Metagenomic screening of cell wall hydrolases, their anti-fungal activities and potential role in wine fermentation

by

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Summary

The grape and wine ecosystem contains fungi, bacteria and yeasts whose interactions contribute to the final wine product. While the non-*Saccharomyces* yeasts are dominant in the early stage of alcoholic fermentation, the later stage is always dominated by *Saccharomyces cerevisiae*. Although their presence in wine fermentation is often short-lived, the non-*Saccharomyces* yeasts are known to produce an array of extracellular hydrolytic enzymes which facilitate the extraction and release of aroma compounds, but might also play a role in microbial interactions.

The present study aimed to investigate the microbial diversity of grape juice and to evaluate the potential of non-*Saccharomyces* yeasts to produce hydrolytic enzymes and display anti-fungal properties. To capture the microbial diversity, culture-dependent (plating) and –independent (Automated Ribosomal Intergenic Spacer Analysis (ARISA)) techniques were used in parallel. The fungal and bacterial ARISA displayed a wider range of operational taxonomic units (OTUs) in comparison to cultivation-based technique, demonstrating that ARISA is a powerful culture-independent technique applicable to ecological studies in wine.

Some of the uncommon yeast isolates derived from our cultivation-based study were subjected to an enzymatic screening process. Hydrolases, such as chitinases, β-1,4-cellulases, β-1,3-1,6-glucanases, β-glucosidases, pectinases and acid proteases were specifically sought. Most of the yeast isolates exhibited chitinase, β-1,4-cellulase as well as β-1,3-1,6-glucanase activities. Only *Metschnikowia chrysoperlae* exhibited β-glucosidase activity. We also retrieved the partial chitinase gene sequences from *M. chrysoperlae*, *Pichia burtonii*, *Hyphopichia pseudoburtonii* that exhibited chitinase activity. Among the isolates, *Pseudozyma fusiformata* exhibited a strong antagonistic activity against the wine spoilage yeasts *B. bruxellensis* AWRI 1499 and *B. anomalus* IWBT Y105. Furthermore, we showed that the killer phenotype of *P. fusiformata* cannot be attributed to a viral encoded dsRNA.

Finally, two metagenomic approaches were employed in an attempt to explore the indigenous microbiome in a more holistic manner, where we adopted whole metagenome Roche GS-FLX 454-pyrosequencing and construction of a fosmid library. The whole metagenome sequencing revealed a wide range of hydrolytic enzymes that showed homology to enzymes from different fungal and non-*Saccharomyces* yeast species. Moreover, the metagenomic library screening resulted in the retrieval of 22 chitinase and 11 β-glucosidase positive fosmid clones originating from yeasts. Two clones of interest, BgluFos-G10 and ChiFos-C21, were subjected to next generation sequencing. BgluFos-G10 revealed 2 ORFs exhibiting homology to glycosyl hydrolase family 16 proteins whereas no ORFs encoding chitinase enzymes could be identified in the ChiFos-C21 clone. However, all the potential ORFs identified exhibited homology to a gene cluster from *Clavispora lusitaniae* ATCC 42720,

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suggesting that the cloned DNA fragments belonged to a yeast species closely related to *C. lusitaniae* or members of the family Metschnikowiaceae.

Overall, our study identified a variety of novel hydrolytic enzymes. However, retrieving the full gene sequences of these identified enzymes would be the immediate follow-up of our study. Moreover, the hydrolytic and antifungal activities exhibited by the yeast isolate could be of major interest in evaluating their potential as biocontrol agents against grapevine fungal pathogens and subsequently the wine spoilage yeasts. It would be interesting to evaluate as well the potential impact of these enzymes under wine making condition and could be our next step of investigation.

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Opsomming

Die druif en wyn ekosisteme bevat swamme, bakterië en giste en die interaksies van hierdie organismes dra by tot die finale wyn produk. Die nie-*Saccharomyces* giste is dominant in die vroeë stadium van die alkoholiese fermentasie, maar die latere fase word altyd gedomineer deur *Saccharomyces cerevisiae*. Alhoewel hulle teenwoordigheid in wyngistings gewoonlik kortstondig is, is die nie-*Saccharomyces* giste bekend vir die produksie van 'n verskeidenheid ekstrasellulêre hidrolitiese ensieme wat die ekstraksie en vrylating van aroma komponente fasiliteer, en ook moontlik 'n rol kan speel in mikrobiese interaksie.

Hierdie studie beoog om die mikrobiese diversiteit van druiwesap te bestudeer en die potensiaal van nie-*Saccharomyces* giste te evalueer ten opsigte van die produksie van hidrolitiese ensieme, asook die demonstrasie van anti-swam eienskappe. Kweking-afhanklike (uitplating), asook –onafhanklike (Automatiese Ribosomale Intergeniese Spasieerder Analise (ARISA)) tegnieke is in parallel gebruik om die mikrobiese diversiteit te bepaal. Die swam en bakteriële ARISA het 'n groter verskeidenheid van operasionele taksinomiese eenhede (OTUe) vertoon in vergelyking met die kweking-gebasseerde tegniek en dit demonstreer dat ARISA 'n kragtige kweking-onafhanklike tegniek is, wat toepasbaar is in ekologiese studies van wyn .

Sommige van die skaarser gisisolate, uit ons kweking -gebasseerde studie was vir ensiemaktiwiteite geskandeer. Daar is spesifiek gesoek vir hidrolases soos chitinases, β-1,4-sellulases, β-1,3-1,6-glukunases, β-glukosidases, pektinases en suur proteases. Die meeste gisisolate het chitinase, β-1,4-sellulase asook β-1,3-1,6-glukunase aktiwiteit vertoon. Slegs *Metschinikowia chrysoperlae* het β-glukosidase aktiwiteit vertoon. Ons het verder die gedeeltelike chitinase geensekwensies van *M. chrysoperlae, Pichia burtonii* en *Hyphopichia pseudoburtonii* wat chitinase aktiwiteit vertoon het, bepaal. Een isolaat, *Pseudozyma fusiformata,* het 'n sterk antagonistiese aktiwiteit teenoor die wyn bederfgiste, *Bretanomyces bruxellensis* AWRI 1499 en *B. anomalus* IWBT Y105 vertoon. Verder het ons gewys dat die killer fenotipe van *P. fusiformata* nie gekoppel kan word aan'n viraal gekodeerde dsRNA nie.

