such as glucans and have a function requiring them to be on the surface of the cell, such as cell wall biogenesis or transport of substrates and metabolites across the cell wall and plasma membrane.

Ding *et al.* [22] and Nombela *et al.* [8] have summarized the different secretion pathways (conventional and unconventional) that proteins can follow in plant, yeast and animal cells (Fig. 2.2). Secretion of proteins via the classical endoplasmic reticulum(ER)-Golgi pathway [blue arrow in Fig. 2.2] is usually driven by an N-terminal signal peptide [46] and is highly conserved in eukaryotes [22]. Most of the secreted proteins are sorted to the ER during synthesis. Once in the rough ER the proteins are folded and moved through the ER and Golgi, where post translational modification takes place. Eventually the proteins are placed into transport vesicles and transported to the cell exterior. Some proteins can enter the ER-Golgi through the post-translational pathway, after they are completely synthesized in the cytosol [46]. Two classes of unconventional protein secretion (UPS) can be defined; the vesicular secretion where proteins are released through membrane bound structures that fuse with each other or are released from the plasma membrane and the non-vesicular secretion, which entails the direct passage of cytosolic proteins across the plasma membrane [22].

Not all unconventional secretion pathways described by Ding *et al.* [22] have yet been described for yeasts; while examples of non-conventional secretion that have been described in yeast include the non-vesicular mode of action and autophagy as a vesicular mode of export.
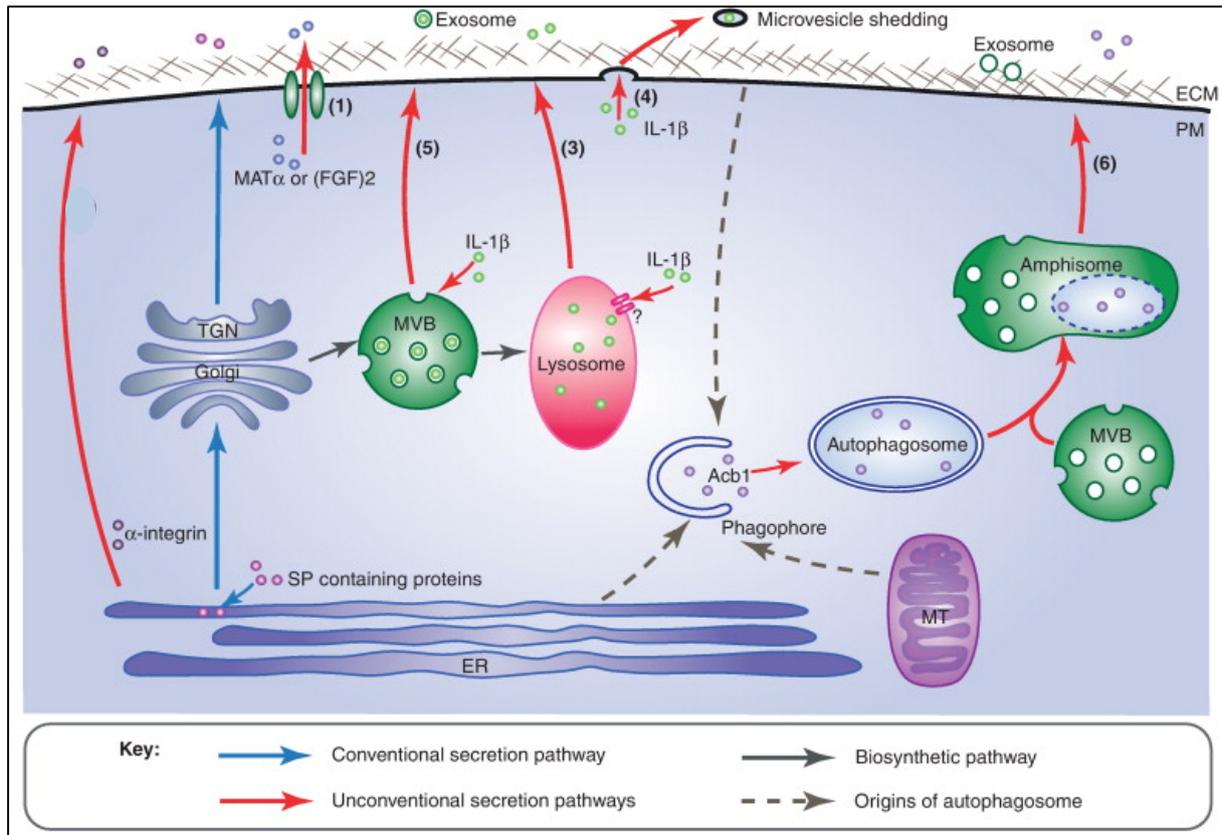
Fig. 2.2 - Depiction of conventional and unconventional protein secretion (UPS) pathways, with representative proteins for each of the UPS pathways included. (1) ABC-transporter; (2) Pathway for membrane proteins in mammals; (3) Lysosome fusion with plasma membrane; (4) Microvesicle shedding; (5) Exosome release; (6) Autophagosome release. Abbreviations: ER, endoplasmic reticulum; ECM, extracellular matrix; MVB, multivesicular body; MT, mitochondrion; PM, plasma membrane; TGN, *trans*-Golgi network; SP, signal peptide [22].

A well-known example of non-vesicular mode of release is that of mating factor MATα via the ABC-transporter system (Fig. 2.2, pathway 1). The vesicular mode of transport is more complex, with Acb1p as the most researched protein that is released from the ER without a signal peptide, and is secreted through autophagy (Fig. 2.2, pathway 6). Autophagy is a process elevated by stress, and in yeast the formation of autophagosome is dependent on the function of a number of proteins in the cytosol [22].

Lysosomes are normally found in mammalian cells, but evidence suggests that the yeast vacuole has the same function as lysosomes in mammals. If we agree with this hypothesis, proteins will enter the lysosome when they are due for degradation and before degradation can happen, the lysosome fuses with the plasma membrane (Fig. 2.2, pathway 3).

When comparing the genome and the secretome of some yeast species, it is clear that we still have a very limited knowledge of the yeast secretome and its plasticity. As summarized in Table 2.1, very few of the proteins predicted from the genome as secreted proteins are actually identified when analysing the secretome of yeast species. The *in silico*

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prediction of secreted proteins may be performed by identifying potential secretion signal sequences and also using known secretion signal sequences, mostly with SignalP software. The prediction does not take into consideration the post-translational modifications that can render these proteins intracellular [1,46,47]. The table also confirms the potential existence of a non-conventional secretion pathway [8,45]. Very few proteins are actually secreted under any given conditions in comparison to the theoretical number; this possibly reveals a large degree of plasticity and adaptability in the yeast.

Table 2.1 - Comparison of yeast genome size, predicted number of secreted proteins and number of proteins detected in extracellular medium as reported in literature

	C. utilis[40]	*P. pastoris*[41]	*K. lactis* [44;46]	*S. cerevisiae*[46]	*Y. lipolytica*[40]	*C. albicans* [45]
Genome size	12.5 Mb	9.4 Mb	10.7 Mb	12.5 Mb	20.5 Mb	14.8 Mb
Predicted ORF's	6417	7935 (5450)	5331 (5076)	5866	6521	6135
Predicted SP	403	88	248 (109)	89-226	388	283 (495)
# Detected proteins	37	20	81-120	74	NR	72
CSP proteins	24	14	34-78	NR	NR	44
UPS proteins	12	4	13-45	13	NR	28

SP: Secreted proteins as predicted by SignalP 3.0; ORF's: Open reading frames; CSP: classical secretion Pathway; UPS: unconventional protein secretion pathway. NR: not reported in literature.

The computational analysis of interaction between transcription factors and the predicted secretome of *Kluyveromyces lactis* confirmed the complexity of the relationship between transcription factors and secreted proteins and also confirmed that the secretome and its expression increases under stress conditions and therefore the number of detected proteins in Table 2.1 is only true for a specific set of conditions. This also accounts for the difference in the number of detected proteins in different studies.

For *C. utilis,* 8 of the 17 identified secretory proteins in the stationary phase were predicted to be localized in the cytosol suggesting that they are either released during cell autolysis or via an unconventional secretion pathway [40].

2.3.3 The yeast cell wall proteome (also referred to as surfome)

Several studies have revealed that the yeast cell surface is composed not only of typical (glycoproteins, chitin, β-1,3-glucan and β-1,6-glucan) but also of atypical (proteins that are

incorporated into the cell wall as a result of stress conditions or unconventional secretion) cell wall proteins [8,48,49]. The yeast cell wall proteome composition is highly dynamic, varying depending on growth conditions such as nutrient availability, temperature, external pH, and oxygen level [48], and complex, with 20% of the yeast genome required for biogenesis of the cell wall [49]. Various proteins from the yeast cell are involved in cell wall function or metabolism and these cell wall proteins are crucial for the physiology of the cell as they participate in morphology change, mating, budding and interactions with other organisms in the cell surroundings [48]. These proteins are able to enter the secretion pathway, but rather than be released into the extracellular medium they are anchored onto the cell wall to perform their function. Most of these proteins therefore possess an anchor molecule or are covalently bound to the polysaccharides in the cell wall [8]. Cell-wall proteins can be divided into three classes, two of these classes are bound to the cell wall polysaccharides through either a direct link (through the γ-carboxyl group of glutamic acids) or an indirect link (generally through a β-1,6-glucan moiety) to the β-1,3-glucan in the cell wall. The third class of cell wall proteins are proposed to attach to the cell wall through a disulphide link with other cell wall proteins as they lack the covalent attachment to the polysaccharides in the cell wall and they can be released from the cell wall of intact cells using a reducing agent [48].

The anchor plays an important role in the functioning of the proteins and allows for efficient transport through the cell and strong immobilization of the protein on the cell surface. It does not interfere with the stability or activity of the expressed protein [23]. A variety of applications in the biotechnological field derive from the possibility to use the anchorage of proteins. A functional protein can be fused to an anchor and then be expressed on the cell surface, making the yeast ideal as a catalyst in many reactions depending on the protein expressed [23].

Trypsin 'shaving' of the yeast cell wall proteins revealed 42 proteins, whose functional classifications was protein biosynthesis, carbon metabolism, stress response and cellular organization [49]. Twelve of these proteins were expressed at the beginning and end of fermentations, with the greatest changes in expression of stress response, protein biosynthesis and cellular organization proteins. Braconi *et al.* [49] found that the levels of proteins on the yeast surface were not always related to their intracellular levels. Proteins such as Sse1p, Eft1p and Rpl5p were surface exposed regardless of their intracellular levels, while other proteins, Pfk2p and Sod1p were not surface exposed while their intracellular levels were high.

Carbon metabolism proteins that were found by Braconi *et al.* [49] to be connected to the cell wall include glyceraldehyde 3-phosphate dehydrogenase (Tdh3p), hexokinase 2 (Hxk2p), 6-phosphofructokinase subunit beta (Pfk2p) and phosphogluconate dehydrogenase

(Gnd1p), all thought to be incorporated into the cell wall upon stress, since they are usually found in the cytosol of yeast cells. Proteins involved in protein biosynthesis are commonly found on the cell surface, although in different studies Braconi *et al.* [49] and Insenser *et al.* [48] found different ribosomal proteins on the surface of *S. cerevisiae* leading Braconi *et al.* [49], to suggest that the incorporation of these proteins into the cell wall might be strain specific.

Mannoproteins that are bound to the cell wall through glycerolphosphatidyl inositol (GPI), have various functions and have varying lengths of mannose chains bound to the serine/threonine (*O*-glycosidic) or asparagine (*N*-glycosidic) [9]. These mannoproteins have been shown to influence wine quality by either protecting the wine against haze formation by binding other proteins or the induction of chemical and sensorial benefits of wine [49]. Charpentier *et al.* [50] suggested that the mannoproteins released by growing and by dead cells during and shortly after alcoholic fermentation may be different and therefore might have altered influences on the wine quality.

2.3.4 Extracellular proteins typically constituting the secretome "sensu stricto"

Extracellular proteins that are well described include invertase, acid phosphatase and α-factor mating pheromone. These proteins were some of the first described yeast proteins as their actions have technological applications [51]. Other extracellular proteins well described in literature are those involved in virulence of the pathogenic yeasts, *Candida glabrata* and *Candida albicans* that secrete yapsin-like proteases and adhesins that play a role in the adhesion of the yeast cells to the mammalian epithelial cells and other proteins involved in phenotypic switching [40,45] and pectinases that are involved in plant invasion [52].

Investigations of secreted proteins in the past have focused on the glycosylated proteins as it was believed that the carbohydrate structures attached to the protein are important in the signal for export of the proteins [45]. Scheiffele and Füllekrug [53] reviewed glycosylation of proteins and the role it plays in transport, concluding that glycosylation is important for folding of proteins and therefore impact the proteins' susceptibility to degradation by proteases.

Other protein complexes that are released from the yeast cell during alcoholic fermentation and ageing on lees, such as mannoproteins and pectinases, have been shown to have some applications in winemaking.

2.4 Potential applications/implications in oenology

2.4.1 Yeast proteins as a potential tool to prevent haze and to stabilize foam

Protein haze in wine is the result of heat unstable proteins or hydrophobic proteins that aggregate together [26,38,54] or interact with other wine constituents such as phenolics and

precipitate [27,39,55]. This phenomenon is not noxious but can cause consumers to reject the product as it is perceived as faulty [13,39].

Traditional methods of ridding the wine of these proteins include the use of bentonite, which might be effective in removing the proteins from wine, but it is not an ideal solution as it can adversely affect the quality of the wine by removing some aroma compounds [13,38,39]. Bentonite treatment also results in significant volume losses in the wine industry due to its swelling and poor settling characteristics [13]. Furthermore, wine after bentonite treatment might still be unstable as bentonite only binds to proteins that are charged at wine pH [39]. Other methods to rid wine of these proteins have been proposed in literature and include ultra-filtration [28], flash pasteurization [56], protease treatment [29,39], polysaccharide adsorbents such as carrageenan and pectin [54] and the use of zirconium oxide [30], but so far, none of these methods has proved to be commercially viable [31]. Recent studies have therefore focused on better understanding the characteristics of these proteins [3,31] so that an efficient and commercially viable method to rid the wine of them can be developed.

The main proteins identified in causing haze are those from the grape involved in pathogen response, namely thaumatin-like proteins and chitinases [13]. This does not mean that they are the only proteins responsible for haze, but they are the best characterized of the haze forming proteins. Nevertheless, other wine compounds, such as phenolics, tannins and glycoproteins from yeasts, have also been implicated in wine haze [27,57]. Hsu and Heatherbell [28] defined the haze forming proteins as those with a low molecular weight (e.g. between 12.5 and 30 kDa) and a pI between 4.1 and 5.8, thus confirming the earlier indication of Mesrob *et al.* [58]. Pocock *et al.* [57] tested various components (phenolics, metal ions, sulphate and anions) of wine to find the factor that is responsible together with proteins for haze formation. Sulphate was identified as the X factor required in wine together with proteins before haze will form. In a similar study, Marangon *et al.* [31] tested the implications of ionic strength (salt concentration) and sulphate on the aggregation of grape proteins involved in haze formation. They reported a strong influence of ionic strength on the aggregation of chitinases, but only to a lesser extent on that of thaumatin-like protein aggregation. Sulphate in the medium had a similar effect on protein aggregation, with chitinases forming a visible haze immediately upon heating and thaumatin-like proteins aggregated only during cooling with particles that formed not visible to the naked eye. Chitinases are therefore more prone to form haze while ionic strength and ionic content have an influence on the haze particle size in model wine.

Enzymatic hydrolysis of the proteins into smaller more soluble molecules can enhance clarification and stabilization of the wine [27,59,60]. Nevertheless, Bakalinsky and Boulton [39] suggested that the hydrolysis of proteins in wine will need a high enzymatic activity in

wine conditions and that the formation of smaller hydrophobic peptides might result in bitter flavour development. The hydrolysis of proteins might also help prevent incomplete fermentations due to assimilable nitrogen deficiency in the must [59,61].

Another method of protecting wine against haze is by adding mannoproteins produced by yeasts. The precise mechanism of haze protection is not completely understood, but is linked to the hydrophobic nature of these moieties. The functions of mannoproteins in the cell are linked to cell wall porosity, water retention, cell-cell adhesion, biofilm formation astringency and enzymatic activity [9]. It was shown that mannoproteins do not prevent heat unstable proteins to precipitate but it reduced the particle size [38]. Mannoproteins can also adsorb undesirable contaminants and contribute to chemical, sensorial and health benefits affecting wine quality [49,62].

Other wine proteins can however be beneficial as in the instance of sparkling wine where the proteins are responsible for foam formation and stability [9,63]. Proteins described in literature that have an influence on foam stability is chitin and mannoproteins released by yeast during the alcoholic fermentation of base wine used for sparkling wine [63].

Foam is formed by gas bubbles that are separated by a thin layer of liquid (the lamella), that accumulates at the air-liquid interface. The foam texture is influenced by the number, size and distribution of the bubbles, with small uniformly distributed bubbles resulting in soft foam. Polypeptides (especially hydrophobic polypeptides) present in wine increase the stability of foam by increasing the surface tension of the bubbles [9,63]. The ethanol and carbon dioxide (CO_2) produced by yeasts during fermentation have opposite effects on the foam production. Ethanol is detrimental to foam formation as it lowers the surface tension of the bubbles causing bubbles to collapse whereas CO_2 increases the amount of foam formed. Yeasts cell walls adhere to gas bubbles and cause a foam layer at the surface of the fermentation vesicle [9].

Foam during the early stages of fermentation is very undesirable as it can slow down the process or totally inhibit fermentation. A thick layer of foam also implies greater void volume at the top of the fermentation vessel which can result in oxidation and spoilage [9]. Foam at the beginning of fermentation can also lead to loss of nutrients equipment failure if the foam rises through the outlet vents of the tank.

Conversely, foam formation at the end of fermentation or in the final product is desired, as in the case of sparkling wines. The foam in sparkling wines behaves characteristically in two phases. In the first phase, foam is abundantly formed by the decompression of carbonic acid and in the second phase, the foam collapses after a few minutes leaving a trace foam collar on the inside of the glass. Sparkling wine foam is sustained by the proteins that are released by the yeast and some grape proteins that survive the fermentation process. Amongst the most important proteins released from the yeast during fermentation are

mannoproteins that are particularly important as their hydrophobic nature causes them to adsorb to foam bubbles [9], stabilizing the foam [9,35,64]. Yeast subjected to stress conditions, such as high osmotic stress during fermentation, can also have a detrimental effect on the formation of foam due to the secretion of either lipids or proteinase A. Lipids promote the fusion of bubbles causing foam to collapse, while proteinase A hydrolyses polypeptides involved in foam stabilization causing foam to dissipate quickly [9].

Studying the effect of bentonite on the foaming potential of sparkling wine, Vanrell *et al.* [25] found a decrease of low molecular proteins in wines treated with bentonite. The low molecular proteins have an influence on the mouth feel perception of the wine and their removal from wine might render the wine "flat" and "watery".

2.4.2 Yeast extracellular enzymes for enhanced wine quality

The process of winemaking and wine quality can be improved by various enzymes [65]. Wine filterability is affected by proteins and polysaccharides such as pectins, glucans and hemicelluloses, that influence the turbidity and viscosity of the wine [66,67]. Polysaccharides influencing wine filterability do not only originate from the grapes, but also from the yeasts during fermentation. The yeast strain used for fermentation can thus have an influence on the haze formation and filterability of the wine [64,66,68]. Degradation and utilisation of these polysaccharides improve wine filterability and clarification [66]. Furthermore, wine aroma and flavour can be enhanced by the hydrolysis of glycosidic aroma precursors [65].

Under winemaking conditions, endogenous pectinase, xylanase and glucanase activities of yeast (*S. cerevisiae*) and grapes are not sufficient to prevent polysaccharide hazes and filter blockages [66]. Exogenous enzymes from fungal and bacterial origins were therefore developed and are commercially available for clarification and processing of wine. These commercial enzymes are typically crude extracts and contain cocktails of esterases, glucanases (cellulases), pectinases (polygalacturonase, pectin methyl esterase, pectin lyase) and xylanases (hemicellulases) in different combinations [66,69-72]. Nevertheless, addition of exogenous enzyme preparations can increase production costs. Moreover, the lack of specificity of these enzymes might induce secondary reactions detrimental to wine flavour [73,74] and colour, such as the production of methanol from pectin methyl esterase activity and oxidation of phenolic compounds [71,75]. Information regarding the enzymatic activities of these commercial enzymes is not readily available [71], and as can be seen in Table 2.2, the description provided by the manufacturer is scientifically cryptic and does not allow easy comparison between enzymes.

These purified enzymes are expensive and application of the enzymes is influenced by their stability and activity that is not always optimal under winemaking conditions. In recent years the attention has therefore shifted towards the use of native yeast enzymes [66].

Table 2.2 - Examples of commercial enzymes available to the wine industry in South Africa and some of the advertised properties. All information was taken from the corresponding company's website.

Company	Product name	Main Enzyme activity	Secondary activity	Source	Advertised action	Secondary action
Anchor yeast	Claristar®	Mannoprotein	-	*S. cerevisiae*	Tartrate stability	-
	Maxifruit	Pectolytic enzymes	Esterase Anthocyanase	*Aspergillus niger*	Improvement in poly-phenol extraction, colour stabilization, aroma extraction, juice extraction, must clarification and wine filtration	Colour stability Wine aroma Reduce maceration time
	Ex color	Pectolytic	Hemicellulases Anthocyanase Cinamyl esterase	*Aspergillus niger*	Polyphenol extraction	Colour extraction Improve wine clarification
Lallemand	Lallzyme® Cuvée blanc	Pectinase with beta-glucosidase activity	-	*Aspergillus niger*	Aroma complexity	Improve wine filterability
	Lallzyme® Ex-V	Pectinases	Cellulase, Hemicellulases	*Aspergillus niger*	Juice extraction	Increase release of aroma compounds
Laffort oenologie	Lafazyme® Arom	Polygalacturonase	Cinnamyl esterase	*Aspergillus niger*	Aroma release	Clarification improves
	LAfase® Fruit	Pectolytic enzymes	-	*Aspergillus aculeatus*	Extraction of phenolic compounds	Increase juice yields

Anchor yeast: http://www.oenobrands.com/en/our-brands/anchor; Lallemand: http://www.lallemandwine.com/spip.php?rubrique3&lang=en; Laffort Oenologie: http://www.laffort.com/en/products

Yeasts belonging to the *Saccharomyces* genus do not secrete enzymes of oenological relevance under winemaking conditions [76,77] as seen for its pectinase [78]. Genera other than *Saccharomyces* thus gained more attention in the last few years due to their potential to contribute positively to wine aroma and quality [69,79-83]. Less information about their proteome and transcriptome is known than for *S. cerevisiae* [5]. Recently, Lomolino *et al.* [84] and Giovani *et al.* [62] concluded, after investigating the proteins of different yeast species, that protein quantities and their ratios differ greatly between yeast species and that non-*Saccharomyces* species released a greater quantity of polysaccharide complexes compared to *S. cerevisiae*.

It is now well acknowledged that other yeast species, such as *Kloeckera/ Hanseniaspora, Candida, Metschnikowia, Torulaspora* and *Pichia,* are also present during fermentation and especially during the early stages [65]. These yeast species have been shown to have the potential of producing extracellular hydrolytic enzymes that might be beneficial during the winemaking process [65,66].

Some of the proteins of oenological interest that have been identified and characterized in recent years include esterases [85-88], pectinases [89], proteases [90] and glucosidases [61,91,92]. A number of enzymes and proteins that are of interest in winemaking are secreted by various oenological yeasts [66,93]. Some of the enzymes produced by the yeast during and shortly after the end of fermentation can be beneficial for the winemaker since they are better adapted and more stable in wine than external enzymes of fungal origin [66]. Pectinases, proteases and glycosidases are some of the enzymes secreted by yeasts that are relevant to wine-making because of their technological effects and their contribution to aroma formation [59,94].

Different studies have investigated enzymes secreted by yeasts, and some of their characteristics are shown in Table 2.3. Most of these extracellular enzymes have optimal pH and temperature that are higher than that of wine fermentations (normally at pH 3.2 - 4.2 and temperature <20°C), however some still retain a great portion of activity at these fermentation conditions, making them more optimal to use than external enzymes from fungal origin.

Table 2.3 - Examples of yeast enzymes identified and their characteristics as reported in literature. Not all these yeast species are found in the wine environment, but they might be related to some of the wine yeasts. The information in red is on yeasts that are not normally found in wine or the wine environment.

Enzyme	Species	Size	T_{opt}	pH_{opt}	pI	Reference
β-glucosidase						
Extracellular	*Schwanniomyces pseudopolymorphus*	100 kDa	40	4	ND	[95]
Extracellular	*Candida peltata* NRRL-Y6888	43 kDa	50	5	ND	[73]
Intracellular	*Metschnikowia pulcherrima*	49kDa	50	4.5	4.2	[96,97]
PGases[a]						
	Saccahromyces pastorianus	43 kDa	50	4.2	5.4	[98]
	Saccharomyces cerevisiae IMI-8b	43 kDa	45	4.5	ND	[98]
	Kluyveromyces marxianus	496 kDa	ND	ND	5.7–6.3	[98]
Pectinesterases						
	Rhodotorula sp.	ND	40	6.0	ND	[98]
Esterase						
Intracellular	*Saccharomyces cerevisiae*	40 kDa	50	7.0	5.0	[85]
Extracellular	*Rhodotorula mucilaginosa*	ND	30	8.0-10.0	ND	[87]
	Saccharomyces cerevisiae	28 kDa	25	ND	ND	[99]
Proteases						
Extracellular	*Metschnikowia pulcherrima*	40.8 kDa	ND	ND	4.2[b]	[90]
	Candida apicola	39.1 kDa	ND	ND	4.3[b]	[90]
	Metschnikowia reukauffi	53.5[b] kDa	40	3.5	ND	[100]
	Yarrowia lipolytica	28-36 kDa	ND	3.1-4.2	3.8-4.9	[100]

ND: not determined; a) polygalacturonases; b) Calculated value based on the deduced protein sequence

2.4.3 Non-*Saccharomyces* yeasts: a neglected reservoir of enzymes?

The under-utilization of the yeast biodiversity and its potential, especially for oenology, is generally accepted [69,101,102]. Previously non-*Saccharomyces* yeasts were often regarded as unfavourable to wine quality due to their production of metabolic products that were associated with off-flavours and the yeasts themselves were mostly isolated from stuck or sluggish fermentations [79]. Over the last few years, the re-evaluation of the role of non-*Saccharomyces* yeast in winemaking has led to the use of controlled mixed fermentations using *Saccharomyces* and different non-*Saccharomyces* yeasts [103]. This re-evaluation came after the major advances research has made in the last few decades on understanding

the ecology, physiology and molecular biology of all yeasts involved in fermentations [79] although there are still large gaps in our knowledge of these yeasts in comparison to *S. cerevisiae*. Yeasts do not co-exist passively in fermentations as was previously thought but rather interact synergistically. These interactions represent a tool for new fermentation technology [79].

Using non-*Saccharomyces* yeasts in pure cultures as starter cultures for fermentation has showed that these yeasts have some beneficial but also some negative fermentation characteristics [86]. The use of mixed cultures for fermentation is then a more practical way of improving wine complexity and enhancing some characteristics of a wine [86,103]. This is possible by taking advantage of several metabolic pathways of the yeasts that have been reported to have a positive effect on wine flavour and aroma [82,86]. Research has shown that using multiple starter cultures for fermentations can give wine more complexity by increasing its ester and glycerol content [79].

Saccharomyces, when used in combination with non-*Saccharomyces* yeasts, can reduce some of the negative characteristics of the non-*Saccharomyces* yeasts [79,104]. Ciani *et al*. [79] summarized the recent results reported by different researchers when using non-*Saccharomyces* and *Saccharomyces* in combined fermentations (Table 2.4). Briefly, *Torulaspora delbrueckii* strains were shown to reduce acetic acid concentration, while *Issatchenkia orientalis* reduces malic acid in wine. *Lachancea* (*formerly Kluyveromyces) thermotolerans* showed a lower production of volatile acidity and higher production of lactic acid, which results in a reduced pH in the final wine. The benefits of using mixed cultures for fermentations are also prevalent in the growth and death kinetics of the different species, with mixed fermentations resulting in a lower biomass production and an increased persistence of non-*Saccharomyces* yeast particularly in low temperature fermentations [104].

Several studies have reported on the benefits of using controlled mixed culture fermentations [86,88,101,103,105]. Garcia *et al.* [105] reported on the improvement of geraniol production in mixed fermentations with *Debaryomyces vanriji* and *S. cerevisiae* due to high levels of β-glucosidase activity in the non-*Saccharomyces* yeast. Investigations of the synergetic effect of *T. delbrueckii* and *L. thermotolerans* in mixed fermentation have also reported (1) reductions in acetic acid content; and (2) reduction in acetaldehyde concentrations and increase in titratable acidity [106,107]. Kurita [88] reported on the positive enhancement of isoamyl acetate when using *Pichia anomala* in mixed fermentation with *S. cerevisiae* and another strain *Pichia kluyveri* produces an increase in varietal thiols when used in mixed fermentations [108].

Table 2.4 - Summarized results of the influence of non-*Saccharomyces* yeasts on wine when used in conjunction with *Saccharomyces cerevisiae* as in review by Ciani *et al.* [79].

Species used	Aim	Process	References[1]
S. cerevisiae *T. delbrueckii*	Reduction of acetic acid production	Sequential cultures	Castelli (1969); Herraiz *et al.* (1990); Ciani *et al.* (2006); Salmon *et al.* (2007); Bely *et al.* (2008)
S. cerevisiae *S. pombe*	Malic acid degradation	Sequential cultures Immobilized cells (batch process) Immobilized cells (continuous process)	Snow & Gallender (1979); Magyar & Panyik (1989); Yokotsuka *et al.* (1993), Ciani (1995)
S. cerevisiae *C. stellata*	Enhancement of glycerol content	Immobilized cells (pretreatment or sequential cultures)	Ciani & Ferraro (1996); Ciani & Ferraro (1998); Ferraro *et al.* (2000)
S. cerevisiae *C. cantarellii*	Enhancement of glycerol content	Mixed or sequential cultures	Toro & Vazquez (2002)
S. cerevisiae *C. stellata*	Improve wine aroma profile	Mixed or sequential cultures	Soden *et al.* (2000)
S. cerevisiae *H. uvarum (K. apiculata)*	Simulation of natural fermentation (improvement of aroma complexity)	Mixed or sequential cultures	Herraiz *et al.* (1990); Zironi *et al.* (1993); Moreira (2005); Ciani *et al.* (2006); Moreira *et al.* (2008); Mendoza *et al.* (2007)
S. cerevisiae *K. thermotolerans*	Reduction of acetic acid production Enhancement of titratable acidity	Sequential cultures	Mora *et al.* (1990); Ciani *et al.* (2006); Kapsopoulou *et al.* (2007)
S. cerevisiae *Issatchenkia orientalis*	Reduction of malic acid content	Mixed fermentation	Kim *et al.* (2008)
S. cerevisiae *Pichia fermentans*	Increased and more complex aroma	Sequential cultures	Clemente-Jimenez *et al.* (2005)
S. cerevisiae *Pichia kluyveri*	Increased varietal thiol	Mixed fermentation	Anfang *et al.* (2009)
S. cerevisiae *Candida pulcherrima*	Improve wine aroma profile	Mixed fermentation	Zohre & Erten (2002); Jolly *et al.* (2003)
S. cerevisiae *Debaryomyces vanriji*	Increase in geraniol concentration	Mixed fermentation	Garcia *et al.* (2002)
S. cerevisiae *Schizosaccharomyces* spp. *Saccharomycodes* spp. *Pichia* spp.	Influence on sensorial and physico-chemical properties of wines	Ageing over the lees during wine maturation	Palomero *et al.* (2009)

1 - For references see the review by Ciani *et al.* [79]

Other non-*Saccharomyces* yeasts associated with grapes and the winery environment and that have been reported to produce extracellular enzymes that are beneficial to production of wine include *D. hansenii, P. galeiformis*, species from the *Rhodotorula* genus and also *P. membranifaciens.* Biocatalytic activities described for non-*Saccharomyces* include proteases, xylanases, and pectinases, especially polygalacturonases, β-glucosidase and β-D-xylosidase activity are widely dispersed among non-*Saccharomyces* [79]. Fernandez *et al.* [59] reported 80% of the 182 non-*Saccharomyces* isolates they tested possessed 1 or more extracellular enzyme activity, with polygalacturonase activity being the most abundant and β-glucosidase activity only found in ~14% of the strains tested. The production of enzymes by yeasts has also been described in olive production, where the enzymes can have a detrimental effect on product quality, such as softening of fruit [109]. These traits can be utilized in a winemaking environment where they are not categorized as detrimental as the same species of yeasts are present in both environments.

Pectinases

Structural polysaccharides interfere with clarification, stabilisation and filtration of must and wine [76,98,110]. Pectin in particular renders the settling of particles and the clarification process very slow [70], and is responsible for increases in filter pressure and consequent clogging of the filter [110].

Pectinases are produced by the microflora on the grapes, especially from the filamentous fungi attacking the grapevine cells but have also been described in yeasts found on the surface of the grape berry, and can be classified according to the mode of action on pectin molecules. These enzymes either de-esterify (pectinesterases, saponification of esterified regions) or depolymerise (polygalacturonases, chain cleavage) specific pectic substrates [66,110].

Pectinolytic enzymes cleave long pectin chains into more soluble segments which facilitate the pressing of the grapes, contribute to clarification of the must and may increase the extraction of substances that contribute to aroma and colour [59,71,111].

Polygalacturonases are the most abundant of the pectic enzymes in grape juice [66,67,112]. Grape and fungal polygalacturonase activity is not affected by catabolite repression and the enzyme is not induced by pectate [66]. Pectinases are divided into exo- and endo-enzymes; the exo-pectinases split off mono- or dimers from the non-reducing end of the polysaccharide chain, the endo-pectinases split the chain randomly, but polygalacturonases cannot catalyse the complete breakdown of pectic acid to galacturonic acid [66].

Blanco *et al.* [67] showed that fermentations by yeasts exhibiting pectinolytic activity had little influence on the viscosity of the wine, although these fermentations had an improved

filtration time. Fermentations done with strains overexpressing *PGU1* (encoding for endo-polygalacturonase) showed various levels of increase in some aroma compounds confirming the potential of polygalacturonases to benefit the aroma profile of wine [113,114], but this results are contradicted in other studies [115,116].

Commercial preparations contain hemi-cellulolytic and cellulolytic activity and sometimes glycosidase and protease activity [66,68,71,110] and therefore these enzyme complexes can sometimes adversely affect the quality of wine due to a lack of specificity and secondary reaction [71,73]. The complex and undefined nature of commercial preparation also makes it difficult to control their effect on wine aroma [77]. Commercial enzymes are used despite these negative possibilities as positive attributes overshadow the negative.

Yeasts that are associated with strong pectinase activity include species from the *Candida*, *Cryptococcus*, *Kluyveromyces and Rhodotorula* genera [89,102,111]. Moyo *et al.* [89] have shown that *Kluyveromyces thermotolerans* is capable of constitutively producing polygalacturonases.

Glycosidases

Free volatiles and various odourless precursors make up the aromatic potential of grape juice. Among these precursors, the glycoconjugated precursors are the most impactful on the wine bouquet [105]. The enzymatic hydrolysis of the glycosidic complexes occurs in two stages; firstly the intersugar linkages are cleaved by sugar specific enzymes (i.e. α-L-arabinofuranosidase; α-L-rhamnosidase) releasing monterpenyl glucosides and then the resulting monoterpenyl are hydrolysed by β-glucosidases to release the monoterpenols [74,77,117]. The two enzymes are both needed for hydrolyses of glycosidic complexes as β-glucosidases do not have endoglucanase activity and can therefore not release disaccharides from the glycosidic complexes [74,77,118]. Secretion of enzymes characterised by predominantly β-glucosidase activity have been described in various *S. cerevisiae* strains, but very few of the diglycosidic enzymes have been identified in the extracellular environment of yeasts.

Arabinofuranosidase

Previous studies have investigated the possible effects of α-L-arabinofuranosidase on wine parameters, with satisfactory results [77,117]. This enzyme indeed hydrolyses the bond between arabinose and glucose of diglycosylated substrates such as grapevine monoterpenes thereby rendering the bond between glucose and the non-sugar moiety more accessible. In combination with β-glucosidase, more of such substrates will be released thus increasing their contribution to the global wine bouquet [77].

Zietsman *et al.* [77] showed that *S. cerevisiae* has no native arabinofuranosidase activity, but when transformed with the α-L-arabinofuranosidase gene from *Aspergillus*

awamori extracellular activity towards *p*-nitrophenol could be detected. The α-L-arabinofuranosidase of *A. awamori* have been showed to have an optimum activity at pH 4 and 55°C, but is stable at pH 3 to pH 7 and temperatures up to 60°C. Heterologous expressed α-L-arabinofuranosidase has characteristics similar to when they are expressed in its organism of origin [77]. α-L-arabinofuranosidase is reversibly inhibited by high glucose concentration but it retains > 60% of its activity at high ethanol concentration (16% v/v). Spagna *et al.* [117] isolated and characterized the α-L-arabinofuranosidase in commercial enzyme preparations and found that it has an optimal temperature of 65°C but at 20°C where it is used it has a relative activity between 10% and 30%. They also reported that enzyme stability in wine (at 25°C) was not very long (1 day) which might make the use of this exogenous enzyme inconsequential.

β-Glucosidases from non-*Saccharomyces* yeasts hydrolyse aroma precursors bound to sugar molecules (terpenyl-glycosides amongst others) and can contribute positively to wine aroma [73,91,92,96,97,105] but are detrimental for colour extraction [71] in red wine when used in high concentrations. Immobilized β-glucosidases have been shown to achieve a rapid and controlled liberation of terpenes. However, as can be seen in Table 2.2, some of the commercial preparations contain secondary activities that might produce negative effects in wine [97].

β-Glucosidases are associated with genera such as *Candida*, *Debaryomyces, Hanseniaspora, Issatchenkia, Metschnikowia* and *Pichia* [91,92,97,102,105,119], with extracellular β-glucosidases produced mainly by strains from the *Candida* spp. and *Debaryomyces* spp. while most of the other species produce intracellular β-glucosidases [91]. Many of the β-glucosidases from non-*Saccharomyces* yeasts have been shown to be inactive at low pH, high glucose and high ethanol, but these enzymes have not been extensively studied and therefore their effects on wine flavour and aroma is not yet fully understood [65,91,92,120,121]. Some studies have shown that the enzyme activity from some yeast is inhibited when glucose is used as carbon source [65,73,91], although the enzymes isolated from *Issatchenkia terricola* and *Schwanniomyces* (formerly *Debaryomyces*) *pseudopolymorphus* showed considerable activity in high glucose medium [92,97]*.*

González-Pombo *et al.* [97] showed that *Issatchenkia terricola* produces a β-glucosidase that is active at acidic pH which is contrary to the findings of other authors who found a decrease in yeast β-glucosidase activity when the pH of the medium decreases [60,91,119].