Ten laaste is twee metagenomiese benaderings, naamlik die volledige metagenoom Roche GS-FLX 454-pirovolgordebepaling en konstruksie van 'n fosmied biblioteek, gebruik om die inheemse mikrobioom op 'n meer holistiese wyse te bestudeer. Die volgordebepaling van die volledige metagenoom het 'n wye verskeidenheid hidrolitiese ensieme aan die lig gebring wat homologie met ensieme van verskillende swamme en nie-*Saccharomyce*s gisspesies getoon het. Verder het die skandering van die metagenomiese biblioteek die isolasie van fosmiedklone van gisoorsprong wat positief is vir chitinase aktiwiteit (22 klone) en β-glukosidase aktiwiteit (11 klone) tot gevolg gehad. Twee van hierdie klone, BgluFos-G10 en ChiFos-C21, is met volgende generasie volgordebepaling ontleed. BgluFos-G10 het twee oopleesrame (OLRe) wat homologie met glikosiel hidrolase familie 16 proteïene het, vertoon maar geen OLRe wat

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chitinase ensieme enkodeer kon in die ChiFos-C21 kloon geïdentifiseer word nie. Al die potensiële OLRe wat geïdentifiseer is, het homologie aan ‘n genepoel van *Clavispora lusitaniae* ATCC 42720 vertoon, wat daarop dui dat die gekloneerde DNS fragmente aan ‘n gisspesie behoort wat naverwant aan *C. lusitaniae* of lede van die Metschinikowiaceae familie is.

In geheel gesien het ons studie ‘n verskeidenheid van nuwe hidrolitiese ensieme geïdentifiseer. Die bepaling van die volledige geenvolgordes van hierdie geïdentifiseerde ensieme sal die onmiddelike opvolg aksie van hierdie studie wees. Verder is die hidrolitiese en anti-swam aktiwiteite wat deur die gisisolate gedemonstreer is, van hoof belang, asook die evaluering van hulle potensiaal as biokontrole agente teen wingerd swampatogene en wyn bederfgiste. Dit sal ook interessant wees om die potensiële impak van hierdie ensieme onder wynmaakkondisies te bepaal, en dit kan dus ons volgende ondersoek stap wees.

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This dissertation is dedicated to my late father Shri. Debabrata Ghosh

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Biographical sketch

Soumya Ghosh was born in the town of Durgapur, West Bengal, India. He matriculated from DAV Model School, Durgapur in 1995. He completed his Bachelor's degree in Microbiology from Garware College, Pune in 1999 and subsequently his Master's degree in Zoology at Pune University in 2001. Thereafter, he worked as a Junior Research Fellow at the Environmental Science Department, Pune University from 2001 to 2003, and then as a Senior Research Fellow at the Post Graduate Institute of Medical Education and Research, Chandigarh, India in 2003. Finally, he accepted a position as Project Assistant at the Institute of Microbial Technology, Chandigarh, India from 2003 to 2004. In 2004, he got a Research Fellow position at the Department of Chronobiology, Biological Center, University of Gröningen, in the Netherlands. From 2005 to 2008, he worked as a Research Fellow at Department of Developmental Biology, Centre of Plant Molecular Biology (ZMBP), Tübingen University, Germany. Thereafter, he continued with same position at the Department of Systems Biology, Technical University Munich, Germany from 2008 to 2010. He enrolled as a PhD student at the Institute for Wine Biotechnology (IWBT), University of Stellenbosch, South Africa in 2011.

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Preface

This dissertation is presented as a compilation of 6 chapters. Each chapter is introduced separately. Chapter 3 is written in the style of the journal into which it was accepted for publication. The other chapters are written in the style of Applied and Environmental Microbiology.

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Chapter 1

GENERAL INTRODUCTION AND PROJECT AIMS

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CHAPTER 1

1.1 Introduction

The wine microbial consortium comprises several genera and species of yeasts and bacteria (1, 7, 10). While non-*Saccharomyces* yeasts dominate the early stage of fermentation, the *Saccharomyces* species are predominantly found in the later stage. The yeast population dynamics is mainly governed by an array of factors like tolerance to ethanol, short generation time of *Saccharomyces* species, accumulation of toxic metabolites, depletion of oxygen and possibly direct cell-to-cell interaction between yeast species (7). Yeast cell wall-degrading enzymes are also thought to play a crucial role in these interactions (6, 7) and they have recently been receiving increasing attention from wine microbiologists. Moreover, from a wine perspective, these enzymes have been proven to be involved in improving the organoleptic properties of wine (10, 15). Although several studies have been conducted to identify and characterize *Saccharomyces cerevisiae*'s extracellular hydrolytic enzymes/killer toxins, those of non-*Saccharomyces* species have been scarcely studied (12). A few cultivation based studies have revealed that wine–related non-*Saccharomyces* yeasts secrete such extracellular enzymes (2, 14). Some of these non-*Saccharomyces* isolates has also been shown to secrete killer toxins (3, 4). In some cases, a link between killer activity and hydrolytic activity, in particular glucanase activity, has recently been established (5, 13).

However, the conventional cultivation based techniques do not provide a comprehensive view of the entire wine microbial consortium. Indeed, unculturable microorganisms are not recovered, thereby representing an unexplored and potentially unexploited reservoir of enzymes/toxins of interest. Identifying these organisms and their extracellular hydrolytic/killer activities would also contribute to our knowledge of their potential role in the dynamics of populations during wine spontaneous fermentation. However so far, most of the enzymes/killer activities detection has been performed by means of traditional cultivation-based approaches (2, 14). This limits our findings since this technique does not permit the recovery and therefore the identification of viable but non-culturable microorganisms or those which are not favoured by the cultivation conditions used during isolation campaigns. In recent years, metagenomic approaches have proved successful in providing a holistic view on the genetic make-up of a given microbial community, especially in an environment where part of the microbiota survives in a viable but not culturable state (8, 9, 11). Therefore, untargeted culture-independent techniques (e.g. metagenomics) would constitute suitable tools to capture the entire genetic information not only to identify microbial populations but also to enable us to understand complex microbial community structures such as those surviving in fermenting grape juice.