Cordero Otero *et al.* [92] have defined β-glucosidases suitable for wine production as those which have a high affinity for grape-derived terpenoid aglycones, as well as optimal

activity at wine pH, resistance to ethanol and glucose inhibition. In their study they identified *S. pseudopolymorphus* and *C. oleophila* as yeasts with extracellular β-glucosidase activity. The β-glucosidase from *S. pseudopolymorphus* showed resistance to ethanol and glucose inhibition. González-Pombo *et al.* [96] showed that *M. pulcherrima* possesses extracellular enzyme activity, although very little and it is induced when cells are grown on cellobiose.

Glycosidase activity in non-*Saccharomyces* yeasts make them ideal candidates for starter cultures either as pure cultures or in combination with *Saccharomyces*, as summarized in Table 2.5.

Esterases

Esters are significant constituents of wine (total of >100 mg/L) and contribute greatly to wine aroma specifically contributing to the fruity characteristics in the fermentation bouquet [82,88,122]. Flavour-active esters are divided into two groups; firstly, the acetate esters such as ethyl acetate (responsible for "fruity" character in wine) and isoamyl acetate ('banana' aromas) and secondly, medium-chain fatty acid ethyl esters, such as ethyl hexanoate ('apple-like' aroma) and ethyl octanoate ('apple' aroma) [74]. The production of esters is dependent on synthesizing and hydrolyzing esterases and the balance of these enzymes in the yeast [88] as well as the concentrations of the co-substrates, acyl-CoA and alcohol [74]. Esterases have the potential to contribute greatly to wine aroma [88,123] as they are responsible for the degradation of hemicelluloses, removing aromatic constituents from the cellulose backbone and increasing in fatty acid content [109]. Ester synthesis in different yeast species differ both in pathway and concentrations [80,88] but very few yeasts have been reported to produce extracellular esterases [87]. Lomolino *et al.* [123] suggested that yeast esterases are more involved in ester breakdown and not so much in ester synthesis. This was confirmed by Domizio *et al.* [104] who reported ester hydrolase activity for several genera of yeast with the activities spread over several levels of intensity.

Lee *et al.* [87] nevertheless described a *Rhodotorula mucilaginosa* strain with extracellular esterase activity. Other literature refers only to cytoplasmic and periplasmic esterase activity in yeasts [72,84,123], but nonetheless most fermentations carried out with non-*Saccharomyces* yeast have had an increase in some of the esters' concentrations [80,82]. Increase in ester concentration might be due to increased intracellular ester synthesis and hydrolysis by one of the proteins (e.g. Atf1p, Atf2p, Eht1p, Eeb1p and Iah1p) identified and characterised previously, [74] or by unknown ester synthases as suggested by [124] in a study with a double deletion of *ATF*1 and *ATF*2 where the deletion strain still produced 50% of the ethyl acetate the wild-type strain produced.

Donaghy *et al.* [72] suggested that esterases might in some cases be a pre-requisite for substrate assimilation in yeast. This might be true as Bronscheuer [99] reported that many

esterases show a wide substrate specificity which will enable access to carbon sources or confirm the role of esterases in the catabolic pathways or in the in vivo detoxification of some toxic compounds found inside the cell [85]. Even with this knowledge esterase reactions and substrates are poorly explored and characterised. Kurita [88] reported that esterase activity is dependent on the oxygen levels in the yeast's environment, suggesting that esterase activity will be repressed during wine fermentation.

Proteases

Enzymatic hydrolysis of the proteins into smaller and more soluble molecules can enhance clarification and stabilization of the wine [59]. It also helps prevent incomplete fermentations due to assimilable nitrogen deficiency in the must [59,61]. Proteases are associated with species such as *Metschnikowia* [90], *Kloeckera* [27,65], *Torulaspora* and *Hanseniaspora* [94,102]. Lagace and Bisson [125] showed that the acid proteases secreted by yeast found on the grape surface can hydrolyze wine proteins when grown in model solutions and conditions that were optimized for the expression of extracellular acid protease activity. Dizy and Bisson [27] found that acid proteases secreted from *Kloeckera apiculata (Hanseniaspora uvarum), Torulopsis magnolia* and *M. pulcherrima* were effective in degrading wine proteins in wine and synthetic solutions. The activity of extracellular proteases is reported to be strain dependent within a specific species as shown by Dizy and Bisson [27] for *Kloeckera* spp*.*

Yeasts also have cytoplasmic proteases responsible for degradation of cellular molecules. Upon autolysis these proteases are released into the extracellular medium, where it is reported that they retain some activity [27].

*Examples of commercialized non-*Saccharomyces *for the wine industry*
Some of the yeast that have been reported to have a positive impact on wine quality have also been added to the collection of commercially available active dried yeast from the different companies that supply products to winemakers around the world and include species *M. pulcherrima* and *T. delbrueckii* as summarized in Table 2.5 [86] (Ganga *et al.,* 2012*,* 8th international cool climate symposium, Tasmania, http://www.winetasmania.com.au/files/poster_ICC_2012_Ganga_final__2_.pdf; Raynal *et al.,* Lallemand technical paper, http://www.lallemandwine.com/IMG/pdf_Sequential_en.pdf). The positive attributes of these yeasts are mostly due to enzyme activity especially aromatic release by glycosidases. Table 2.5 also highlights some of the claimed properties these commercialized yeasts.

Table 2.5 - Examples of commercial non-*Saccharomyces* starter cultures available to winemakers around the world. The table also highlights some of the reported benefits of using the specific non-*Saccharomyces* yeasts.

Manufacturer	Commercial name	Species	Claimed characteristics
Chr Hansen	Prelude	*T. delbrueckii*	To be used in co-inoculation with winemakers' preferred *S. cerevisiae*. PRELUDE increases the body, softens the palate and rounds the mouth feel of wines. It is particularly suitable for premium and high premium wines from Chardonnay, Sauvignon blanc or Semillon and some winemakers have started to use it successfully in rosé and red wines.
	Melody / harmony	*L. thermotolerans + T. delbrueckii + S. cerevisiae*	MELODY has a stronger effect than HARMONY. They are both used for white, rosé and red wines depending on the desired effects.
	Rhythm / Symphony	*L. thermotolerans + S. cerevisiae*	RYTHM has a stronger effect than SYMPHONY and the two blends are mainly used in red and rosé wines and base white wines for sparkling wines.
Lallemand	Level² TD	*T. delbrueckii + S. cerevisiae*	Enhances aromatic and mouthfeel complexity of white musts (Chardonnay, Chenin, Semillon, Ugni blanc, Melon, Maccabeu grapes). The *S. cerevisiae* was carefully chosen for its ability to interact positively with the *T. delbrueckii* TD219 and to carry out a reliable alcoholic fermentation finish. Level² TD enhances the high quality grapes by improving mouthfeel, aromatic complexity and by smoothing aggressive character. Level² TD upgrades wine quality of lower quality must. Sequential inoculation enables controlled development of wines aromatic complexity by favouring the perception of certain esters without overwhelming the wines.

	LAMAP L1781	*M. pulcherrima*	The *M. pulcherrima* LAMAP L1781 strain is particularly promising for its α-arabinofuranosidase enzyme activity, which stimulates the liberation of thiols and terpenes in white wines. Wines produced in fermentation trials with a complementary *S. cerevisiae* presented a greater concentration of volatile thiols judged to be richer and more complex in aromatic gustatory terms.
Laffort Oenologie	ZYMAFLORE Alpha TD n. Sacch	*T. delbrueckii*	Great aromatic purity and complexity as well as a high production of volume on the palate. ZYMAFLORE Alpha TD n. Sacch is an excellent choice for making expressive and full bodied wines. 10% vol of average alcohol production observed. ZYMAFLORE Alpha TD n. Sacch should be used with a S. cerevisiae to reproduce the natural ecosystem of musts in fermentation and to ensure a complete alcoholic fermentation.

Another benefit of using non-*Saccharomyces* yeasts is the control of spoilage microorganisms. In order to exploit the beneficial potential of non-*Saccharomyces* yeasts while limiting the potential spoilage it must be known what the effect of wine making practices is on these yeasts as well as the metabolic characteristics of these yeasts [101].

*Killer Toxins and non-*Saccharomyces *yeasts in the control of undesirable microorganisms*

Traditionally, chemical preservatives, such as sulphur dioxide, are used in wine to control the growth of microorganisms during storage and ageing. These chemical preservatives might lead to potential faults with the final product or consumer sensitivity. Sulphur dioxide is the most used preservative but consumer allergies have led to a greater search for natural antimicrobial agents with no known allergic reactions [103]. Sulphur dioxide is also not always fully efficient in controlling growth of some spoilage yeasts such as *Brettanomyces bruxellensis*. It has recently been demonstrated that some non-*Saccharomyces* yeasts would have the potential to control the growth of these undesirable microorganisms as some yeasts produce toxic proteins or glycoproteins that inhibit the growth of fungi, bacteria and other yeasts [109,126].

The importance that yeast killer systems could play in the control of spontaneous and spoilage microflora is now increasingly of interest to the wine industry as selected killer yeast strains can be used as the inoculated strain and then repress the growth of undesirable strains [103] and so enable better control over undesirable characteristics in the wine, such as excessive hydrogen sulphite, volatile acidity and other off-flavours [127]. *S. cerevisiae* killer toxins have been the focus of studies for the last three decades [127,128], but they have limited anti-yeast spectra, which is mostly restricted to sensitive *Saccharomyces* strains and a few other sensitive yeast species such as *Candida glabrata* [127]. Thus, these killer toxins are not effective against wild yeasts such as *Kloeckera/Hanseniaspora, Pichia, Brettanomyces, Zygosaccharomyces* and *Saccharomycodes,* which represent the main targets of antimicrobial agents used in winemaking [103]. The *Saccharomyces* killer toxins have been well studied and characterized. Three killer toxins from viral origin have been identified and two chromosomal killer toxins have also been described [129]. The mode of action for the viral killer toxins is similar, as they have similar sequences. The chromosomal killer genes however show little homology with other killer genes and Magliani *et al.* [129] concluded that it will therefore have a different mode of action.

The isolation of killer yeast strains from various oenological sources, including grape berries, wine and cellar equipment in different regions of the world has been reported in literature [86,103,129]. Hidalgo and Flores [130] showed an increase in frequency of killer yeasts during fermentation while the frequency of sensitive yeasts decrease, supporting the hypothesis that killer yeasts will survive longer and therefore drive the fermentation [127].

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Non-*Saccharomyces* yeast that have been reported to produce killer toxins include *Kluyveromyces phaffi (*reclassified as *Tetrapisispora phaffi)*, which has a wide anti-*Hanseniaspora* activity [131], *Kluyveromyces wickerhamii* (both not usually found in wine or on the grape surface), *Pichia anomala* (reclassified as *Wickerhamomyces anomalus*) [104,132] and *Pichia membranifaciens* [133]. The mycocins produced by these yeasts have been shown to control the undesirable *Brettanomyces/Dekkera* species under wine making conditions [132,133]. Other species of yeasts more commonly found in the wine environment that show killer activity include, *L. thermotolerans, T. delbrueckii* and *H. anomala* [130]. Characterisation of the *T. phaffi's* killer toxin showed that it is a glycosylated protein whose N-terminal region showed no correlation with other reported killer toxins but similarities to β-1,3-glucanase of *S. cerevisiae* and β-1,3-transferase of *Candida albicans* [103]. The receptor sites for most characterised killer toxins from non-*Saccharomyces* species is either mannoproteins or specifically β-1,6-glucan although pustulans has been described as the receptor site for the killer toxin produced by *Kluyveromyces wickerhamii.* Magliani *et al.* [129] reviewed the yeast killer system and concluded that most yeast killer toxins, no matter what their receptor site is, increase membrane permeability causing a loss of ions from the cell.

Kluyveromyces lactis, (a yeast found in food and used industrially for protein production) also produces killer toxins through two cytoplasmic plasmids although the mode of action is still poorly understood [129]. The killer toxins secreted by *K. lactis* inhibits growth in a wide range of susceptible yeasts from the genera, *Zygosaccharomyces, Torulopsis, Kluyveromyces* and *Candida* by arresting the cell cycle in the G1 phase [129]. It differs from other killer toxins in that is does not act as an ionophore and thus the infected cells can still maintain a membrane potential although they are no longer viable.

When investigating the inhibitory effect yeast products have on malolactic bacterial growth, Comitini *et al.* [126] found that a proteinaceous factor is secreted by the *Saccharomyces* yeasts. The action of this factor is highly strain dependent as only 3 of the 8 *Saccharomyces* strains tested showed an inhibitory effect on 2 of the *Oenococcus oeni* strains tested and a stimulating effect on another *O. oeni* strain. The activity of the protein factor was observed for the low molecular fraction (smaller than 10 kDa) as well as the fraction bigger than 10 kDa, and the action of the higher molecular weight fraction was impaired when treated with proteases or heat, where on the other hand no inhibitory effect was observed when the low molecular weight fraction was treated with proteases. This suggests that the antimicrobial effect is the result of various metabolic products secreted by the yeasts and not necessarily the effect of a specific killer toxin. Comitini *et al.* [126] also suggested that the bacteriocidal effect of this proteinaceous factor is concentration dependent, with lower concentration inhibiting some growth and higher concentrations completely inhibiting growth.

Arroyo-López *et al.* [109] suggested that killer activity of yeasts studied in the olive industry might be related to the pH and salt content (in particular NaCl) of the medium, this will also hold true for yeasts in the wine industry as similar yeasts are found in both environments.

Pérez-Nevado *et al.* [128] studied other methods of cell death in mixed fermentations with *S. cerevisiae and H. guilliermondii.* They suggest other toxins than the traditional killer toxin as a mechanism of cell death, since the strains used in this study was either killer sensitive (*Saccharomyces*) or killer neutral (*Hanseniaspora*) for the classical killer toxins, K1, K2 and K28. Cultures of *Hanseniaspora* died off in mixed fermentations with *Saccharomyces*, but in pure culture fermentations these *Hanseniaspora* cultures survived even when reaching considerable ethanol levels.

2.5 Conclusion

The wine protein content is dynamic and contributed by various sources throughout the wine making process. The majority of proteins found in wine are from the grape berry, with a smaller contribution from the yeasts present on the grape berry and during the alcoholic fermentation. Other contributors to the wine proteome include lactic acid bacteria, additives such as commercial enzyme preparations and fining agents (albumin and casein). Regardless of their representation in the total amount, each of these proteins impacts on wine properties.

Considering the growing trend in developing and/or selecting new wine yeasts that can improve the quality of wine, the ability of non-*Saccharomyces* yeasts to release proteins and enzymes that are active in winemaking conditions can be used as a criterion for the selection of potential new starter cultures. The selection of the best yeasts to use as starter cultures must be based on the knowledge that these yeasts will have the most beneficial impact on the final product either as pure cultures or in combination with other fermentative yeasts. The best candidates will be those that present the best global activities for the abovementioned characteristics and also survive fermentation.

To date, limited knowledge of the proteins active in fermentations is available despite the vast amount of information on enzyme production by non-*Saccharomyces* yeasts [59,65,66,92]. Until recently, the targeted approaches used to identify and characterize enzymes produced by non-*Saccharomyces* yeasts were limited to whatever activity was sought and did not consider the interactions and influence of different yeasts and their enzymes on each other; in particular, in co-inoculations and spontaneous fermentations. In fact, different interactions of yeast in mixed fermentations have been described, but only with regards to the impact they have on the aroma of wine (as summarized in Table 2.4) [79]. In most cases, the contribution of each of the yeasts cannot be conclusively described.

Therefore, a more holistic approach is needed to understand the contribution of individual yeasts toward the wine proteome and enzyme pool. The impact one yeast can have on the other yeast in fermentation also requires further investigation in order to assess whether their secretomes (both nature and amount of proteins thereof) are influenced by each other and whether they ultimately alter wine properties.

2.6 References

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CHAPTER 3

Research Results

Investigating the secretome of non-*Saccharomyces* yeasts during alcoholic fermentation

Chapter 3 - Investigating the yeast secretome in fermentation

3.1 INTRODUCTION

Over the years, various aspects in which yeasts can contribute to wine quality have been reported in literature from the production of metabolic compounds such as glycerol, ethanol, acetic acid etc. to the more complex contributions such as haze stability and complex aroma profiles [1-6]. The use of yeasts has progressively developed from naturally occurring yeast (so-called spontaneous fermentations) to specific starter cultures of specific strains (most belonging to *Saccharomyces cerevisiae* species) back to the use of non-*Saccharomyces* species, but in a more targeted and controlled way. Non-*Saccharomyces* yeasts used to be regarded as spoilage microorganisms in winemaking, but have recently gained new attention as research indicates that some of these species can be beneficial in winemaking, mostly when used in combination with *S. cerevisiae* [3;7].

These non-*Saccharomyces* yeasts have been shown to produce various oenologically important extracellular enzymes when screened on specific media [7-10]. To understand the implication and involvement of these enzymes during alcoholic fermentation and ageing, it is critical to know which enzymes are secreted and what their characteristics are. However, targeted screening for specific enzymatic activities limits our knowledge of the global contribution of non-*Saccharomyces* yeasts as relevant proteins or enzymes can easily be missed. Moreover, enzymes with specific functions may have other functions of relevance in oenology as seen with glucanases exhibiting killer activity [11]. It is therefore crucial to investigate the full secretome using untargeted approaches.

A few studies have in recent years isolated and characterized proteins secreted by yeasts, mostly those that are of clinical or technological importance, including *Kluyveromyces lactis*, *Pichia pastoris* and *Candida albicans* [12-15]. The yeast secretome is dependent on nitrogen and carbon sources, and small variations in these sources can have an influence on which proteins are secreted and at what levels [12,13]. The exoproteome includes proteins that are secreted through the conventional secretion pathway (ER-Golgi) and also proteins that have a function in the cytosol and that are secreted through unconventional secretion pathways as reviewed by Ding *et al.* [16].

Protein analyses in wine have focused mostly on proteins responsible for haze formation and haze preventions [17-21] and foam stability in sparkling wines [22-25], with very few studies focusing on the yeast proteome and how it may influence wine properties [22,26-29].

The secretome of wine related non-*Saccharomyces* yeasts is poorly characterized and further investigation is required to understand how it may influence the organoleptic and technological properties of wine. In this study, non-*Saccharomyces* yeasts that produce extracellular enzymes under wine making conditions were identified. Isolation and characterization of the proteins released from two non-*Saccharomyces* yeasts during alcoholic fermentation were performed, by visualizing the protein profile on 1D SDS-PAGE and 2D SDS-PAGE and identifying the secreted proteins in the fermentations with mass fingerprint analyses. The goal of this study is to investigate the proteins secreted by non-*Saccharomyces* yeasts during alcoholic fermentation and potentially identify the proteins that are differentially secreted as results of yeast interactions in mixed culture fermentations.

3.2 MATERIALS AND METHODS

3.2.1 Yeast strains and growth conditions

Yeast strains

Various strains of *Metschnikowia pulcherrima*, *Lachancea thermotolerans*, *Cryptococcus flavescens*, *Hanseniaspora uvarum/clermontiae*, *Candida azyma*, *Hanseniaspora vineae*, *Rhodotorula mucilaginosa*, *Hanseniaspora opuntiae/guilliermondii*, *Candida zemplinina*, *Pichia galeiformis/manshurica* and *Issatchenkia orientalis* were screened for specific enzyme activities. *Schwanniomorphus polymorphus* var. *africanus* CBS 8047[1] [30], *Metschnikowia pulcherrima* FOEB L0642[2] [31] and *Saccharomyces paradoxus* RO88 [32] were used as positive controls for the enzyme screenings. *Saccharomyces cerevisiae* VIN13, a commercial wine yeast culture from Anchor Yeast, was used as control during fermentation studies. Strain FOEB L0642 was kindly provided by Prof I. Masneuf-Pomarède (Université Bordeaux Segalen, France) and strain RO88 by Prof S. Redžepović (University of Zagreb, Croatia).

Species confirmation

All species identifications were confirmed by sequencing the 5.8S-ITS rDNA region. The PCR was carried out using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [33]. The PCR reactions were carried out in a total reaction volume of 50 µl with 100 ng of genomic DNA. The final concentrations in the reaction mix were 0.5 µM ITS1 and 0.5 µM ITS4, 1 x reaction buffer, 250 µM dNTPs from TaKara (Separations, Randburg, South Africa), 2.5 mM $MgCl_2$, 1 U Phusion Taq Polymerase

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[1] CBS - Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

[2] FOEB – Faculté d'Œnologie de Bordeaux, Université Bordeaux Segalen, Bordeaux, France.

from Thermo Scientific (Inqaba Biotechnologies, Johannesburg, South Africa). The mixture was subjected to an initial denaturation of 5 min at 94°C, thereafter, 40 cycles consisting of a denaturation of 30 s at 94°C, annealing of 30 s at 51°C, extension of 45 s at 72°C and a final extension of 7 min at 72°C. 5µl of the PCR products were visualized on a 1% agarose gel containing ethidium bromide. The PCR products were sent to the Central Analytical Facility of the University of Stellenbosch (South Africa) for sequencing.

Sequence results were compared against the NCBI nucleotide database using BLAST algorithm and identifications were confirmed when the sequence coverage and maximum percentage of identification was higher than 98% (Query cover > 98%, Max ID % > 98%).

Chemicals

The highest purity grade chemicals were used throughout the experiments. All chemicals were from Sigma-Aldrich (Germany) or Merck (Germany) unless otherwise stated.

Growth conditions

All yeasts were maintained on Wallerstein Laboratory (WL) nutrient agar or Yeast Extract Peptone Dextrose (YPD) media. All cultures were grown aerobically in YPD broth at 30C before enzyme screenings. Pre-cultures for the fermentations were grown in a rich medium progressively increasing the sugar concentration and volume as shown in Table 3.1. Cells were collected for transfer by centrifuging for 5 min at 8000 rpm and dissolving the cell pellet in the fresh medium. On day 6 the cells were harvested, washed and inoculated into synthetic grape juice medium for fermentations.

Table 3.1 - Composition of preculture media used to acclimatise yeasts to high sugar environment, before inoculating into synthetic juice medium for fermentation.				
	% Yeast extract	% Peptone	% Glucose	Final volume
Day 1	1	1	2	10 ml
Day 2	1	1	2	50 ml
Day 3	1	1.3	3	150 ml
Day 4	1	1.5	4	500 ml
Day 5	1	2	5	1000 ml

3.2.2 Fermentations

Fermentations were carried out in triplicate in 600 ml synthetic grape juice medium adapted from Henschke and Jiranek [34] and Bely *et al.* [35] as defined in Table 3.2. The fermentations were carried out at 20C under self-induced anaerobic conditions. Single culture fermentations were inoculated with a final cell density of 2 x 10^7 cfu/ml. Multi-starter fermentations were carried out by inoculating non-*Saccharomyces* together with

S. cerevisiae VIN13 as starters. Non-*Saccharomyces* yeasts were inoculated at 2 x 10^7 cfu/ml, while VIN13 cultures were inoculated 24 h later at 2 x 10^5 cfu/ml. The inoculum ratio of non-*Saccharomyces*/*S. cerevisiae* was thus 100:1.

Table 3.2 - Chemical composition of grape juice like medium. pH was adjusted to 3.5 with 2 M KOH.

Carbon Sources			Trace Elements		
	Glucose	100 g	Manganese chloride	$MnCl_2.4H_2O$	200 µg
	Fructose	100 g	Zinc chloride	$ZnCl_2$	135 µg
Acids			Ferric chloride	$FeCl_2$	30 µg
	KH Tartrate	2.5 g	Cupric chloride	$CuCl_2$	15 µg
	L-Malic acid	3 g	Boric acid	H_3BO_3	5 µg
	Citric acid	0.2 g	Cobalt nitrate	$Co(NO_3)_2.6H_2O$	30 µg
Salts			Sodium molybdate	$NaMoO_4.2H_2O$	25 µg
	K_2HPO_4	1.14 g	Potassium iodate	KIO_3	10 µg
	$MgSO_4.7H_2O$	1.23 g	**Vitamins**		
	$CaCl_2.2H_2O$	0.44 g		Myo-Inositol	100 mg
Nitrogen Sources				Pyridoxine.HCl	2 mg
	NH_4Cl	120 mg		Nicotinic acid	2 mg
Alanine	ALA	100 mg		Ca Pantothenate	1 mg
Arginine	ARG	750 mg		Thiamin.HCl	0.5 mg
Asparagine	ASN	150 mg		PABA.K	0.2 mg
Aspartic acid	ASP	350 mg		Riboflavin	0.2 mg
Glutamine	GLN	200 mg		Biotin	125 µg
Glutamic acid	GLU	500 mg		Folic Acid	0.2 mg
Glycine	GLY	50 mg	**Lipids / oxygen**		
Histidine	HIS	150 mg		Ergosterol	10 mg
Isoleucine	ILE	200 mg		Tween 80	0.5 ml
Leucine	LEU	300 mg			
Lysine	LYS	250 mg			
Methionine	MET	150 mg			
Phenylalanine	PHE	150 mg			
Proline	PRO	500 mg			
Serine	SER	400 mg			
Threonine	THR	350 mg			
Tryptophan	TRP	100 mg			
Tyrosine	TYR	20 mg			
Valine	VAL	200 mg			

Progress of the fermentations was monitored through weighing the flasks daily. Effective end of fermentation was assumed when the weight loss became constant over 3 days (i.e. < 0.5 g weight loss overnight). After 25 days, all fermentations were terminated, regardless of whether the sugars were fully depleted. At day 25, Fourier Transform Infrared (FT-IR)

spectrum readings were collected with the Winescan^{TM} FT120 instrument, software version 2.2, (Foss Analytical, Hillerød, Denmark), to determine the concentrations of major metabolites.

3.2.3 Screening for enzyme activities

For all screenings, the yeast cultures were grown aerobically in 5 ml YPD broth for 12 h and 10 µl of the overnight culture spotted on the selected agar plates.

Protease Activity

To determine acid protease activity, skim milk medium was prepared according to Bilinski *et al.* [36]. Shortly, 70 ml of 0.05 M citrate phosphate buffer at pH 3.5 was prepared by adding 44.2 ml disodium phosphate to 25.8 ml 0.1 M citric acid. Skim milk to a final concentration of 100 g/l was dissolved in the citrate phosphate buffer. Sixty milliliters phosphate buffer was added to the skim milk solution and heated in the microwave, until warm but not boiling. Minimal medium containing 4.8 g glucose and 3.36 g YNB was prepared with 9.6 g bacteriological agar in 480 ml water and after autoclaving was added to the skim milk solution. The pH was adjusted using 6 N hydrochloric acid and plates poured.

M. pulcherrima FOEB L0642 was present as a positive control on each plate [31]. The plates were incubated at 30°C for 5 days and observed for any halo formation. A clear halo surrounding the colony was taken as positive protease activity.

β-Glucosidase Activity

β-Glucosidase activity was determined by spotting the yeast onto a selective medium as described by Strauss *et al.* [7] with some modifications.

The selective medium contained 10 g/l yeast extract, 20 g/L peptone, 5 g/l arbutin and the pH adjusted to 3.5. After autoclaving, 20 ml of a 1% filter sterilized ammonium ferric citrate solution and 20 g/L prepared bacteriological agar was added.

Overnight cultures were spotted on the plates together with the positive control *S. polymorphus* var*. africanus* DSM 8047 and incubated at 30°C for 5 days and then observed for a dark brown halo which indicates that the yeast isolate produces extracellular enzyme [30].

Pectinase Activity

Pectinase activity was determined by plating the yeasts strains onto agarose plates containing 0.5% (w/v) polygalacturonic acid, 0.8% (w/v) Type II Agarose (Sigma-Aldrich, Germany) and 40 mM ammonium acetate (pH 4.0). The colonies were washed off and the plates flooded with 6 M HCl. Observations of a white halo around the colony revealed positive activity. *S. paradoxus* RO88 was used as a positive control [32].

Killer activity

The strains selected for the fermentation study were also screened for killer toxin production and for sensitivity by spotting 5 x 10^3 cells (producer yeast) on sterile white grape juice agar (preservative free white grape juice, 1% yeast extract, 15 g/l bacteriological agar, pH 4.5) seeded with 1 x 10^6 cells/ml of overnight culture (sensitive yeast). Plates were incubated at 20°C for 5 days and killer toxin production by the spotted culture and sensitivity by the seeded culture was observed as a clear halo around the spotted culture.

3.2.4 Haze protection potential

The potential of the yeast to protect wine against the formation of haze during storage was tested using the method described by Pocock and Waters [21]. Shortly, 1 ml cell-free wine samples were supplemented with a concentration range of BSA (0-2 g/l) and 0.5 g/l potassium sulphate. The absorbance (OD) of all samples was measured at 520 nm before incubating samples at 80°C for 2 h and then cooling it at 4°C overnight. Before reading the final absorbance at 520 nm samples were warmed to room temperature. The haze formation potential of each sample was calculated by subtracting the initial OD from the final OD. The haze assay was performed in triplicate and factorial analysis of variance performed in STATISTICA version 10 (StatSoft, Inc. (2011), www.statsoft.com), using a significance level of 5%.

3.2.5 Protein collection

Extracellular proteins were collected by adding cell free samples from the fermentations to ice-cold acetone in an equal volume. Proteins were precipitated from the sample overnight at -20°C and collected by centrifugation for 30 min at 7000 rpm and 4°C. The protein pellet was dried overnight in an open container at -80°C. The dried protein pellet was resuspended in 50 mM citrate phosphate buffer (pH 3.5) and filter sterilized with 0.22 µm PES filters (Stargate Scientific, Johannesburg, South Africa).

Protein samples were further concentrated and desalted by ultra-filtration using Amicon centrifugal filter devices with a 10 kDa pore size (Millipore, Merck, South Africa). Protein concentrations were determined using the Pierce BCA protein assay reagent kit from Thermo Scientific (Separations, Johannesburg, South Africa) using BSA as standard.

3.2.6 Protein analyses

1D Gel electrophoresis

Seventy micrograms of protein was loaded onto a discontinuous sodium dodecyl sulphate bis-acrylamide gel. The SDS-PAGE gel consisted of a 4% polyacrylamide stacking gel (in

125 mM Tris-HCl, pH 6.8, 0.1% SDS) casted over a 12% resolving polyacrylamide gel (in 375 mM Tris-HCl, pH 8.8, 0.1% SDS). Classic Laemmli buffer (4% SDS, 20% Glycerol, 10% 2-mercaptoethanol, 0.004% Bromophenol blue and 0.125 M Tris-HCl, pH approximately 6.8) was used as sample buffer. The electrode chambers were filled with Tris-Glycine buffer, pH 8.3, containing 50 mM Tris, 200 mM glycine, 0.1% SDS. Electrophoresis was conducted at 120 V until the dye front reached the bottom of the gels. Staining was carried out in the microwave with Coomassie brilliant blue R250 in 50% [v/v] ethanol, 10% [v/v] acetic acid and destained with 12.5% [v/v] isopropanol and 12% [v/v] acetic acid according to the protocol described by de Beer *et al.* [37]. SDS-PAGE gel lanes were excised and sent to the proteomics laboratory at the Central Analytical Facility of Stellenbosch University (South Africa) for mass fingerprint analysis.

In gel digestion

Gel lanes were cut into smaller pieces and washed with water followed by 50% (v/v) acetonitrile and 50 mM ammonium bicarbonate. The gel pieces were incubated in acetonitrile until the gel pieces turned white and then dried *in vacuo*. Proteins were reduced with 10 mM dithioerythritol (DTT) followed by a wash step in ammonium bicarbonate, and then acetonitrile before being alkylated by 55 mM iodoacetamide. Following alkylation the gel pieces were washed with ammonium bicarbonate followed by acetonitrile before being dried in vacuo. The gel pieces were digested with 100 µl of 10 ng/µl trypsin solution overnight. The resulting peptides were extracted twice with 70% acetonitrile in 0.1% trifluoroacetic acid and then in 100% acetonitrile and then dried. The dried peptides were dissolved in 5% acetonitrile in 0.1% formic acid.

Mass spectrometry

Peptide analysis was carried out on a Thermo Scientific EASY nLC II connected to a LTQ Orbitrap Velos mass spectrometer. (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray source. A total of 10 µl of trypsin digested sample was injected in a capillary chromatography system. Peptide mixtures were separated on an EASY-column (2 cm, ID 100 µm, 5 µm, C18) pre column followed by XBridge BEH130 Nanoease column (15 cm, ID 75 µm, 3.5 µm, C18) with a constant flow rate of 300 nl/min. Peptides were eluted with a solvent gradient from 5-17% B in 5 min, 17-25% B in 90 min, 25-60% B in 10 min, 60-80% B in 5 min and kept at 80% B for 10 min. Solvent A was 100% water in 0.1% formic acid and solvent B was 100% acetonitrile in 0.1% formic acid.

The mass spectrometer was operated in data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Data were acquired using the Xcaliber software package. The precursor ion scan MS spectra (m/z 400-2000) were acquired in the

Orbitrap with resolution R = 60000 with the number of accumulated ions being 1 x 10^6. The 20 most intense ions were isolated and fragmented in linear ion trap (number of accumulated ions 1.5 x 10^4) using collision induced dissociation. The lock mass option (polydimethylcyclosiloxane; m/z 445.120025) enabled accurate mass measurement in both the MS and MS/MS modes. In data-dependent LC-MS/MS experiments, dynamic exclusion was used with 60 s exclusion duration. Mass spectrometry conditions were 1.8 kV, capillary temperature of 250°C, with no sheath and auxiliary gas flow. The ion selection threshold was 500 counts for MS/MS and an activation Q-value of 0.25 and activation time of 10 ms.

Data analyses

Post run the proteins were identified using Thermo Proteome Discoverer 1.3 (Thermo Scientific, Bremen, Germany); the tandem mass spectra were submitted to the Mascot search algorithm (Matrix science, London, UK) and searched against the NCBI and Swissprot *Saccharomyces cerevisiae,* Uniprot *Clavispora*, *Candida, Yarrowia* and *Lachancea thermotolerans* databases, using a fixed modification of carbamidomethyl cysteine and variable modifications of oxidized methionine, N-acetylation and deamidation. Precursor mass tolerance was set to 10 ppm and fragment mass tolerance to 0.8 Da. Two missed tryptic cleavages were allowed. Proteins were considered positively identified with at least 2 unique tryptic peptides per protein and a Mascot score threshold of 20. Peptide validation was performed with Percolator with a maximum delta Cn of 0.5 and decoy database searches with a FDR of 0.02 and 0.05 with validation based on the q-value.

2D Gel electrophoresis

2D-PAGE was performed as previously reported [38] with minor modifications. Samples containing about 300 µg of protein were solubilised in 2D rehydration buffer (8 M Urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte$^®$ 3/10 ampholyte, 0.002% bromophenol blue; Bio-Rad) and applied onto linear IPG strips (pH 3-10, 17 cm, Bio-Rad,) where it was allowed to rehydrate passively for 16 h. The first dimension was carried out on an IEF Cell (Bio-Rad) at 20°C using the following run parameters: 250 V for 15 min, linearly increasing the voltage to 10000 V during 3 h, focusing was finalized at for a total of 40 kvh. Immobilized pH gradient strips were reduced (2% DTT) and then alkylated (2% iodoacetamide) in equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS). The second dimension was carried out on homogeneous SDS-12% acrylamide gel. Electrophoresis was conducted at 16 mA/gel constant current for 30 min and 24 mA/gel constant current for four and a half hours in a Protean II cell (Bio-Rad). After electrophoresis gels were stained with a silver nitrate protocol adapted from Blum *et al.* [39]. Gels were briefly fixed in 50% methanol, 10% acetic acid solution for 30 min and 5% methanol for 15 min. Thereafter, they were rinsed 3 times in

mQ H_2O (Millipore, Billerica, MA), each rinse 5 min, before sensitizing in 0.2 g/l sodium thiosulphate solution for 2 min. The excess sodium thiosulphate was again rinsed off with mQ H_2O and the gels then incubated in cold 2% silver nitrate solution for 10 min. Gels were developed in developer solution (3% (w/v) sodium carbonate, 0.002% (w/v) sodium thiosulphate and 0.0185% (v/v) formaldehyde) for 10 min, and the developing reaction stopped by incubating gels in di-sodium ethylene diamine tetra-acetic acid (14 g/l, Na_2-EDTA). All gels were stored in mQ H_2O until imaging could be done. Gel images were obtained using the Molecular Imager® PharosFX™ system (Bio-Rad).

3.3 Results and Discussion

3.3.1 Enzyme activity screen

Previously isolated strains (Jooste LS and Divol B, unpublished data) were screened on substrate specific agar plates for extracellular enzyme activities and identities confirmed by sequencing the 5.8S-ITS rDNA region. Enzyme activities observed are summarized in Table 3.3. Only one strain showed pectinase activity and this is in agreement with previously published studies, reporting that this activity occurs rarely in non-*Saccharomyces* yeasts [7,40]. Protease activity was more abundant in yeasts while extracellular β-glucosidase activity was only observed for a limited number of yeasts and mainly for strains of the *Metschnikowia* and *Hanseniaspora* genera.

Table 3.3 - Results of extracellular enzyme screening on substrate specific agar plates at pH 3.5 of various isolated yeast species. The strains in bold font were used for further experiments.				
IWBT #	Possibilities	β-Glucosidase	Pectinase	Protease
1002	*Cryptococcus flavescens*	+	-	-
1005	*Metschnikowia pulcherrima*	+	-	+
1013	*Hanseniaspora uvarum/clermontiae*	+	-	+
1014	*Candida azyma*	++	-	+
1017	*Lachancea thermotolerans*	-	-	-
1021	*Hanseniaspora vineae*	+	-	-
1027	*Rhodotorula mucilaginosa*	+	-	+
1028	*Saccharomyces cerevisiae/paradoxus*	-	+	-
1034	*Hanseniaspora vineae*	+	-	-
1035	*Hanseniaspora opuntiae/guilliermondii*	+	-	+
1038	*Lachancea thermotolerans*	-	-	-
1039	*Hanseniaspora opuntiae/guilliermondii*	+	-	+
1048	*Hanseniaspora uvarum/clermontiae*	+	-	-

1065	*Metschnikowia pulcherrima*	+	-	+
1072	***Metschnikowia pulcherrima***	**+**	**-**	**+**
1074	*Rhodotorula mucilaginosa*	+	-	+
1102	*Metschnikowia pulcherrima*	+	-	++
1106	*Candida zemplinina*	-	-	-
1107	*Metschnikowia pulcherrima*	+	-	++
1108	*Metschnikowia pulcherrima*	+	-	++
1111	*Candida zemplinina*	-	-	-
1112	*Metschnikowia pulcherrima*	+	-	++
1113	*Metschnikowia pulcherrima*	+	-	++
1114	*Metschnikowia pulcherrima*	+	-	++
1115	*Metschnikowia pulcherrima*	+	-	+
1120	*Metschnikowia pulcherrima*	+	-	+
1123	*Metschnikowia pulcherrima*	+	-	++
1124	*Metschnikowia pulcherrima*	+	-	+
1125	*Metschnikowia pulcherrima*	+	-	+
1129	*Pichia galeiformis/manshurica*	-	-	+
1132	*Candida azyma*	-	-	+
1207	*Metschnikowia pulcherrima*	-	-	+
1211	*Candida zemplinina*	-	-	-
1213	*Metschnikowia pulcherrima*	-	-	++
1216	*Candida zemplinina*	-	-	-
1217	*Metschnikowia pulcherrima*	-	-	++
1228	*Issatchenkia orientalis*	-	-	+
1240	*Lachancea thermotolerans*	-	-	-
1256	*Pichia manshurica*	-	-	-
1270	*Metschnikowia pulcherrima*	-	-	+
1295	*Lachancea thermotolerans*	+	-	-
1326	***Lachancea thermotolerans***	**+**	**-**	**-**

+ Growth was observed; ++ A halo around the spot was observed; - No activity observed.