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1.2 Project aims

The overall aim of this project was to apply a set of targeted and untargeted approaches to determine the microbial diversity of a specific grape juice and to evaluate the functional potential of the wine microbiome with focus on hydrolytic enzymes and antifungal compounds. To achieve this, three main objectives were set as follows:

I. Determination of the wine microbial consortium by using a cultivation independent technique (Automated Ribosomal Intergenic Spacer Analysis) in conjunction with the traditional culture-based study of samples collected at different stages of wine fermentation.

II. Characterisation of uncommon yeast isolates with regards to different extracellular hydrolytic enzymatic activities: chitinases, glucanases, β-glucosidases, acid proteases, pectinases and killer activities.

III. Whole wine metagenome sequencing, construction of the wine metagenomic fosmid library and subsequent evaluation of the library through functional screening for hydrolases and killer toxins.

1.3 References

1. **Barata, A., M. Malfeito-Ferreira, and V. Loureiro.** 2012. Changes in sour rotten grape berry microbiota during ripening and wine fermentation. Int J Food Microbiol **154:**152-61.
2. **Charoenchai, C., G. H. Fleet, P. A. Henschke, and B. E. N. Todd.** 1997. Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. Aust J Grape Wine Res **3:**2-8.
3. **Comitini, F., and M. Ciani.** 2011. *Kluyveromyces wickerhamii* killer toxin: purification and activity towards *Brettanomyces/Dekkera* yeasts in grape must. FEMS Microbiology Letters **316:**77-82.
4. **Comitini, F., J. De Ingeniis, L. Pepe, I. Mannazzu, and M. Ciani.** 2004. *Pichia anomala* and *Kluyveromyces wickerhamii* killer toxins as new tools against *Dekkera/Brettanomyces* spoilage yeasts. FEMS Microbiol Lett **238:**235-40.
5. **Comitini, F., N. Di Pietro, L. Zacchi, I. Mannazzu, and M. Ciani.** 2004. *Kluyveromyces phaffii* killer toxin active against wine spoilage yeasts: purification and characterization. Microbiol **150:**2535-41.
6. **Dicks, L. M. T., S. Todorov, and A. Endo.** 2009. Microbial interactions. Biology of Microorganisms on Grapes, in must and in wine**:**335-347.
7. **Fleet, G. H.** 2003. Yeast interactions and wine flavour. Int J Food Microbiol **86:**11-22.
8. **Handelsman, J.** 2004. Metagenomics: application of genomics to uncultured microorganisms. Microbiol Mol Biol Rev **68:**669-85.

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9. **Hess, M., A. Sczyrba, R. Egan, T. W. Kim, H. Chokhawala, G. Schroth, S. Luo, D. S. Clark, F. Chen, T. Zhang, R. I. Mackie, L. A. Pennacchio, S. G. Tringe, A. Visel, T. Woyke, Z. Wang, and E. M. Rubin.** 2011. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. Science **331:**463-7.
10. **Jolly, N. P., C. Varela, and I. S. Pretorius.** 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res **14:**215-37.
11. **Li, L. L., S. R. McCorkle, S. Monchy, S. Taghavi, and D. Van der Lilie.** 2009. Bioprospecting metagenomes: glycosyl hydrolases for converting biomass Biotech Biofules **2:**1-11.
12. **Liu, G. L., Z. Chi, G. Y. Wang, Z. P. Wang, Y. Li, and Z. M. Chi.** 2013. Yeast Killer toxins, molecular mechanisms of their action and application Crit Rev Biotechnol**:**1-13.
13. **Santos, A., D. Marquina, J. A. Leal, and J. M. Peinado.** 2000. (1 -> 6)-beta-D-glucan as cell wall receptor for *Pichia membranifaciens* killer toxin. Appl Environ Microbiol **66:**1809-1813.
14. **Strauss, M. L. A., N. P. Jolly, M. G. Lambrechts, and P. van Rensburg.** 2001. Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. J Appl Microbiol **91:**182-190.
15. **van Rensburg, P., and I. S. Pretorius.** 2000. Enzymes in Wine making: Harnessing natural catalysts for efficient biotransformations - A Review. S Afr J Enol Vitic **21:**52-73.

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Chapter 2

LITERATURE REVIEW

Fungal hydrolases and their impact on wine microbial interactions and winemaking

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CHAPTER 2: Literature Review

Fungal hydrolases and their impact on wine microbial interactions and winemaking

2.1 Introduction

The fermentation of grape juice is a biological process involving a complex microbial network in which several genera and species of microorganisms (mainly yeasts and bacteria) interact. These microorganisms that constitute the wine microbial consortium (WMC) originate from grape surfaces, winery equipments and insects such as fruit flies, bees and wasps that act as vectors of dispersion (48). Unripe berries typically harbour microbial population up to 10^3 cfu/g berry while ripe berries may contain 10^4-10^6 cfu/g berry (47, 119) (Figure 1A). However, the microbial population may increase up to 10^8 cfu/g of berry on damaged grapes (8, 46). The early stage of spontaneous alcoholic fermentation is characterized by sequential development of yeasts typically dominated by non-*Saccharomyces* yeasts (10^7 cfu/mL) of the genera *Cryptococcus*, *Debaryomyces*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia* and *Rhodotorula* with *Kloeckera*/*Hanseniaspora* and *Starmerella* being the most dominant genera (70). At this stage of fermentation, *Saccharomyces cerevisiae* is present at a very low level usually around 50 cfu/mL (47). With the progress of fermentation, the non-*Saccharomyces* yeast population declines and the population of *S. cerevisiae* (10^7-10^8 cfu/mL) rapidly gains dominance (Figure 1B). The decline of non-*Saccharomyces* yeasts has been attributed to several factors including selective pressure exerted by increasing levels of ethanol and organic acids, low pH values, low oxygen availability, depletion of certain nutrients, as well as possible yeast-yeast interactions (e.g. killer toxins and other microbial peptides) (46).