It can also be seen in Table 3.3 that enzyme activity is strain dependent and this also confirms what has been reported in literature [7,30].

One *Metschnikowia pulcherrima* strain and one *Lachancea thermotolerans* strain (highlighted in Table 3.3), was selected for further fermentations and analyses. The two strains selected showed some enzyme activities and are representative from each species. These two species have previously been reported to benefit wine aroma and quality when used in fermentations [41,42]. *M. pulcherrima* is a poor fermenter, while *L. thermotolerans* is known to be a strong fermenter. Fermentation capacity of yeast species has been shown to

be strain dependent, with strains from the same species fermenting at different rates and resulting in different organoleptic characteristics in the final product [41]. *L. thermotolerans* was also selected as the proteome of this species has been fully characterized.

The two strains selected for fermentations together with *S. cerevisiae* VIN13 were also screened for the production of or sensitivity towards killer toxins. None of the strains showed any sensitivity towards killer toxins from the other strains on the assay plates (data not shown).

3.3.2 Fermentations

Fermentations were carried out in synthetic grape juice medium using individual cultures of *S. cerevisiae* (Sc), *M. pulcherrima* (Mp), *L. thermotolerans* (Lt) and mixed cultures of *S. cerevisiae* with *M. pulcherrima* (MpSc) and *S. cerevisiae* with *L. thermotolerans* (LtSc). The progress of these fermentations is reported in Fig. 3.1. Fermentations started within 1 to 4 days respectively for Sc and MpSc. Mp cultures fermented very slowly as was evident from the daily weight loss and it did not ferment to dryness (defined as less than 5 g/L sugar in wine). This coincides with previous results reported in literature that certain species of non-*Saccharomyces* and in particular *M. pulcherrima* are not strong fermenters [41,43].

As can be seen in Fig. 3.1, MpSc fermentations did not ferment to dryness either and fermented slightly slower than the pure Sc. A delay in the onset of the MpSc fermentation is observed. The MpSc overall fermentation kinetics is assumed to be a result of *S. cerevisiae* that drives the fermentation as *M. pulcherrima* has been reported to be a weak fermenter [41]. Although the fermentation curve shows a greater accumulated weight loss for MpSc than for Sc in the final stages of fermentation, the residual sugar in MpSc, at day 25, was still more than that of Sc as can be seen in Table 3.4. The difference in accumulated weight loss at the end of fermentation for these two fermentations is not significant as seen with the large error bars and might simply be due to technical error. MpSc has the pattern of Mp for the first 4 days of fermentation and then that of Sc, suggesting that the fermentation is driven by Mp for the first days and that Sc then takes over, probably due to a decline in the number of Mp present and metabolically active in the fermentation. The fermentation kinetics observed correlate well with a similar study of Comitini *et al.* [44], observing a decline in the population of *M. pulcherrima* after 3 days in a mixed culture fermentation with *S. cerevisiae.*

No significant difference could be determined in the fermentation kinetics of the Lt and LtSc fermentations. The mixed culture fermentation (LtSc) had the same fermentation kinetics as the pure Lt until day 15, where after it seemed to accelerate and followed the trend of the Sc kinetics. This might again suggest a decline in non-*Saccharomyces* population, although much later and not as definite as in the case of MpSc. Comitini *et al.*

[44] and Gobbi *et al.* [42] reported similar trends when studying *L. thermotolerans* in mixed starter culture fermentations.

From this data, a greater contribution from Lt towards the proteome of the LtSc fermentation would be expected compared to the contribution of Mp to the MpSc fermentation. Quantitative analyses of proteins present in mixed culture fermentations will nevertheless be required to understand the contribution of the different yeasts to the proteome of wine.
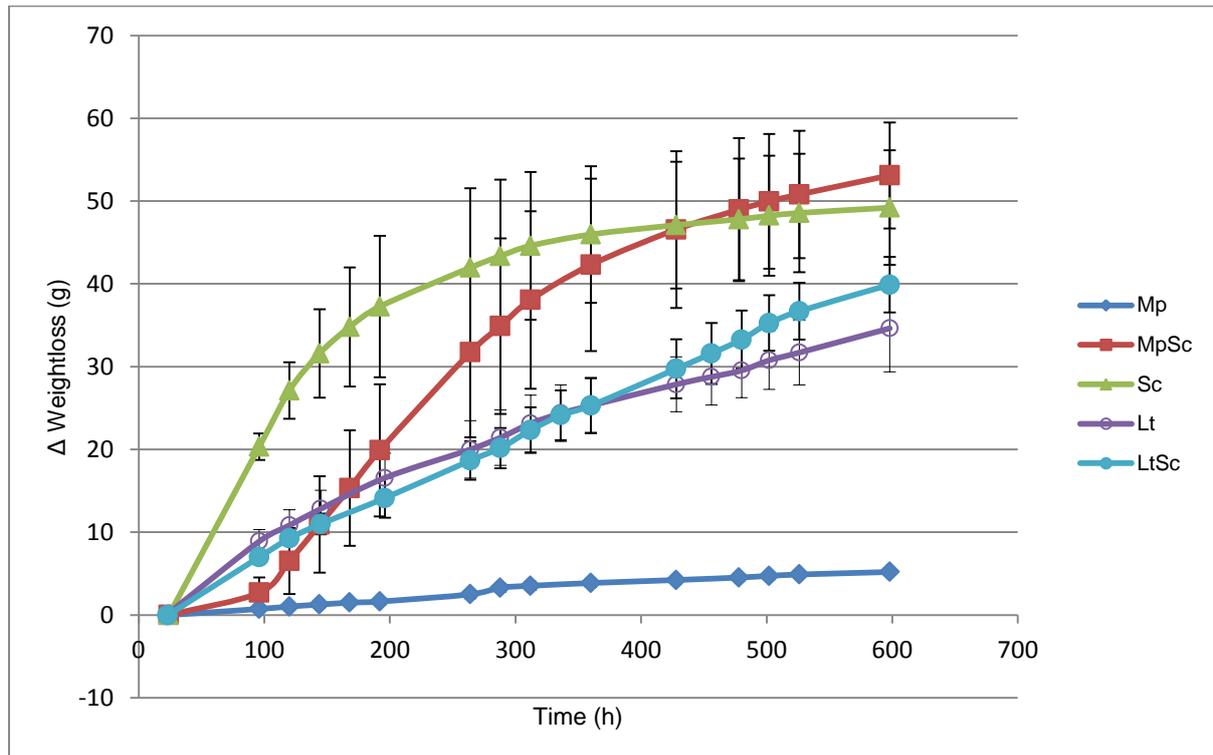

Fig. 3.1 - Curve of accumulated weight loss over time for the first 600 hours after inoculation (25 days). Mp – *Metschnikowia pulcherrima;* **MpSc –** *Metschnikowia pulcherrima + Saccharomyces cerevisiae;* **Sc –** *Saccharomyces cerevisiae;* **Lt –** *Lachancea thermotolerans;* **LtSc -** *Lachancea thermotolerans + Saccharomyces cerevisiae.*

Chemical analyses of the medium after 25 days are displayed in Table 3.4. After 25 days, Sc reached sugar concentrations of below 8 g/l and little weight loss could be observed between successive days; this corresponds to the end of the alcoholic fermentation. At this point, Mp had only fermented about 25 g/l sugar resulting in 1% (v/v) ethanol. MpSc fermented the most sugar among the mixed fermentations, with only 21 g/l left after 25 days and an ethanol content of 10.7% (v/v). This is comparable to the results published by Zohre and Erten [43], showing similar compositions for pure *S. cerevisiae* and mixed culture (*S. cerevisiae* and *M. pulcherrima*) fermentations in grape juice. In 25 days, Lt had fermented 130 g/l sugar resulting in 7.2% ethanol. The residual sugar left in the LtSc fermentation was 51.4 g/l and the ethanol content 8.9% (v/v). This is probably due to the presence of *S. cerevisiae*. Nevertheless, the presence of *S. cerevisiae* slowed the

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fermentation, initially. As can be seen in Table 3.4, the pure non-S*accharomyces* and the mixed fermentations resulted in lower volatile acidity but no significant difference was observed for total acidity.

Table 3.4 - FT-IR results for major metabolites after 25 days in fermentations. All compounds measured in g/l, except ethanol which was measured in % (v/v).									
		pH	Volatile Acid	Total Acid	Malic Acid	Glucose	Fructose	EtOH	Glycerol
Lt	Avg	3.25	0.4	4.5	3.5	32.8	45.0	7.2	9.0
	StDev	0.04	0.0	0.0	0.1	6.3	6.1	0.8	0.8
LtSc	Avg	3.14	0.4	4.6	3.3	18.6	32.8	8.8	7.4
	StDev	0.03	0.0	0.0	0.1	4.3	5.4	0.6	0.5
Mp	Avg	3.58	0.5	4.6	4.1	96.9	95.9	1.0	17.6
	StDev	0.08	0.0	0.2	0.3	7.7	8.0	0.1	1.7
MpSc	Avg	3.04	0.5	4.4	2.6	5.8	15.4	10.7	6.3
	StDev	0.04	0.0	0.2	0.3	5.2	11.7	0.9	0.9
Sc	Avg	3.05	0.6	4.3	2.5	2.8	5.0	11.9	5.7
	StDev	0.03	0.0	0.1	0.1	2.7	2.5	0.6	0.4

Avg – average values for triplicates; StDev – Standard deviation between triplicates; Mp – *Metschnikowia pulcherrima;* MpSc – *Metschnikowia pulcherrima + Saccharomyces cerevisiae;* Sc – *Saccharomyces cerevisiae;* Lt – *Lachancea thermotolerans;* LtSc - *Lachancea thermotolerans + Saccharomyces cerevisiae.*

3.3.3 Isolation and analyses of proteins from extracellular medium

Proteins isolated from extracellular medium were quantified as reported in Table 3.5. The concentration of the proteins harvested from the different fermentation varied greatly, even between biological replicates. This might be due to the crude method of harvesting proteins, as some proteins might be lost in the denatured fraction due to denaturation of proteins or binding thereof to functional proteins. The sensitivity and specificity of the BCA protein determination kit might be a possible other explanation, as the presence of sugars and other macromolecules that are isolated together with the proteins can influence the colour development and thus the determined concentration. As can be seen in Table 3.5, the standard deviations between replicates are more than 15% of the average concentrations.

Table 3.5 - Concentrations of proteins (µg/ml) harvested from various fermentations as determined with the Pierce BCA protein assay reagent kit using BSA as standard.					
	Mp	MpSc	Sc	Lt	LtSc
Avg	8846	5917	8795	5746	3939
Stdev	3836	3246	3194	2722	603

Avg – average values for triplicates; StDev – Standard deviation between triplicates; Mp – *Metschnikowia pulcherrima;* MpSc – *Metschnikowia pulcherrima + Saccharomyces cerevisiae;* Sc – *Saccharomyces cerevisiae;* Lt – *Lachancea thermotolerans;* LtSc - *Lachancea thermotolerans + Saccharomyces cerevisiae.*

The proteins were visualized on a one dimensional SDS-PAGE gel and some differences between the different fermentations could be observed (Fig. 3.2). The Mp sample had a clear band between 15 and 25 kDa that was not observed for any of the other samples (although a smear in this region is visible for the MpSc sample) and also more bands around the 35 kDa weight. The MpSc and LtSc protein profiles looked similar to that of Sc even though some of the bands from the Mp and Lt fermentations, respectively, could also be seen (indicated by the black arrows in Fig. 3.2(a) and (b)), suggesting that both yeasts contributed to the secretome of MpSc and LtSc, despite a stronger contribution of *S. cerevisiae.* A stronger contribution from *S. cerevisiae* was nevertheless expected in the mixed culture samples due to the possible early decline (after day 4) of Mp and the later (day 15) decline of Lt in the mixed culture fermentations. Protein bands in the mixed culture samples, indicated by black arrows in Fig. 3.2(a) and (b) are possibly contribution from the non-*Saccharomyces* yeasts in these fermentations. Some proteins visible in the pure culture fermentation samples completely disappear in the mixed culture fermentation, indicating that there are some interactions between the yeasts present in the mixed culture fermentation.
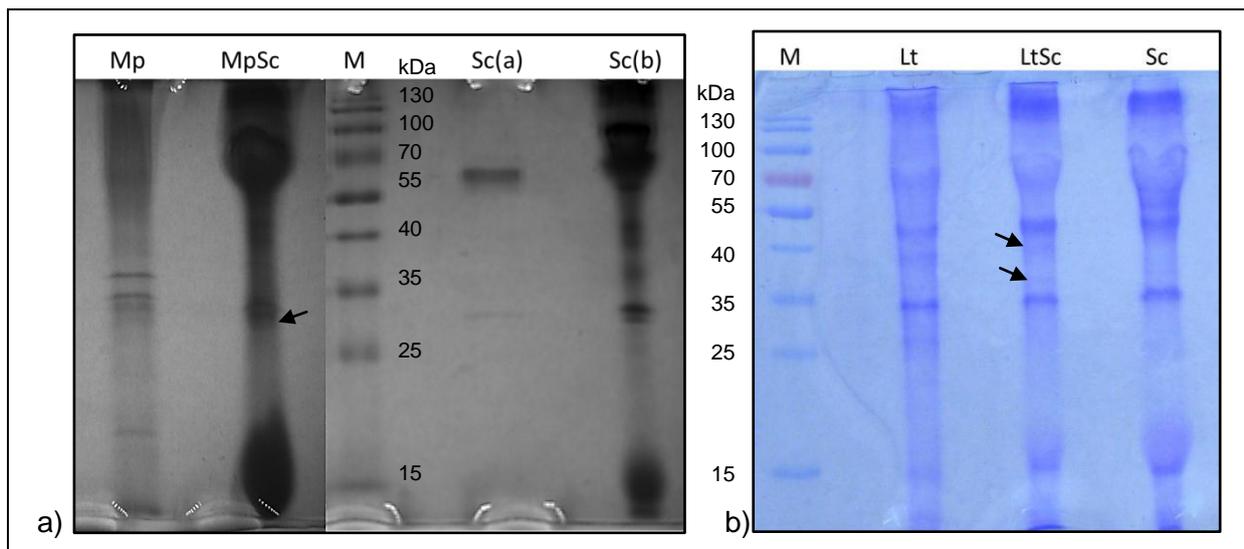

Fig. 3.2 - 1D SDS-PAGE of proteins collected from fermentations. A) Proteins from pure *Metschnikowia pulcherrima* (Mp)*,* pure *Saccharomyces cerevisiae* (Sc) and the mixed culture (MpSc) fermentations stained with silver nitrate. B) 1D SDS-PAGE gel image of proteins isolated from the pure *Lachancea thermotolerans* (Lt), pure *Saccharomyces cerevisiae* (Sc) and mixed culture (LtSc) fermentations stained with Coomassie blue. M refers to the molecular weight marker PageRuler pre-stained protein ladder (Thermo). Sc(a) indicates diluted sample of Sc(b), while arrows indicate protein bands that were possibly from the non-*Saccharomyces* in the fermentation.

Entire lanes were excised and proteins identified by MS/MS as described in the materials and methods.

To confirm the presence of proteins from both yeasts in the mixed culture fermentations and to better visualize the differences between samples, the secretome was analysed using 2D SDS-PAGE.

2D SDS-PAGE

Protein profiles were compared by 2D SDS-PAGE, where the proteins were separated in the first dimension on 17cm ReadyStrip ® IPG strips (Bio-Rad), the second dimension separation according to molecular weight was in 12% acrylamide/bis-acrylamide. When comparing the images, differences between fermentation were clear. Most proteins isolated from the extracellular medium were visible on the 2D gels in the vicinity of pI 3.5 to pI 5 over the whole range of molecular weights. This is in accordance with literature reporting soluble proteins in wine to have a low pI between 4.1 and 5.8 and low molecular weight (20 – 30 kDa) [45,46]. Although the origin of the proteins in these studies is undefined, they are most probably yeast and grape berry proteins that contribute to these fractions.

The contribution of different yeasts to the secretome of mixed culture fermentations can be seen when comparing Fig 3.3 (a), (b), (e) and (c), (d), (e); a limited number of proteins can be observed for the Mp (a) fermentation and the MpSc (b) shows a higher number of proteins which is clearly a combination of protein spots from (a) and the pure Sc (e), confirming the stronger contribution of *S. cerevisiae* in MpSc due to the early decline of *M. pulcherrima*. Although the image quality for (e) is not optimal, proteins seen in the pI 9 – 10 region are not all visible in the same region in the Mp (a) image. Proteins visible in the 35 kDa region on image (a) are also visible on image (b), but not all these are visible on image (c). This might be due to poor separation across the pI range in (e).

The number of proteins visible in (d) is greater than either (c) or (e) even though proteins from both (c) and (e) can be seen in (d). Although staining differences might play a role in the differences observed, it clearly shows that Lt (c) contributes a greater number of proteins to LtSc (d) than Mp (a) contributes to MpSc (b). This was expected as Mp declines quicker in the mixed culture fermentation MpSc, as can be seen in Fig. 3.1, while Lt is present for longer during the LtSc fermentation.

c)

d)
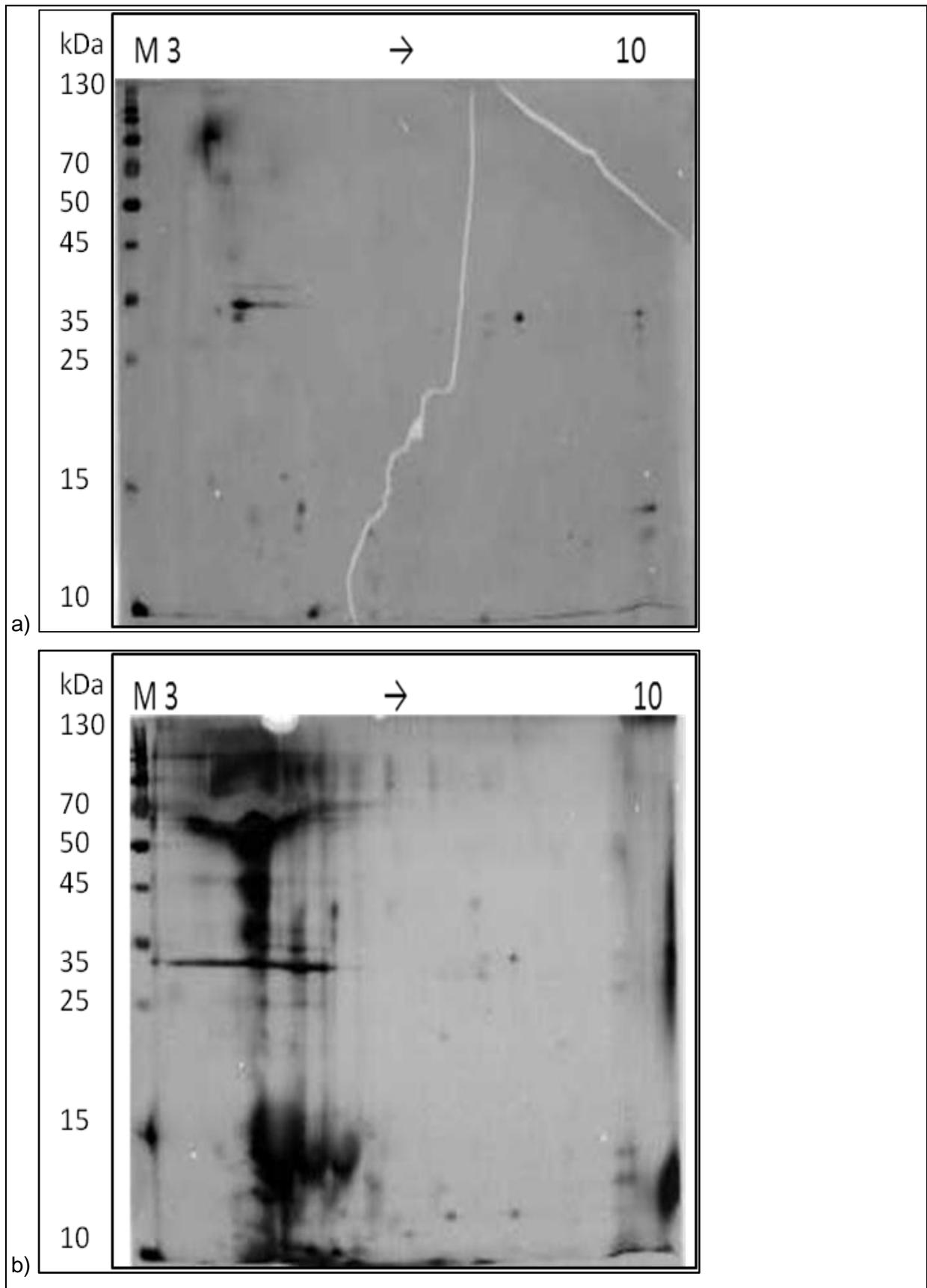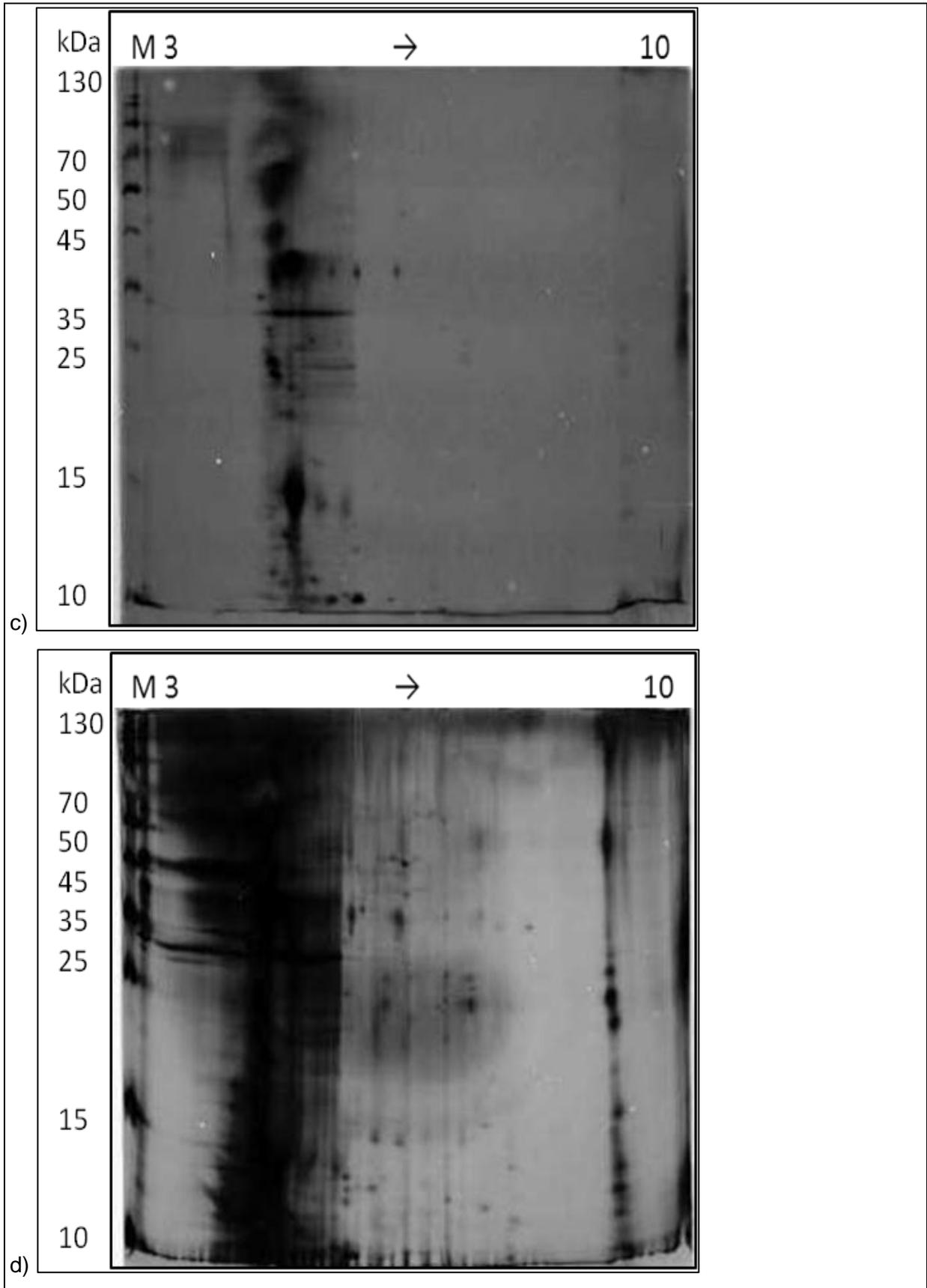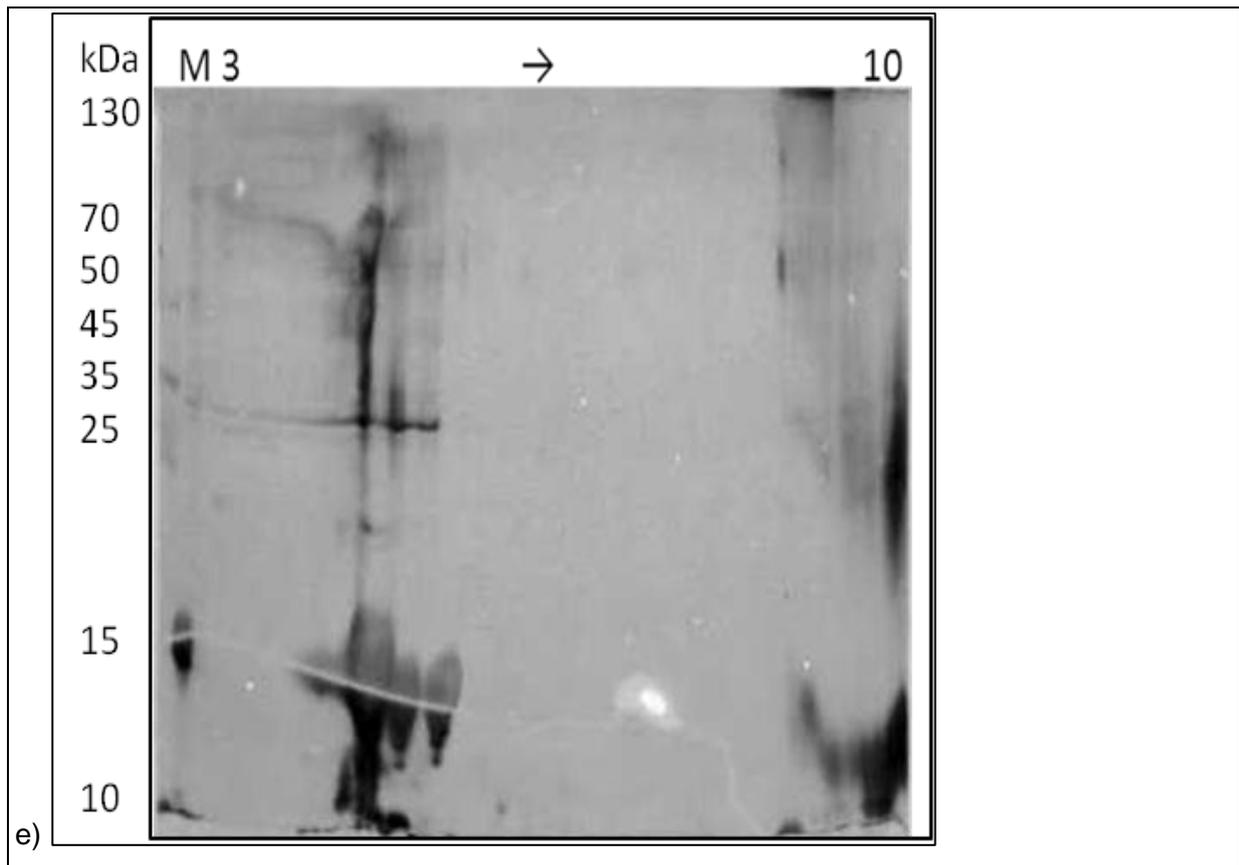
Fig. 3.3 - 2D SDS-PAGE images of proteins collected from the extracellular medium of fermentations different non-*Saccharomyces* yeast and *Saccharomyces cerevisiae.* A) Proteins from pure *Metschnikowia pulcherrima* [Mp]*,* b) Proteins from the mixed culture (*Metschnikowia pulcherrima* and *Saccharomyces cerevisiae)* [MpSc] fermentation, c) *Lachancea thermotolerans* [Lt] extracellular proteins, d) proteins from the mixed culture fermentation (*Lachancea thermotolerans* and *Saccharomyces cerevisiae)* [LtSc], e) *Saccharomyces cerevisiae* pure fermentation [Sc]. PageRuler pre-stained protein ladder was loaded on the left hand side of each gel (M) and 3/10 indicate the orientation of the IPG strip.

MS/MS analyses

Proteins were identified by mass spectrometry as described. Briefly, entire gel lanes were excised from the 1D SDS-PAGE gels and all embedded proteins were digested with trypsin in the gel. The resulting peptides were then extracted from the gel pieces. Proteins were identified by Thermo Proteome Discoverer 1.3 (Thermo Scientific, Bremen, Germany) by searching the tandem mass spectra in the Mascot search algorithm (Matrix science, London UK) against the NCBI and Uniprot databases. Only proteins identified by 2 or more unique tryptic peptides were used for further analyses. Sc peptide mass spectra were searched against NCBI *Saccharomyces cerevisiae* and Lt against Uniprot *Lachancea thermotolerans.* Mp mass spectra was searched against *Uniprot Clavispora, Candida, Yarrowia* as the proteome of *M. pulcherrima* has not yet been fully annotated and closely related yeast species, based on the phylogeny of partial LSU (26S) DNA sequences are *Clavispora*

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lusitaniae and *Yarrowia lypolytica* [47]. Species of the genus *Candida* were included as *M. pulcherrima* is the teleomorph of *Candida pulcherrima*.
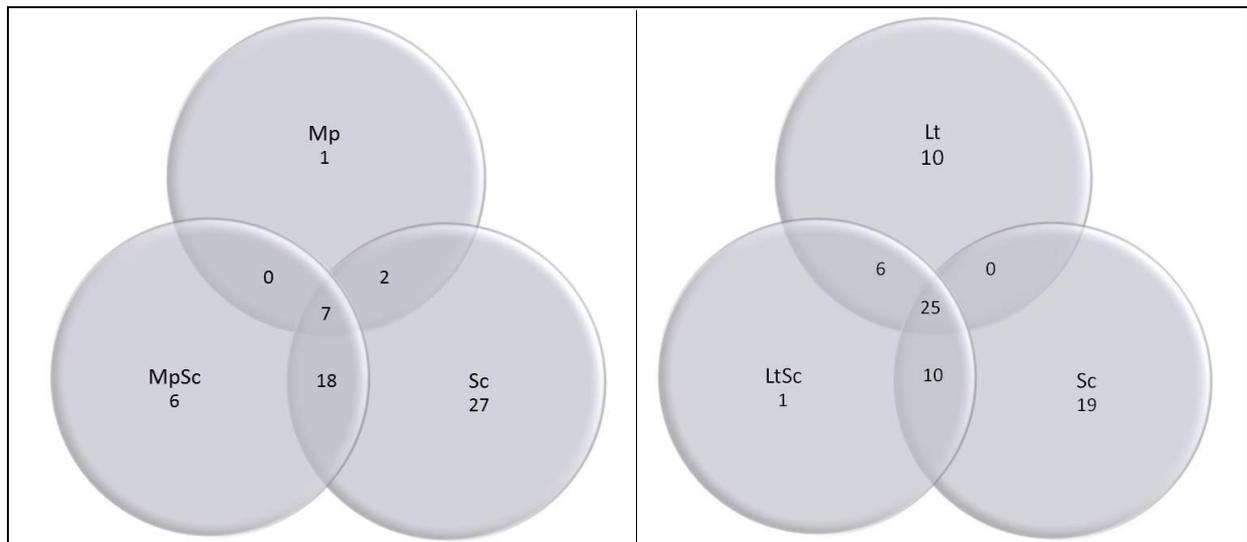
Fig. 3.4 - Venn diagrams showing the number of proteins identified in every one of the fermentations as well as the number of identified proteins that are shared between fermentations. Mp – *Metschnikowia pulcherrima;* **MpSc –** *Metschnikowia pulcherrima + Saccharomyces cerevisiae;* **Sc –** *Saccharomyces cerevisiae;* **Lt –** *Lachancea thermotolerans;* **LtSc -** *Lachancea thermotolerans + Saccharomyces cerevisiae.*

The number of proteins identified differs greatly between fermentations as can be seen in Fig. 3.4 and Table 3.6; this is in correlation with what was previously reported [12]. Table 3.6 summarizes the proteins identified in fermentations. In the pure culture fermentations, "X" indicating the fermentation in which a specific protein or homologue thereof was identified. In the mixed fermentations, LtSc and MpSc, the proteins possibly contributed by the different yeasts are indicated by "L" for proteins from *L. thermotolerans*, "M" for proteins from *M. pulcherrima* and "S" for proteins secreted by *S. cerevisiae.* Where "LS" and "MS" are indicated, homologous proteins from both species were identified.

M. pulcherrima seems to secrete far fewer proteins than either *S. cerevisiae* or *L. thermotolerans* in pure culture fermentations, with only 10 proteins identified in comparison to the 54 and 41 for the other two species respectively. This is possibly due to the lack of biomass formed during fermentation as the Mp fermentation did not progress beyond 10 g/l of sugar as can be seen in Fig. 3.1 and/or the limited number of proteins identified can be due to poor identification because of the attempt to use the proteomes of other species for identification, as more than 10 proteins can be seen on the 2D gel image (Fig. 3.3 (a)). Overall the proteins secreted by Sc, Mp and Lt and their functions are similar, with the majority of proteins identified in all three fermentations involved in cell wall biogenesis.

A total of 61 unique proteins (Table 3.6) were identified in the extracellular medium of Mp*,* Sc and MpSc, of which only 7 homologous proteins were identified in all three wines. These proteins are Crh1p, Ygp1p, Ccw14p, Exg1p, Ecm33p, Gas1p and Bgl2p all involved

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in cell wall biogenesis/degradation as glycosidases, hydrolases or transferases. In MpSc, 31 proteins were identified of which 18 were also identified in the pure Sc fermentation and 6 were only identified in the mixed culture fermentation, namely Eno1p, Tal1p, Tdh3p, Egt2p, a K2 killer toxin precursor and a phospholipase that is similar to YMR006Cp. The previous assumption (from alcoholic fermentation kinetics, 1D and 2D gels) of a greater contribution from Sc towards the proteome of MpSc is confirmed with these numbers. Mp and Sc have 2 proteins in common that were not identified in the mixed culture fermentation. The 2 proteins are Pst1p and Gas5p, a cell wall mannoprotein and a 1,3-β–glucanosyltransferase, respectively. A single protein that was identified only in the pure Mp fermentation could not be named as no homologue was found in any annotated proteome. The protein was identified as a putative uncharacterised protein from *C. lusitaniae* a closely related species of *M. pulcherrima*.

A total number of 71 proteins were identified in the Lt*,* Sc and LtSc fermentations of which 25 are present in all 3 wines and 1 is unique to the LtSc fermentation. This protein was identified as a homologue of ScAcb1p, an acetyl-coenzyme A binding protein*.* Of the 71 identified proteins, another 6 were identified in the pure Lt and LtSc fermentations and 10 were identified in the LtSc and pure Sc. Ten proteins were identified only in the Lt fermentation, while in the pure Sc fermentation 19 unique proteins were identified. The latter were not identified in either the pure Lt or LtSc fermentations.

When comparing the proteins identified across all five fermentations, 4 proteins were identified in all wines and originated from all yeasts: Ecm33p, Gas1p, Bgl2p and Exg1p. The presence of these proteins in all wines indicates their critical role in cell wall biogenesis, as they are secreted by all yeast and this is not influenced by interactions of yeasts on one another. Of these 4 proteins, three were in the core secretome suggested by Buerth *et al.* [12] namely, the glucanases Bgl2p, Exg1p (isoform of Exg2p) and transglucosylase Gas1p. The other proteins in the suggested secretome of Buerth *et al.* [12] were only identified in some of the fermentations: Scw11p and Pry2p only in Lt and an isoform Pry3p in Sc, Cts1p in Lt, LtSc and Sc, Exg2p only in Sc, Scw4p, Tos1p and Uth1p in all but Mp (maybe because the *M. pulcherrima* proteome is not known and peptide homology was not close enough to the reference proteomes used), ScTdh3p was identified only in MpSc, and an isoform Tdh1p was identified in Sc. Tdh3p is found in exponentially growing cells whereas the presence of Tdh1p found in the cell wall proteome of cells in the stationary phase suggested that it might be involved in other processes than glycolysis [48]. Pry1p was not identified in any of our fermentations although we identified two of its isoforms, Pry2p and Pry3p. The possibility exist that all these proteins could have been present in all fermentations but as the identification of proteins was accepted with two or more unique peptide they were maybe not identified due to low abundance.

A number of *S. cerevisiae* proteins identified in Sc or LtSc but not Lt were BLAST searched to see if the *L. thermotolerans* proteome contains homologues. No homologues were identified for among others Exg2p, Scw10p, Spr1p and Vel1p, although *L. thermotolerans* possess other isoforms of these proteins, clarifying why they could not be found in the Lt and LtSc fermentations.

In Table 3.6, it is seen that several cell wall proteins are found in the wine. These were expected as protein 'shaving' is a natural process that occurs due to cell wall reorganization, and most of these cell wall proteins are involved in carbohydrate metabolism and cell wall biogenesis or degradation. Several intracellular proteins were also identified. This phenomenon may be attributed to autophagy or autolysis of yeast cells during alcoholic fermentation. Although we expected intracellular proteins (as reported previously in literature) the number of intracellular proteins was slightly unexpected. Intracellular proteins identified in this study are mostly related to glycolysis, and a few with other functions were also identified (amino acid metabolism, electron transport and proteolysis). Autolysis might be responsible for some of the intracellular proteins identified, especially those involved in proteolysis. Another unexpected protein that was identified only in the MpSc fermentation is the killer toxin precursor for K2 killer toxin from *S. cerevisiae.* The activity screen on agar plates showed no sensitivity of *M. pulcherrima* towards the killer toxin produced by *S. cerevisiae,* although this might only be on agar plates and the cells might behave differently in liquid medium. Killer toxins produced by *S. cerevisiae* could be one of the reasons contributing to a decline in the population of *M. pulcherrima* and the delayed start of alcoholic fermentation in the MpSc fermentation. The occurrence of the *S. cerevisiae* killer toxin precursor in the MpSc fermentation and not in the LtSc fermentation suggests biological competition between the *M. pulcherrima* and *S. cerevisiae* that is not present between *L. thermotolerans* and *S. cerevisiae.* The presence of killer toxins in the MpSc fermentation could explain the fermentation kinetics seen in Fig. 3.1, while the prolonged presence of Lt in the LtSc fermentation is in accordance with other studies also reporting the late decline of the Lt population in LtSc mixed fermentations [42]. The killer toxins of *S. cerevisiae* have a limited anti-yeast spectrum, mostly against other *S. cerevisiae* and a few *Candida* species [49-51] (including the anamorphic form of *M. pulcherrima*), due to a receptor-mediated process by interacting with receptors, R1 in mannoprotein or β-1,6-glucan in the cell wall of sensitive strains [11,51,52]. Hidalgo and Flores [51] showed that non-*Saccharomyces* yeasts isolated from must and wine are sensitive to killer toxins from various yeast species. Further studies (e.g. liquid assays) are needed to investigate the potential impact of K2 on *M. pulcherrima* during fermentations.