Alcoholic fermentation is usually followed by malolactic fermentation (MLF), an important process in some wines as it is necessary for reducing acidity. In addition, MLF may enhance the sensory properties and improvethe microbial stability of wine (34). MLF is performed by Gram-positive and micro-aerophilic lactic acid bacteria (LAB), which are classified into two groups based on their catabolic end products. The homo-fermentative LAB produce only lactic acid as the sole product of sugar metabolism whereas, the hetero-fermentative LAB produce CO_2 and acetate along with lactic acid (78). The LAB population in grape must and wine mostly comprises *Lactobacillus hilgardii*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Oenococcus oeni*, *Leuconostoc mesenteroids, Pediococcus damnosus* and *Pediococcus parvulus* (Figure 1B). *Oenococcus oeni* is often the main bacterium conducting MLF, as it is best adapted to must and wine (78).

The wine bacterial population also includes acetic acid bacteria (AAB) which are characterized as Gram-negative, aerobic, catalase-positive rods belonging to the family *Acetobacteraceae* (50)

and are categorized into four genera: *Acetobacter*, *Acidomonas*, *Gluconobacter* and *Gluconacetobacter* (39, 103).

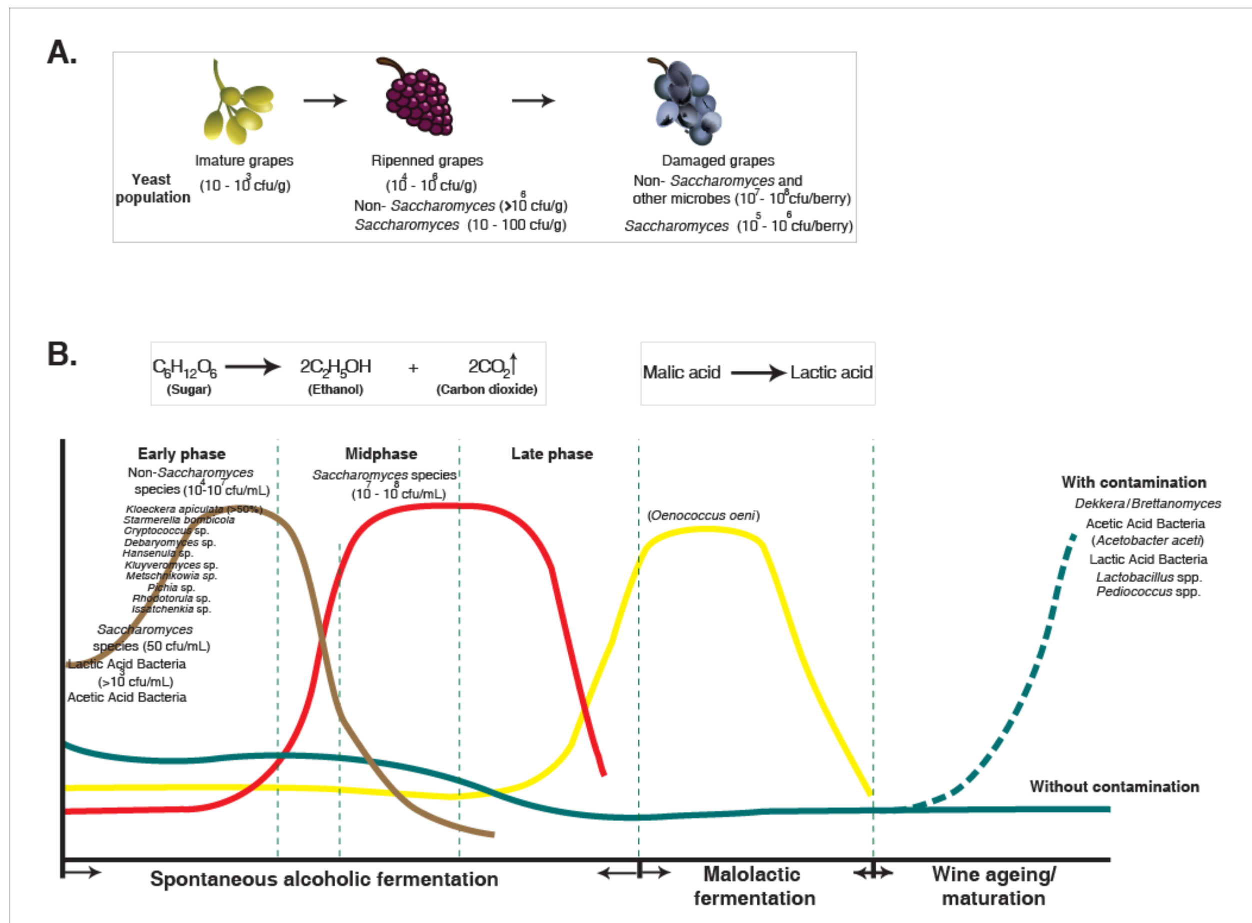

Figure 2.1 A representation of the wine microbial dynamics; (A) Depicts yeast population titers during different developmental stages of the grape berry and also of the damaged grape; (B) Shows the evolution of the wine microbial consortium throughout fermentation and during storage. Only major microorganisms are shown in the Figure. Other yeast and bacterial species may occur.

Gluconobacter oxydans usually dominates the AAB population (10^2-10^3 cfu/g) on the healthy grape berry surfaces while on damaged grapes, *Acetobacter aceti* and *Acetobacter pasteurianus* are typically dominant with levels reaching up to 10^5-10^6 cfu/g (7). The AAB population declines rapidly at the onset of alcoholic fermentation (<100 cfu/mL) due to the limited supply of O_2 (38, 139). However, under certain circumstances, these AAB can result in the spoilage of wine (9). The most common spoilage caused by AAB occurs in stuck fermentation or during wine maturation/storage when the wine is exposed to air. During this stage, wine spoilage yeasts such as *Brettanomyces bruxellensis* and *Zygosaccharomyces bailii* may occur and produce undesirable off-flavours as reviewed previously (6).

Apart from the common LAB and AAB populations, the grape must microbiota may also include other minor bacterial species of the genera *Chryseobacterium*, *Methylobacterium*,

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Sphingomonas, *Arcobacter*, *Naxibacter*, *Ralstonia*, *Frigoribacterium*, *Pseudomonas*, *Zymobacter* and *Acinetobacter* that do not play a significant role in wine fermentation (14).