The presence of Bgl2p and other β-glucosidases (Utr2p, Dse4p, Crh1p, Exg1p and Exg2p to name a few) might be responsible for the glucosidase activity observed and

reported in Table 3.3 for the isolates selected. Although a greater variety of glycosidases were identified in Sc, the non-*Saccharomyces* glycosidases might show greater substrate specificity towards arbutin. This would explain why β-glucosidase activity was observed for the non-*Saccharomyces* yeasts but not for *S. cerevisiae* on the screening plates. Further investigations are however required to support this hypothesis. Alignment of the homologous proteins showed minor changes in the amino acid sequences, which might be connected to the substrate specificity of these proteins; heterologous expression of the various non-*Saccharomyces* genes will need to be performed to confirm the hypothesis. Glucosidase activity in non-*Saccharomyces* yeasts might also be due to the activity of an unidentified or uncharacterised protein. Apart from glucosidases, very few other proteins of oenological interest have been identified, with invertase (Suc2p) being the only one that has been described in literature to have an influence on wine quality. Although the *M. pulcherrima* strain used in this study has previously been reported to possess the Mp*APR*1 gene encoding an acid protease, the protease was not identified in either the Mp or MpSc fermentations although it was specifically sought [53]. This might be due to the low abundance of *M. pulcherrima* proteins in fermentations as seen in Fig. 3.4 or the fact that *M. pulcherrima* does not secrete this protein when fermenting the medium used in this study.

The identification of proteins that are only present in the mixed culture fermentations, MpSc and LtSc, suggests that the presence of a second organism alters the secretome of a specific species in the fermentation although the proteins identified (Tal1p, Acb1p, YMR006Cp, Egt2p and a K2 killer toxin precursor protein) could not be grouped together according to a specific biological process. Furthermore, proteins of *L. thermotolerans,* involved in glycolysis as well as Adh1p and Pdc1p, involved in the production of ethanol from pyruvate were detected in the Lt and LtSc fermentations but no homologous proteins of *S. cerevisiae* were found in LtSc. This strongly suggests that the presence of *L. thermotolerans* in the fermentation directly impacts on *S. cerevisiae* and its primary metabolic activity i.e. sugar metabolism and alcoholic fermentation. This would explain the sluggish fermentation kinetics observed in LtSc compared to Sc. Further investigation is required to confirm an inhibition of glycolysis, possibly due to the down regulation of the genes encoding glycolysis-related enzymes.

Changes in the secretome in the presence of another micro-organism might be linked to the availability of nutrients, as changes in carbon and nitrogen availability we know to have an influence on the secretion of some proteins [12,54].

Table 3.6 - Identified proteins, from all fermentation sets, grouped according to the biological process in which they are involved.									
Accession	Protein[a]	Cell compartment	Molecular function	Description	Sc*	LtSc*	Lt*	MpSc*	Mp*
Amino Acid Metabolism									
P05694[1]; C5DE81[2]	Met6	Cytoplasm; Plasma membrane	Transferase	[1]5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase OS=*Saccharomyces cerevisiae*; [2]KLTH0C07018p OS=*Lachancea thermotolerans*	X(2)	L(2)			
P06169[1]; C5DC94[2]	Pdc1	Cytoplasm; nucleus	Decarboxylase; Lyase	[1]Pyruvate decarboxylase isozyme 1 OS=*Saccharomyces cerevisiae*; [2]KLTH0B01188p OS=*Lachancea thermotolerans*	X(8)	L(7)	X(5)		
Carbohydrate Metabolism									
C5DGT1	Dcw1	Plasma membrane	Catalytic activity; Mannosidase activity	KLTH0D08008p OS=*Lachancea thermotolerans*				X(2)	
P29029[1]; C5DN90[2]	Cts1	Cell wall; Secreted	Glycosidase; Hydrolase; Endochitinase	[1]Endochitinase OS=*Saccharomyces cerevisiae*; [2]KLTH0G15070p OS=*Lachancea thermotolerans*	X(2)	L(2)	X(3)		
259145860; C8Z6V4	Utr2	Cell wall	Hydrolase; transferase	Probable glycosidase CRH2 OS=*Saccharomyces cerevisiae*	X(3)				
C5DHU8	Scw11	Cell wall	Catalytic activity; Cation binding	KLTH0E07282p OS=*Lachancea thermotolerans*				X(2)	
P53753[1]; C5DDY9[2]; 323335774[1]; 323303170[1]	Dse4	Cell wall; Secreted	Glycosidase; Hydrolase	[1]Endo-1,3(4)-beta-glucanase 1 OS=*Saccharomyces cerevisiae*; [2]KLTH0C04906p OS=*Lachancea thermotolerans*	X(15)	L(2)S(3)	X(2)	S(12)	
C5DNJ6	Acb1	Secreted	Acyl-CoA-binding; Fatty acid transport	KLTH0G17578p OS=*Lachancea thermotolerans*		L(2)			
P32334	Msb2	Cell membrane; Membrane	Osmosensor activity	Protein MSB2 OS=*Saccharomyces cerevisiae*	X(2)				
Cell redox Homeostasis									
C5DK27	Grx2		Oxidoreductase	KLTH0F01276p OS=*Lachancea thermotolerans*				X(3)	
Cell wall biogenesis/degradation									
P38616[1]; C5DCW7[2]; 190409145; 151944378; 323335922	Ygp1	Secreted		[1]Protein YGP1 OS=*Saccharomyces cerevisiae*; [2]KLTH0B06380p OS=*Lachancea thermotolerans*	X(5)			S(2)	X(2)
B5VL27	Cis3	Cell wall; Secreted	Constituent of cell wall	Cell wall mannoprotein CIS3 OS=*Saccharomyces cerevisiae*	X(2)	S(2)			
P53301; 323354858	Crh1	Cell wall; Membrane; Secreted	Glycosidase; Hydrolase	Probable glycosidase CRH1 OS=*Saccharomyces cerevisiae*	X(10)			MS(7)	X(3)
P15703[1]; C5DDA8[1];	Bgl2	Cell wall; Secreted	Glycosidase;	[1]Glucan 1,3-beta-glucosidase OS=*Saccharomyces cerevisiae*;	X(11)	L(5)S(8)	X(7)	MS(11)	X(3)

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Table 3.6 - Identified proteins, from all fermentation sets, grouped according to the biological process in which they are involved.									
Accession	Protein[a]	Cell compartment	Molecular function	Description	Sc*	LtSc*	Lt*	MpSc*	Mp*
323304773[1]			Hydrolase	[2]KLTH0B09658p OS=*Lachancea thermotolerans*					
B5VE42[1]; C7GQJ1[1]; C5DIP5[2]; 323356050[1]	Ecm33	Cell membrane; Cell wall; Membrane; Secreted	Cell wall organization	[1]Cell wall protein ECM33 OS=*Saccharomyces cerevisiae* ; [2] KLTH0E14124p OS=*Lachancea thermotolerans*	X(7)	L(2)S(5)	X(3)	MS(2)	X(6)
P23776[1]; C5E2Q9[2]; 6323331[1]; 37926403[1]	Exg1	Cell wall; Secreted	Glycosidase; Hydrolase	[1]Glucan 1,3-beta-glucosidase I/II OS=*Saccharomyces cerevisiae*; [2]KLTH0H06974p OS=*Lachancea thermotolerans*	X(20)	L(17)S(15)	X(20)	S(18)	X(3)
P52911	Exg2	Cell membrane; Membrane	Glycosidase; Hydrolase	Glucan 1,3-beta-glucosidase 2 OS=*Saccharomyces cerevisiae*	X(3)				
P22146[1]; C5DDB4[2]; C5DDB5[2]; 323332206[1]	Gas1	Cell membrane; Cell wall; Membrane; Secreted	Transferase	[1]1,3-beta-glucanosyltransferase GAS1 OS=*Saccharomyces cerevisiae*; [2]KLTH0B09812p OS=*Lachancea thermotolerans*	X(9)	L(4)S(8)	X(5)	MS(9)	X(2)
Q03655; 323303438; 323307655	Gas3	Cell wall; Membrane; Secreted	Transferase	Probable 1,3-beta-glucanosyltransferase GAS3 OS=*Saccharomyces cerevisiae*	X(9)	S(2)		S(7)	
Q08193[1]; C5DLE7[2]; 323346603[1]	Gas5	Cell wall; Membrane; Secreted	Transferase	[1]1,3-beta-glucanosyltransferase GAS5 OS=*Saccharomyces cerevisiae*; [2]KLTH0F12474p OS=*Lachancea thermotolerans*	X(9)	L(3)S(6)	X(4)		X(3)
P39005[1]; C5DKG0[2]; 323332950[1]; 151944956[1]	Kre9	Cell wall; Secreted		[1]Killer toxin resistance/Cell wall synthesis protein KRE9 OS=*Saccharomyces cerevisiae*; [2]KLTH0F04378p OS=*Lachancea thermotolerans*	X(2)	L(2)S(2)	X(2)	S(2)	
Q04951; 259148895	Scw10	Cell wall; Secreted	Glycosidase; Hydrolase	Probable family 17 glucosidase SCW10 OS=*Saccharomyces cerevisiae*	X(10)	S(4)		S(5)	
P53334[1]; C5DDB7[2]; 6321718[1]; 151943551[1]	Scw4	Cell wall; Secreted	Glycosidase; Hydrolase	[1]Probable family 17 glucosidase SCW4 OS=*Saccharomyces cerevisiae*; [2]KLTH0B09878p OS=*Lachancea thermotolerans*	X(14)	L(6)S(7)	X(7)	S(8)	
P32603; 323307216	Spr1	Secreted	Glycosidase; Hydrolase	Sporulation-specific glucan 1,3-beta-glucosidase OS=*Saccharomyces cerevisiae*	X(2)	S(2)		S(2)	
P40472[1]; C5DMI9[2]; 71064095[1]	Sim1	Cell wall; Secreted		[1]Protein SIM1 OS=*Saccharomyces cerevisiae*; [2]KLTH0G09306p OS=*Lachancea thermotolerans*	X(4)	L(8)S(4)	X(8)	S(3)	
C5DNP7	Act1	Cytoplasm; cytoskeleton	ATP Binding	KLTH0G18832p OS=*Lachancea thermotolerans*			X(2)		
P28319[1]; C5DGI9[2]; 4814(CAA46969.1)[1]	Cwp1; Yju1	Cell wall; Membrane; Secreted	Constituent of cell wall	[1]Cell wall protein CWP1 OS=*Saccharomyces cerevisiae*; [2]KLTH0D05676p OS=*Lachancea thermotolerans*	X(8)	S(6)	X(2)	S(7)	
323335957; E7LZ38	Egt2	Cell wall	Cellulase	Egt2p OS=*Saccharomyces cerevisiae* (strain VIN13)				S(3)	
A7A003[1]; C5DKP3[2]; 323347641[1]	Uth1	Cell wall; Membrane; Secreted; mitochondrion		[1]Protein UTH1 OS=*Saccharomyces cerevisiae*; [2]KLTH0F06358p OS=*Lachancea thermotolerans*	X(4)	L(7)S(3)	X(6)	S(3)	

Table 3.6 - Identified proteins, from all fermentation sets, grouped according to the biological process in which they are involved.									
Accession	Protein[a]	Cell compartment	Molecular function	Description	Sc*	LtSc*	Lt*	MpSc*	Mp*
DNA Repair									
P61864; P0CH08	Ubi1	Cytoplasm/ Nucleus	Ribonucleoprotein;	Ubiquitin OS=*Saccharomyces cerevisiae*	X(4)	S(3)			
C5DHN8[1]; 6322989[2]; C4YAP5[3]; 323308175[2]; P0CG63[2]	Ubi4	Cytoplasm/ Nucleus		[1]KLTH0E05852p OS=*Lachancea thermotolerans* ; [2]Polyubiquitin OS=*Saccharomyces cerevisiae;* [3]Ubiquitin OS=*Clavispora lusitaniae*	X(2)		X(2)	M(2)S(2)	
Electron Transport									
P22217; 6323072	Trx1	Cytoplasm; Golgi apparatus; Membrane; Nucleus	Electron carrier; oxidoreductase	Thioredoxin-1 OS=*Saccharomyces cerevisiae*	X(4)			S(2)	
P22803[1]; 323714537[2]	Trx2	Cytoplasm; Golgi apparatus; Membrane; Nucleus	Electron carrier; oxidoreductase	[1]Thioredoxin-2 OS=*Saccharomyces cerevisiae*; [2]Chain A, Crystal Structure Of Mxr1 From *Saccharomyces Cerevisiae* In Complex With Trx2	X(4)			S(4)	
C5DC97	Trx		Electron carrier; oxidoreductase	Thioredoxin OS=*Lachancea thermotolerans*		L(4)	X(5)		
Glycolysis									
P00942[1]; C5DDZ8[2]	Tpi1	Mitochondrion; Plasma membrane	Isomerase	[1]Triosephosphate isomerase OS=*Saccharomyces cerevisiae*; [2]Triosephosphate isomerase OS=*Lachancea thermotolerans*	X(8)	L(2)	X(2)		
C5DNB9; C5DCC7	Gap1		Oxidoreductase	Glyceraldehyde-3-phosphate dehydrogenase OS=*Lachancea thermotolerans*		L(5)	X(8)		
C4Y6W3	Eno1	Cell surface; Secreted	Lyase	Enolase 1 OS=*Clavispora lusitaniae*				M(4)	
P00925[1]; C5DD59[2]	Eno2	Cytoplasm	Lyase	[1]Enolase 2 OS=*Saccharomyces cerevisiae*; [2]Enolase OS=*Lachancea thermotolerans*	X(3)	L(11)	X(10)		
P14540[1]; C5DGT9[2]	Fba1	Cytosol/ Mitochondrion	Lyase	[1]Fructose-bisphosphate aldolase OS=*Saccharomyces cerevisiae*; [2]KLTH0D08250p OS=*Lachancea thermotolerans*	X(4)	L(5)	X(4)		
P00950[1]; C5DMB1[2]	Gpm1	Cytosol/ Mitochondrion	Isomerase	[1]Phosphoglycerate mutase 1 OS=*Saccharomyces cerevisiae*; [2]KLTH0G07392p OS=*Lachancea thermotolerans*	X(4)	L(3)	X(5)		
P00560[1]; C5E3H6[2]	Pgk1	Cytoplasm	Kinase; Transferase	[1]Phosphoglycerate kinase OS=*Saccharomyces cerevisiae*; [2]Phosphoglycerate kinase OS=*Lachancea thermotolerans*	X(6)	L(7)	X(5)		
P00549[1]; C5DG09[2]	Pyk1; Cdc19	Plasma membrane	Kinase; Transferase	[1]Pyruvate kinase 1 OS=*Saccharomyces cerevisiae*; [2]Pyruvate kinase OS=*Lachancea thermotolerans*	X(2)	L(3)	X(2)		
P00360	Tdh1	Cytoplasm	Oxidoreductase	Glyceraldehyde-3-phosphate dehydrogenase 1 OS=*Saccharomyces cerevisiae*	X(6)				
3720; P00359	Tdh3	Cytoplasm	Oxidoreductase	Glyceraldehyde-3-phosphate dehydrogenase 3 OS=*Saccharomyces cerevisiae*				S(2)	

Table 3.6 - Identified proteins, from all fermentation sets, grouped according to the biological process in which they are involved.									
Accession	Protein[a]	Cell compartment	Molecular function	Description	Sc*	LtSc*	Lt*	MpSc*	Mp*
C4Y1M4	Tal1	Cytoplasm	Transferase	Transaldolase OS=*Clavispora lusitaniae*				M(3)	
P00724[1]; C5DDR9[2]; 296178357[1]	Suc2	Cytoplasm; Secreted	Glycosidase; Hydrolase	[1]Invertase 2 OS=*Saccharomyces cerevisiae*; [2]KLTH0C03278p OS=*Lachancea thermotolerans*	X(4)	L(5)	X(7)	S(2)	
Lipid Metabolism									
P39105[1]; C5DEN4[2]; 323347117[1]	Plb1	Cell membrane; Membrane	Hydrolase; Lipase	[1]Lysophospholipase 1 OS=*Saccharomyces cerevisiae*; [2]LTH0C10670p OS=*Lachancea thermotolerans*	X(4)	L(5)	X(6)	S(3)	
Q03674	Plb2	Cell wall; Membrane; Secreted	Hydrolase; Lipase	Lysophospholipase 2 OS=*Saccharomyces cerevisiae*	X(3)				
207342449; B5VPD0	YMR006C		Phospholipase	YMR006Cp-like protein [*Saccharomyces cerevisiae* AWRI1631]				S(5)	
Proteolysis/Protein Metabolism									
Q12303; 323347469	Yps3	Cell membrane; Membrane	Aspartyl Protease; Hydrolase; Protease	Aspartic proteinase yapsin-3 OS=*Saccharomyces cerevisiae*	X(2)			S(2)	
C5DER2	Prb1		Hydrolase; Protease Serine Protease	KLTH0C11396p OS=*Lachancea thermotolerans*		L(3)	X(4)		
C5DD93	Prc1	Vacuole	Serine carboxypeptidase	KLTH0B09328p OS=*Lachancea thermotolerans*			X(6)		
C5DMX3	Yps1		Aspartyl Protease; Hydrolase; Protease	KLTH0G12386p OS=*Lachancea thermotolerans*		L(4)	X(4)		
323334011; E7KB69	Yps7		Aspartyl Protease	Aspartic proteinase yapsin-7 OS=*Saccharomyces cerevisiae*	X(2)				
P07267[1]; C5DF06[2]; 14278413[1]	Pep4	Vacuole	Aspartyl Protease; Hydrolase; Protease	[1]Saccharopepsin OS=*Saccharomyces cerevisiae*; [2]KLTH0D11264p OS=*Lachancea thermotolerans*	X(8)	L(7)S(3)	X(5)		
Sterol Transport									
P47033	Pry3	Cell wall; Membrane; Secreted		Cell wall protein PRY3 OS=*Saccharomyces cerevisiae*	X(2)				
E7KRI2; 323347524	Dan2			Dan2p [*Saccharomyces cerevisiae* Lalvin QA23]	X(2)				
C5DIB0				KLTH0E11088p OS=*Lachancea thermotolerans*		L(3)	X(5)		
Other Function									
Q12140	Bsc1			Bypass of stop codon protein 1 OS=*Saccharomyces cerevisiae*	X(2)				
E7QII2; 323353722; 323336411	Ccw14			Covalently-linked cell wall protein 14 OS=*Saccharomyces cerevisiae*	X(3)			M(2)	X(2)
C5DFQ4				KLTH0D17006p OS=*Lachancea thermotolerans*			X(2)		
C5DK26				KLTH0F01254p OS=*Lachancea thermotolerans*		L(2)	X(2)		