2.2 Characterizing microbial diversity in wine

The diversity and dynamics of the WMC has been the subject of many investigations due to its direct influence on wine quality. Several methods/techniques have been developed and implemented for this purpose over the years. These methods are broadly classified as cultivation -dependent and -independent; their advantages and disadvantages are briefly described in Table 2.1. The most common methods employed in wine fermentation will be discussed in detail below.

2.2.1 Cultivation-dependent techniques

The complex microbial ecosystem of wine was first studied in 1866 using the technique of optical microscopy (120). This method was considered to be the first level of identification that enables us to visualize the cells and assign tentative identities based on their size and morphology. Identification was then accomplished through microscopy in conjunction with biochemical tests such as assessing oxidase activity, glucose fermentation and nitrate assimilation ability for bacteria (162) and evaluating the assimilation and fermentation of carbon compounds, assimilation of nitrogen compounds, vitamin requirement, high osmotic pressure, as well as acid production for yeasts (173). Following the discovery of PCR (112), molecular techniques such as PCR-Restriction Fragment Length Polymorphism (RFLP) were introduced to explore the wine microbial consortium.

PCR-RFLP involves the amplification of the phylogenetic marker genes such as 16S rRNA gene for bacteria and the ITS-5.8S rRNA-ITS2 gene (51) or the D1-D2 domains of the 26S rRNA gene of fungi (85). A restriction digestion of the amplified marker genes is carried out using endonucleases such as *Hae*III, *Hinf*I, *Cfo*I. Based on the different banding profiles the isolates are distinguished from each other and further identified by sequencing (42). In addition, the amplified genes of representative isolates can be sequenced and identified by comparing with sequences in known databases such as GenBank (3, 158). This enables us to identify isolates that although exhibiting identical colony characteristics on cultivation media may belong to different species. Thus, PCR-based culture-dependent techniques opened new doors in microbial ecology. Although RFLP is discussed as an example, several other molecular techniques are used for microbial ecology/taxonomic studies as listed in Table 2.1 (25).

The greatest limitation of PCR-RFLP and other similar techniques is that it only allows for the identification of cultivable microorganisms. During alcoholic fermentation, certain species outgrow the others (118) and the microbial populations that are numerically less abundant become difficult to recover through cultivation. Adaptation to the culture medium may also hinder the growth of certain cells since the transfer from their specific environment to a rich cultivation medium

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constitutes a shock that some cells may not be able to overcome (153). Also, because of the deprivation of certain nutrients from their growing environment or sudden adverse conditions such as the presence of an inhibitor, a change in the pH or temperature, some microorganisms enter into a viable but non-culturable (VBNC) state (137). These microorganisms are sub-lethally injured or viable but weakly metabolically active. They momentarily lose their ability to form colonies on solid cultivation media (72). In wine, acetic acid bacteria have been reported to enter into a VBNC state when they are deprived of O_2. Lactic acid bacteria and certain yeast species also enter into such a physiological state when exposed to sulphites (107). These cells can then only be enumerated by culture-independent techniques such as fluorescence microscopy. These VBNC cells cannot be isolated by the routine laboratory techniques that are commonly based on cultivation. For all these reasons, a high risk of underestimation of the microbial diversity occurs when using plating as a means to enumerate and identify live microorganisms in complex microbial environments such as wine (64). Moreover, cultivation is laborious and time consuming. In addition, the time required for the growth of the colonies causes delay and creates an additional bias as some species grow faster than others. *Saccharomyces* spp. indeed take approximately 2 days to grow in comparison to certain non-*Saccharomyces* yeasts that require more days to form visible colonies (69).

In order to circumvent these limitations, culture-independent techniques have been developed and optimized in an attempt to better characterize the microbial diversity of complex and dynamic ecosystems.

2.2.2 Cultivation independent techniques

The use of culture–independent techniques to monitor microbial population diversity and dynamics in wine has been growing since the beginning of the 21st century. These techniques involve the direct extraction of the nucleic acids present in a given sample. Various culture independent techniques such as DNA-DNA hybridization, whole cell hybridization, RT-qPCR, D/TGGE (Denaturing/Temperature Gradient Gel Electrophoresis) are employed to investigate the grapevine and wine microbiota (Table 2.1). The DNA-DNA hybridization (127, 151) or whole-cell hybridization (4) with taxon-specific probes were also used, giving a first overview of the entire microbial community including culturable microorganisms as well as those in VBNC state. Among all these, DGGE is the most commonly used molecular fingerprinting technique to investigate the microbial diversity throughout the grape ripening process and fermentation (128, 149) and therefore, the following paragraph will give a brief account of this technique.

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2.2.2.1 Denaturing Gradient Gel Electrophoresis

In both DGGE (45, 114) and TGGE (134, 136) the DNA fragments generated by PCR are of same length but vary in their nucleotide sequences and are separated based on decreased electrophoretic mobility of the partially melted double-stranded DNA molecule in a polyacrylamide gel consisting of a linear gradient of DNA denaturants (mixture of urea and formamide) or temperature. The ITS-rRNA region is typically the target of the PCR preceding DGGE but other genomic DNA regions have also been used for PCR-DGGE (128, 130). For instance, a study that investigated the bacterial diversity during the malolactic fermentation of wine made use of the *rpo*B gene as the target phylogenetic marker gene for PCR-DGGE (138). In most studies, the bands are excised and the DNA is eluted. Thereafter, the DNA fragments are sequenced and analyzed for identification of the community members (113), based on comparison with previously established databases.

PCR-DGGE has been used to monitor the diversity and dynamics of yeast and bacteria from fruit-set in the vineyard and throughout fermentation of different types of wine including red, white, and botrytized wines (108, 125, 128-130). Studies employing DGGE were the first to clearly demonstrate microbial dynamics during grape berry development (125, 129). While there were correlations between the yeast community evolution and berry development, the same could not be observed for bacteria(129). The berry surface was shown to harbor a diverse community of basidiomycetous yeasts and biofilm forming ascomycetous yeasts as well as the yeast-like fungus *Aureobasidium pullulans* during the early developmental stages and that this population is gradually replaced by fermentative yeasts as the berry reaches full ripeness (125, 129). Furthermore, it was shown that the yeast dynamics during fermentation are very similar in different wines and consistent with what has been observed through culture-dependent studies, with the non-*Saccharomyces* yeast population showing a decline towards the middle of fermentation, while the bacterial population dynamics might differ. For instance, Renouf and colleagues (130) demonstrated that the bacterial diversity in white wines was higher and the population remained for longer periods in white wine fermentation than in red wine. DGGE also confirmed observations from culture-dependent studies which show that *Saccharomyces* spp. are minor species on the grape surface (<10%).

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Table 2.1 List of different techniques for microbial diversity studies with advantages and disadvantages

Technique principle	Technique	Advantages	Disadvantages
Microscopy	Visualization of microbial cells	Visualization of cell morphology, viability and abundance of the microorganism	Biochemical tests are required to confirm the identification of microorganisms
Cultivation based PCR-independent	Growing microbial cells on synthetic media	Complete description of colony characteristics, pure culture could be obtained	Less abundant microbes could not be grown easily and unculturable microorganisms are not retrieved. Misinterpretation of the microbial biodiversity in complex ecosystems
Cultivation based PCR-dependent	PCR-RFLP	More accurate method for microorganism identification by restriction digestion	RFLP data is non-interpretable when applied on complex microbial mixtures and their restriction products get superimposed
Culture independent PCR dependent	DGGE/TGGE	Microorganisms in complex ecosystems could be detected in a short period of time	Microorganisms cannot be detected at a low titre level, identical Tm of PCR products of two microorganism might lead to possible co-migration on the gel, casting of the gel is technically challenging
	ARISA	Very sensitive technique, sequences with a single nucleotide changes could be identified, microbial abundance and diversity studied in a short period of time	Cannot identify the microorganism because the DNA fragments cannot be retrieved from the capillary electrophoresis
	NGS	High throughput analysis of complex microbial communities using short DNA amplicons, analyze 100-1000 samples on single platform	Poor read quality gives inaccurate taxonomical assignment and alpha diversity assignment for microbial communities
	SSCP	Restriction enzymes are not required, resolved bands can be isolated and sequenced	High concentrations of single stranded DNA might cause re-annealing of the DNA
Culture independent and PCR independent	DNA-DNA hybridization	Specific probes identify group of microorganisms	Limited to indentifying a small number of known species
	FISH	Identify several species using a set of fluorophore-labeled probes	Cannot identify the non-viable cells

Overall DGGE tends to reveal higher species diversity than culture-dependent methods. However, this method also has some limitations. For instance, its detection limit decreases to 10^4 cfu/ml in the presence of a high *S. cerevisiae* population (25). This is also a challenge in performing an inventory of yeast species on the berry surface due to differences in the ratio of major and minor species which can sometimes exceed a 1000 fold, thus making the detection of the minor species difficult, while inefficient DNA extraction might limit the retrieval of certain yeasts e.g. *Cryptococcus* species (125, 128). Possible co-migration of DNA fragments that have a certain amount of sequence variation may prevent the isolation of individual bands. The existence of sequence micro-heterogeneity could also lead to overestimating certain microbial populations, as shown in the previous study (82). Nevertheless, DGGE remains an important tool deciphers the microbial diversity in wine as it can reveal more diversity. For instance, Mills and colleagues (108)

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showed that some yeast species such as *Hanseniaspora* spp. that could not be distinguished through culture dependent studies due to similar colony characteristics could be resolved by DGGE. However, some deficiencies associated with this method including possible failure to detect common yeasts such as *Metschnikowia pulcherrima* even when present at concentrations above the detection threshold for PCR-DGGE, as highlighted in a previous study (108) suggests that this method will always need to be applied in conjunction with other methods.

2.2.2.2 Automated Ribosomal Intergenic Spacer Analysis

Another culture independent technique which is frequently used for determining the microbial diversity and estimating the microbial population is ARISA (Automated Ribosomal Intergenic Spacer Analysis). This technique has been widely used on various habitat like soil, aquatic environments and human gut (81). Recently, it has been successfully implemented on Slovakian wine matrix to assess yeast diversity and population dynamics (18, 23, 83, 178). The studies successfully identified yeast isolates from different wineries by using ARISA, therefore demonstrating the suitability of the technique. Furthermore, using this technique the authors also monitored the yeast population dynamics at different stages of fermentation (18). The authors highlighted that this technique is rapid, effective, inexpensive and useful to analyze a large number of samples. Once again, the ITS (Intergenic Spacer) region is used as a 'barcode' for the fungal and eubacterial taxonomy. A PCR-based amplification of the ITS region with the oligo-nucleotide primers in which one of the primers (usually the forward primer) is labelled with fluorescent markers such as FAM (Carboxy-fluorescein) (44, 61). Thereafter, the amplified labelled PCR products along with a size standard are subjected to capillary electrophoresis (e.g. ABI310XI genetic analyzer) to obtain an electropherogram of different fragment lengths and intensities. Genotyping software packages such as GeneMapper 4.1 software (5) convert the fluorescent electropherogram (operational taxonomic units- OTUs) into peaks indicating the fluorescent intensity which are further considered for calculating the fragment size by comparing with the size standard. The fluorescence intensity of each of these peaks indicates the abundance of each of the microorganisms present in the sample. Although this technique provides information about the microbial diversity and abundance in a relatively short period of time, reliable taxonomic assignment of the peaks remains a challenge (Table 2.1).

The PCR-based and culture-independent techniques provide extensive information regarding the species present in the environment but a large amount of genetic information is missed because of its targeted approach. Therefore they often fail to provide enough information regarding the genetic functionality of the microbes in a complex community.