Table 3.6 - Identified proteins, from all fermentation sets, grouped according to the biological process in which they are involved.									
Accession	Protein[a]	Cell compartment	Molecular function	Description	Sc*	LtSc*	Lt*	MpSc*	Mp*
C5DKI7[1]; C5DKI8[2]	Hsp150	Cell wall	Constituent of cell wall	[1]KLTH0F05016p OS=*Lachancea thermotolerans*; [2]KLTH0F05060p OS=*Lachancea thermotolerans*				X(2)	
61952; Q87020	KIL-k2	Secreted		K2 killer toxin precursor [Killer virus of *S. cerevisiae*]				S(2)	
C5DEE3[2]; 151941952[1]; A6ZXS8[1]	Npc2			[1]Phosphatidylglycerol/phosphatidylinositol transfer protein OS=*Saccharomyces cerevisiae*; [2] KLTH0C08492p OS=*Lachancea thermotolerans*	X(2)	L(5)	X(5)		
C5E330	Pry2	Secreted		KLTH0H09834p OS=*Lachancea thermotolerans*				X(4)	
A6ZY20; 187470953	Pst1; Hpf2	Cell membrane; Cell wall; Membrane; Secreted		Cell wall mannoprotein PST1 OS=*Saccharomyces cerevisiae*	X(10)	S(8)			X(5)
P38288[1]; C5DIE9[2]; 323338696[1]	Tos1	Secreted		[1]Protein TOS1 OS=*Saccharomyces cerevisiae*; [2]KLTH0E11968p OS=*Lachancea thermotolerans*	X(6)	S(2)L(2)	X(3)	S(6)	
A6ZTT3; A6ZPL5; 171704597	Vel1	Cytoplasm		Protein VEL1 OS=*Saccharomyces cerevisiae*	X(2)	S(2)		S(2)	
207346916; B5VFN0	YDL037C			YDL037Cp-like protein [*Saccharomyces cerevisiae* AWRI1631]	X(2)				
207345583; B5VI84	YGL258W			YGL258Wp-like protein [*Saccharomyces cerevisiae* AWRI1631]	X(2)				
C5DML3	YIL108W			KLTH0G09856p OS=*Lachancea thermotolerans*			X(2)		
P40442	YIL169C	Membrane	Signal transducer	Putative uncharacterized protein YIL169C OS=*Saccharomyces cerevisiae*	X(2)				
P46992; 323304417	YJL171C	Cell membrane; Membrane		Uncharacterized protein YJL171C OS=*Saccharomyces cerevisiae*	X(3)	S(2)		S(2)	
C5DFV9	Zps1			KLTH0D00308p OS=*Lachancea thermotolerans*			X(3)		
P46955; 968906	Nca3	Mitochondrion		Protein NCA3, mitochondrial OS=*Saccharomyces cerevisiae*	X(4)	S(6)			
P00330[1]; C5DNB7[2]	Adh1	Cytoplasm; Plasma membrane	Oxidoreductase; Metal binding;	[1]Alcohol dehydrogenase 1 OS=*Saccharomyces cerevisiae*; [2]KLTH0G15686p OS=*Lachancea thermotolerans*	X(4)	L(2)	X(2)		
C4YB98				Putative uncharacterized protein OS=*Clavispora lusitaniae*					X(2)

a) The *Saccharomyces cerevisiae* homologue for the identified proteins; L, S, M - refers to the possible origin of the protein in mixed fermentations, whereas MS and LS indicate that the possibility exist that both inoculated cultures contribute to the existence of the protein in culture fermentations. X indicates the presence of the specific protein in the pure culture fermentations. *The numbers in brackets indicate the number of unique peptides used to identify the proteins.

3.3.4   Haze formation potential

Extracellular yeast proteins (i.e. yeast mannoproteins) are known to have varying protein haze reducing abilities and as the protein content of all our wines differ we investigated whether this will influence haze formation.

The potential of the yeast proteins to influence wine haze was tested by spiking samples with different concentrations (0 – 2 g/l) of BSA and 0.5 g/l potassium sulphate (Fig. 3.5).
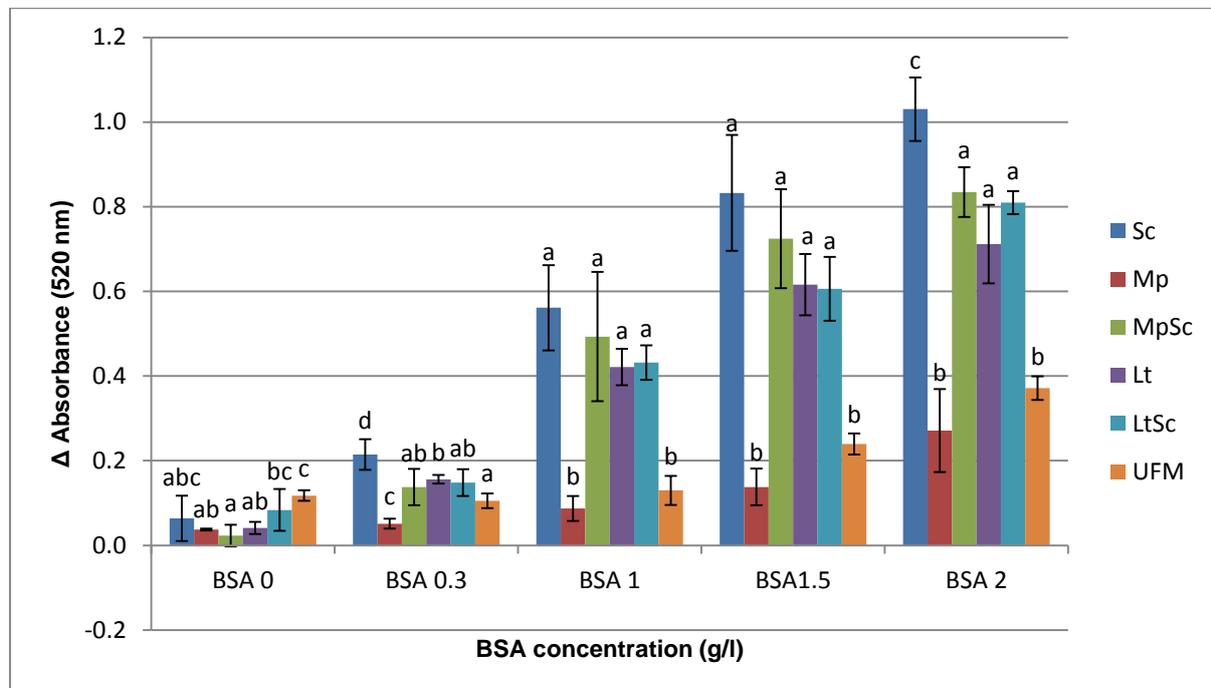

Fig. 3.5 - Haze formation index of wines fermented with different yeasts. A range (0 – 2 g/l) of BSA and 0.5 g/l potassium sulphate was added to medium before heat treatment. Higher index shows increase in haze formation after heat treatment. Error bars indicate standard deviation between three replicates, and the letters denote a significant difference on a 5% significance (p < 0.05) level. Mp – *Metschnikowia pulcherrima;* **MpSc –** *Metschnikowia pulcherrima + Saccharomyces cerevisiae;* **Sc –** *Saccharomyces cerevisiae;* **Lt –** *Lachancea thermotolerans;* **LtSc -** *Lachancea thermotolerans + Saccharomyces cerevisiae;* **UFM – unfermented synthetic medium.**

At the highest protein concentration, all non-*Saccharomyces* strains showed the potential to lower the potential of wine to form haze in comparison to VIN13. *M. pulcherrima* showed the highest potential to prevent haze formation at high BSA concentrations. This might be due to the high residual sugars in the sample as a result of poor fermentation since a similar trend can be seen for the unfermented medium or that *M. pulcherrima* did not secrete as many proteins that contribute to haze formation as the other yeasts. As discussed in section 3.3.4 (and seen in Fig. 3.5), the total number of proteins identified in the Mp fermentation is much lower than the number of proteins identified in any of the other fermentations.

At lower BSA concentrations the potential of different yeasts to inhibit haze formation was less distinct; all fermentations showed the same potential to inhibit haze formation in

wine. The mixed culture fermentation with *M. pulcherrima* and *S. cerevisiae* showed a significant inhibition at lower BSA concentration but at higher BSA concentration, it was not significantly different from the *L. thermotolerans* pure and mixed fermentations.

Out of the 3 haze protection factor proteins Hpf1p, Hpf1'p and Hpf2p described in literature [55], only Hpf2p (alias Pst1p) was identified in the current study; it was present in Sc, LtSc and Mp. Hpf2p of *M. pulcherrima* is present in Mp but not in MpSc, yet the haze protection is greater in Mp than in Sc or MpSc, therefore the lower protein haze formation in the Mp fermentation might be a result of MpHpf2p secretion. A more likely reason for the peculiar result observed for Mp is that Mp did not ferment much. As a result, the medium composition did not change much hence the similarity of haze intensity with unfermented juice (UFM). Unlike that of Mp, the chemical composition of the other samples was more similar and the haze obtained for these samples could therefore be compared with one another. Another protein identified that has been described to exhibit haze protective activity is invertase, (Suc2p) [29,55], it was identified in all fermentations except for Mp. The absence of Suc2p in the Mp fermentation suggests that haze protection by specific protein factors is a complex phenomenon that will need to be studied in more detail.

As far as we know this is the first study looking at the contribution that non-*Saccharomyces* yeasts make toward the formation of haze in wine and the results suggest that these yeasts may have stronger haze protective ability than *S. cerevisiae.* Specific proteins of non-*Saccharomyces* should maybe be investigated for their haze protection ability.

3.4 Conclusion

Over the years, much has been learned about the contribution of non-*Saccharomyces* yeasts towards various aspects of wine quality. The contribution to quality is mainly due to the activity of enzymes produced by these yeasts or their ability to metabolize metabolic compounds produced by the traditional wine yeast, *S. cerevisiae.*

Proteins produced and secreted by yeasts during and shortly after alcoholic fermentation are as important to wine quality as intracellular proteins, especially with regards to haze formation, foam stability in sparkling wine and the release of aroma compounds from grape precursors.

Different non-*Saccharomyces* yeasts have a similar secretome under fermentative conditions, although the number of proteins varies greatly, confirming the suggestion of Buerth *et al.* [12] regarding a core secretome, shared between yeasts of different species. Although we only identified 3 of their core proteins in all the fermentations, the other proteins in the proposed core secretome or isoforms thereof were found in some of the fermentations, but the possibility exists that the abundance of these proteins was very low in the other fermentations and therefore they were not identified. The contribution of different yeasts to the proteome in mixed culture fermentations are shown in this study, with a

correlation between the fermentation kinetics, seen in Fig. 3.1, and the proteins identified (summarized in Table 3.6). The contribution of Mp to the MpSc is much less than the contribution of Lt to LtSc. This correlates with the potential early decline of Mp in the MpSc and the later decline of Lt in the LtSc. The number of proteins identified in individual fermentations could also be correlated with the fermentation kinetics, as the number of proteins identified for Mp, which did not ferment very well, was less than the number of proteins identified in Sc, the fermentation that went to dryness.

Proteins of oenological interest that have been identified in this study include the various β-glucosidases, endochitinase and invertase. All of which have been reported in literature to have potential implications on wine quality. The impact of the proteins from non-*Saccharomyces* yeasts on wine quality will need to be further investigated, preferably in real grape juice to fully understand the potential of these proteins. Heterologous expressions of the genes will also further our knowledge about the substrate specificity of the enzymes that are homologous in *S. cerevisiae* and non-*Saccharomyces* but show different activities on plate assays.

The presence of non-*Saccharomyces* yeasts in the fermentation seems to lower the potential of the final product to form haze. In order to confirm this observation, the assay should however be carried out using the same matrix to minimize the impact of chemical differences in the wine. More strains and species of non-*Saccharomyces* yeasts could also be included and real grape juice could offer a more realistic view.

This study has contributed to our knowledge of non-*Saccharomyces* yeasts (in particular *M. pulcherrima* and *L. thermotolerans*) and the potential they have in grape juice fermentations. Further studies will need to be done to improve our understanding of the yeast-yeast interactions, although with this study we could confirm that some non-*Saccharomyces* yeast (*L. thermotolerans*) could survive along with *S. cerevisiae* in the fermentation and that the non-*Saccharomyces* contributed towards the protein pool even when its population declined rapidly upon inoculation with *S. cerevisiae.*

3.5 Acknowledgements

3.6 References

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CHAPTER 4

General discussion and conclusions

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4.1 General discussion

Proteins secreted by yeasts in wine have a variety of effects on wine quality. Some proteins are beneficial, such as extracellular yeast enzymes that can hydrolyse the glycosidic bond of grape aroma precursors and release aroma compounds [1-4] and mannoproteins preventing haze in white wine [5,6] and stabilizing foam in sparkling wines [7-9]. Most non-*Saccharomyces* yeasts naturally occurring in the wine environment (e.g. on grape berry surface and cellar equipment) have been shown to secrete enzymes of oenological interest [10-12]. Not all secreted enzymes are, however, active under winemaking conditions. Targeted approaches to identify and characterise the enzymes of oenological interest have limited our broad knowledge on how non-*Saccharomyces* can globally contribute to wine quality. Therefore, the need to investigate the full secretome of yeasts under winemaking conditions was identified. This study set out to identify non-*Saccharomyces* yeasts that secrete enzymes active under winemaking conditions and to characterize their full secretome using mass fingerprinting in pure and mixed culture with *S. cerevisiae* under fermentative conditions. The qualitative data obtained were correlated to the visual observations of 1D and 2D SDS-PAGE gels and to the fermentation kinetics. Finally, the influence of yeast secretomes on the potential of wine to form haze was also investigated.

A number of yeasts previously isolated from grape juice, exhibited enzyme activities during screening on agar plates. Two non-*Saccharomyces* yeasts were selected for fermentation studies: one *M. pulcherrima* reported to be a poor fermenter, but showing beneficial impact on wine quality and one *L. thermotolerans* a strong fermenter, also reported to be beneficial and whose proteome is annotated. Fermentation with *M. pulcherrima* was slow with only 25 g/l of sugar fermented in 25 days, resulting in 1% (v/v) ethanol. The multistarter fermentation (MpSc), inoculated sequentially with *M. pulcherrima* and *S. cerevisiae*, showed a delayed onset with rapid weight loss only starting 3 days after inoculating with *S. cerevisiae.* Thereafter the fermentation kinetics were similar to that of the Sc pure culture fermentation. Fermentations with *L. thermotolerans* showed no distinct lag phase after inoculation, although the fermentation progressed slower than for *S. cerevisiae* pure cultture. The multi-starter fermentation (LtSc) showed similar fermentation kinetics to that reported by Comitini *et al*. [13] and Gobbi *et al*. [14], following the kinetics of pure *L. thermotolerans* for the first ±15 days, where after it follows the fermentation kinetics of *S. cerevisiae.* A greater contribution of *L. thermotolerans* to the protein content of LtSc than that of *M. pulcherrima* in MpSc was therefore expected.

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1D SDS-PAGE revealed similar protein profiles for all fermentations, with protein bands visible across the whole spectrum of molecular weights. Contributions of both yeasts present, to the protein profile of mixed culture fermentations could be observed and this contribution was confirmed with 2D SDS-PAGE. Proteins secreted by yeasts during alcoholic fermentation of synthetic grape juice have a low pI between 3.5 and 6, similar to that reported for soluble proteins in wine [15].

The number of proteins secreted by yeasts in fermentations varied greatly, with proteins involved in glycolysis and cell wall metabolism among the most secreted. Four proteins secreted by all yeasts in all fermentation were identified as Bgl2p, Ecm33p, Exg2p and Gas1p, all playing a critical role in cell wall biogenesis. Three of these proteins have previously been suggested to be part of the core secretome of yeasts [16]. Other proteins from the suggested core secretome or isoforms thereof were only identified in some of the fermentations, with Sc containing the most and Mp the least. Various isoforms of the same protein were identified in different fermentations, e.g. for Tdh1p that was identified in Sc and the isoform Tdh3p identified in MpSc. Boucherie *et al.* [17] found Tdh3 in exponentially growing cells, whereas Tdh1 was identified in the stationary phase, suggesting that Tdh1 may be involved in processes other than glycolysis. Should this hypothesis be correct and possibly also applicable to other enzymes, this could explain the presence of so many intracellular proteins in the secretome at the end of fermentation, before autolysis played a significant role in the release of proteins into the medium.

The influence of yeast interactions on the biology of the yeasts sharing the same environment and on the secretion of proteins was evidenced by the presence of proteins only identified in mixed culture fermentations, such as *L. thermotolerans* proteins in LtSc and pure Lt fermentations, while the *S. cerevisiae* homologues were only found in the pure Sc fermentation and not LtSc. This was observed for proteins involved mainly in glycolysis but also for those involved in lipid and protein metabolism indicating a reason for the slower fermentation kinetics of the LtSc fermentation. Fermentation kinetics of LtSc was slightly slower than the Lt fermentation and much slower than Sc until day 15 when *S. cerevisiae* possibly took over and the fermentation progressed more rapidly than the Lt pure culture fermentation.

A variety of glycosidases were identified in all the fermentations, with *S. cerevisiae* secreting the most. No unique β-glucosidases were identified in the non-*Saccharomyces* fermentations thus suggesting the β-glucosidase activity observed on plate assays (and in other studies, [18-20]) to be a function of an unidentified β-glucosidase or that of a homologous protein secreted by non-*Saccharomyces* with greater substrate specificity towards arbutin (as was observed in our study) and possibly the grape glycosylated precursors as this is not the usual substrate for these cell wall glucosidases. The K2 killer

toxin precursor from *S. cerevisiae* identified in the MpSc fermentation indicates biological competition between *M. pulcherrima* and *S. cerevisiae* and could contribute to the rapid decline of the *M. pulcherrima* population in the MpSc fermentation.

The *M. pulcherrima* strain used in this study has previously been reported to possess the Mp*APR*1 gene encoding an acid protease, and although it was specifically sought, the protease was not identified with mass fingerprint analyses.

The extracellular proteins are known to have varying influence on the ability of wine to form protein haze; therefore we investigated whether the difference in protein composition observed in this study would have any influence on haze formation. Non-*Saccharomyces* yeasts showed a potential to lower the haze formation potential of wine, with *M. pulcherrima* exhibiting the highest inhibition and mixed culture fermentations also showing lowered haze formation in comparison to *S. cerevisiae.* The potential of *M. pulcherrima* to inhibit haze formation will need to be investigated further as it was unclear whether the inhibition was due to the low number of proteins secreted by *M. pulcherrima* or a specific protein, such as Hpf2p, secreted or an influence of the medium composition that has not changed much due to the fermentation that did not progress very well, as the haze potential of Mp showed a similar trend as the haze potential of unfermented medium.

4.2 Potential further investigations

The untargeted approach used in this study to characterize the secretome of yeasts in fermentations broadened our knowledge and understanding of the non-*Saccharomyces* yeasts studied and their interactions with *S. cerevisiae* in a wine like medium and also the role and influence of various proteins secreted by these yeasts on wine properties, although it also raised a few questions.

Similarities in the secretome of yeasts under fermentative conditions, confirm the importance of certain proteins for the general function and survival of the yeasts. Although the number of proteins identified for different yeasts varied greatly, most of the core proteins suggested by Buerth *et al.* [16] were identified in all the fermentations. Further investigation is needed to understand the influence of two yeasts on one another and the proteins secreted in fermentation, even though the contribution of both yeast to the protein pool of mixed culture fermentations was clearly observed. Correlating the secretome with the transcriptome (similar to Rossouw *et al.* [21] studied intracellular proteins and transcriptome in parallel) should be envisaged.

The study should be broadened to other non-*Saccharomyces* to identify conserved and divergent behaviours in the secretion of proteins and the influence these proteins have on wine properties.

The potential of non-*Saccharomyces* yeasts to lower the potential of protein haze formation in wine was indicated in this study, and further investigation with more strains and species is needed to confirm if non-*Saccharomyces* have a stronger ability than *S. cerevisiae* to protect wine against haze. The haze protection ability of specific non-*Saccharomyces* proteins also needs to be investigated.

Studies in synthetic medium give a good indication of how yeasts and their proteins interact, but to really understand the impact on wine properties and quality, experiments will need to be repeated in real grape juice.

4.3 References

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