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2.2.2.3 High-Throughput rRNA amplicon sequencing

More recently, high throughput Next Generation Sequencing (NGS) has been used as a molecular tool for phylogenetic analysis. DNA is directly extracted from the matrices and the rRNA-encoding genes amplified for taxonomical classification. Microbial diversity in grape must and wine has been investigated using different sequencing platforms (14, 15, 33, 123). As it can be expected, these approaches revealed much higher diversity compared to other culture-independent studies. In fact, David and colleagues (33) demonstrated this by comparing yeast diversity retrieved through ITS-RFLP and DGGE during fermentation. In this study more than 16 yeast species were identified by rRNA amplicon sequencing in comparison to 5 and 7 by ITS-RFLP and DGGE, respectively from the grape berry surface. Moreover, as expected, the diversity decreased during fermentation as detected by all the techniques, but a disparity was noticed in the abundance of the individual species as detected by NGS in comparison to culture dependent techniques. This observation suggest that there is a high probability of misinterpretation of results derived from cultivation based approach (33). More recently, the 454-pyrosequencing of rRNA amplicons of the metagenomes sampled from the grapevine leaves were conducted during the vegetative cycle. The result indicates the abundance of Ascomycetous fungi in comparison to Basidiomycetous. The authors also identified a high diversity of Proteobacteria, Fimicutes and Actinobacteria and found the yeast-like fungus *A. pullulans* and Enterobacteriocae in abundant (123).The abundance of *A. pullulans* in different grapevine tissues is consistent across all methods and confirms that this fungus a well-established resident organism on grapevine. Identical study conducted previously (14) to determine the bacterial diversity and has compared the depth of NGS with the cultivation based technique, Terminal Restriction Fragment Length Polymorphism (TRFLP). The study used the bacterial 16S rRNA gene as barcode to demonstrate the bacterial communities of the fermenting must and clearly highlighted the minor bacterial population along with the dominant LAB species which was never shown before. For instance, the identification of the members from the group of *Sphingomonas* and *Methylobacterium* after 51 days of fermentation clearly shows that these bacteria are capable of surviving well in the wine fermentation. More recently a study (14) using the same approach demonstrated that the microbial diversities of the fermenting must depends on the grape variety and the site and location of the vineyard. For instance, both the fungal and bacterial communities varied across the different grape growing regions. Additionally the authors also demonstrated that the climatic features have a deep influence on the vine grape microbiota which ultimately influences the microbial communities in the grape must. This study clearly evidences a link between the vineyard environment and the grape vine/must microbial consortium. Nevertheless, as discussed above, all these techniques are usually targeted to specific genes and therefore, a large amount of genetic information is missed. Whole metagenome sequencing approaches on the other hand provide an opportunity to capture the entire genetic information available. These not only identify the microbial populations but also

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enable us to understand the microbial community structure and function in a given ecosystem. Whole metagenomic sequencing approaches have improved the retrieval of novel extracellular enzymes, peptides and other biocatalysts from various environments but are yet to be applied in the wine ecosystem.

2.3 Microbial enzymatic activity during wine fermentation

The yeasts and bacteria that constitute the WMC produce an array of metabolites such as terpenoids, esters, higher alcohols, glycerol, acetaldehyde, acetic acid, succinic acid of oenological interest that have shown to contribute to the aroma properties of the finished wine (69). Apart from free volatile flavour compounds that are present in the grape berries, most of them are released through enzymatic hydrolysis of the odourless non-volatile precursor compounds (163). Studies have shown that these biochemical reactions are driven by hydrolytic enzymes (glycosidases, β-glucosidases, esterases, lipases, pectinases, etc.) which do not only originate from grapes but mostly from yeasts and bacteria. Although these enzymes have their own biological functions in modelling the yeast cell wall structure, except for lipases, glycosidases (β-xylosidases, arabinofuranosidases), β-1,4-glucanases, glucosidases, pectinases and esterases, they may indeed catalyze various reactions on substrates present in grape juice. These enzymes from the pre-fermentation stage, through fermentation, post-fermentation and aging, play a pivotal role in the biotransformation of grape juice to wine (163). Studies have screened these extracellular enzymes from culturable wine non-*Saccharomyces* yeast isolates (21, 155). Recent reports have also suggested that some of these yeasts display antagonistic activities against other yeasts, probably by damaging their cell wall as reviewed earlier (90). It is hypothesized that these yeasts might play some role in driving the microbial population dynamics. A few studies also showed that killer activity may be mediated through hydrolytic enzymatic properties (24, 26, 66).

2.3.1 Hydrolysis of grape macromolecules

Yeasts secrete a wide range of extracellular enzymes (98). Some of these enzymes were found to have potential applications in the biotechnological sector; therefore their diversity and characteristics have been and are still actively researched. Various environments are being explored to isolate yeasts and enzymes that would be adapted for various industrial applications.

In wine, it has been reported that the presence of selected non-*Saccharomyces* yeasts contributes positively to the sensory properties and chemical complexity of the final product (40, 57, 150). Unlike *S. cerevisiae*, several non-*Saccharomyces* yeasts secrete an array of enzymes (e.g. glucanases, glucosidases, proteases, and pectinases) that are active under winemaking conditions (Table 2.2) (70). For instance, studies have shown that *Hanseniaspora* spp., *Debaryomyces* spp., *Candida* spp., *Pichia* spp. and *Torulaspora* spp. produce extracellular hydrolytic enzymes such as glucosidases, pectinases and proteases (21, 155). Moreover, the

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secretion of extracellular enzymes such as glucosidases, pectinases, proteases, amylases and xylanases of oenological relevance was demonstrated in pure and mixed fermentations of *S. cerevisiae*, *T. delbrueckii* and *H. vineae*. These findings suggested that, although non-*Saccharomyces* yeasts are outnumbered by *S. cerevisiae,* their enzymes might be still active to the end of the fermentation. More importantly, these enzymes are found to be active at high glucose concentration as well (104). These extracellular enzymes catalyze different types of reactions in must/fermenting grape juice (Table 2.2). For instance, the hydrolysis of the non-volatile precursors from grapes carried out by glycosidases releases the volatile compounds, thereby improving the wine aroma (21). β-Glucosidases can catalyze the release of grape terpenes, thiols from their sugar moiety, thereby making these compounds fragrant, contributing to the aroma of wine. Pectinases (e.g. polygalacturonases) lower the viscosity of the grape juice, increases the juice extraction and improve wine clarification and facilitate the filtration (163). Moreover, pectinases also play a major role in the extraction of polyphenolic compounds such as anthocyanins and proanthocynadins from the grape skin and seed cell wall, respectively (117), maintaining the sensory balance of wine and the mouth feel as well. Although not yet commercialized, some of the non-*Saccharomyces*' proteolytic activities have been shown to hydrolyse proteins, including those responsible for haze formation, ensuring the protein stability of the finished wine (21). It has been reported that although filamentous fungi do not participate in the wine fermentation, they secrete different enzymes such as pectinases (163). In fact, most of the commercially prepared enzymes are derived from bacteria and fungi (43).

2.3.2 Fungal cell wall degrading enzymes

Yeast and filamentous fungi produce a cocktail of hydrolytic enzymes which are closely associated with the cell wall. These enzymes mainly include glucanases and chitinases some of which also exhibit transglycosylase activity. These enzymes are pivotal in maintaining cell wall plasticity and are involved in the breakage and re-forming of bonds within and between polymers leading to the re-modelling of the cell wall during growth and morphogenesis (1). However, these enzymes have also been shown to be necessary in mycoparasitic interactions. Mycoparisitism is a well-established relationship between fungal species where one fungus parasitizes the other either by producing haustoria and penetrating into the host to absorb nutrients from living fungal hyphae (biotrophism) or by invading and destroying the fungal cell wall and feed on the dead cell contents (necrotrophism). Several enzymes belonging to classes of chitinases (161), α-(1,3)-, β-(1,4)-, β-(1,3)- and β-(1,6)-glucanases (36, 144) and proteinases (124) are reported to be mainly involved in mycoparisitism or induced under mycoparisitism-related growth conditions. Mycoparisitism is extensively studied in filamentous fungi, but this phenomenon has also been demonstrated in yeasts. Several yeasts, including *M. pulcherrima*, *Candida oleophila*, *Pichia guilliermondii* and *A. pullulans* have been shown to exhibit antagonistic behaviour against grape associated filamentous

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fungi such as *Botrytis cinerea* and *Penicillium* spp. Extensive production of extracellular cell wall lytic enzymes is thought to promote attachment of the yeast/mycoparasitic cells to fungal hyphae and partial degradation of the mycelia of the prey (1, 179).

Similarly, *Pichia membranifaciens* FY-101 was shown to display antagonistic activities against *B. cinerea* on the grapevine plantlets. This antagonistic action has been shown to be mediated through extracellular β-1,3-glucanases (102). Recent studies seem to suggest that cell wall hydrolytic enzymes play a significant role in yeast-yeast interactions especially interference, competition since several non-*Saccharomyces* yeasts have been shown to secrete killer toxins that also display glucanase activity (91). However, the association of the yeast killer toxin and the hydrolytic cell degrading enzymes is not a well-established relation. Also, even though there is growing evidence to support the possible involvement of cell wall degrading enzymes in the action of yeast antagonists, it is not known whether these enzymes are active during wine fermentation and if they influence yeast dynamics.

Table 2.2 Yeast derived enzymes of oenological interest and their primary physiological role (21, 46, 70, 155, 161, 163)

Enzymatic activities	Catalytic activity	Primary biological functions	Oenological relevance	Producing yeast
Chitinase	β-1,4-glycosidic bonds between N-acetyl glucosamine residues	Cell wall recycling during ageing, autolysis and cell wall remodelling during active growth	Unknown	*M. pulcherrima*, *M. fruticola*, *C. albicans*, *Rhodotorula glutinis*, *Lodderomyces elongisporus*
Glucanase	β-1,3-, β-1,3-1,6-glycosidic linkages glucans	Re-modelling of the cell wall during growth and morphogenesis	Hydrolyzes non-volatile glycosidic precursors of grapes to odorous volatiles; increases wine flavour and aroma	*Starmerella bombicola*, *C. hellenica*, *Kloeckera apiculata*, *Pichia farinosa*, *P. kluyverri*
Glucosidase	β-1,4-D-glycosidic linkages	Typically involved in cell wall maintenance, cell septation	Hydrolyzes non-odorous glycosidic precursors of grapes to odorous volatiles; enhances wine flavour and aroma	*M. pulcherrima*, *K. apiculata*, *W. anomalus*
Proteolytic	-CO-NH-peptide linkages	Intracellular protease are involved in degradation of damaged and unneeded proteins; extracellular proteases are involve in release of assimilable nitrogen, pathogenesis	Decrease the protein content and brings stability to wine	*S. bombicola*, *M. pulcherrima*, *K. apiculata*, *Debaryomyces hansenii*
Pectinase	α-D-1,4-linked galacturonic acid residues	No function	Increase juice extraction from grapes by lowering the viscosity, improve wine clarification and filtration	*S. bombicola*, *C. oleophila*, *M. pulcherrima*, *C. valida*, *K. apiculata*

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2.3.3 Yeast killer toxins

According to a previous study (94), yeast killer activity occurs via the production of exotoxins that interact with specific cell wall receptors on the sensitive cells of same or congeneric species. A substantial amount of studies over the years have enriched our knowledge of killer toxins, in particular, their nature, structure, synthesis and mode of action. Killer toxins were first identified in *S. cerevisiae* (12) and later in other yeasts as well. *S. cerevisia*e's killer toxins are characterized as low molecular glyco-proteinaceous compounds that display killing properties against sensitive cells of the same or different yeast genera. These killer strains are immune to their own toxin but may be sensitive to the other types of toxins (152, 177). Most of these killer toxins are protease sensitive, heat labile (maximum temperature tolerance 25°C) and active only under acidic pH (16, 17, 101, 168). These killer toxins are encoded by cytoplasmically inherited dsRNA viruses, linear dsDNA plasmids and nuclear genes as well (94, 99).

2.3.3.1 Non-*Saccharomyces* killer toxins and their killer phenotypes

Non-*Saccharomyces* toxin-producing killer strains have been identified in the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Pichia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Ustilago*, *Torulopsis*, *Williopsis*, *Zygosaccharomyces*, *Aureobasidium*, *Zygowilliopsis* and *Mrakia* (90, 100). These genera display killer activity towards a wider range of species although the specific sensitive species vary tremendously depending on killer species or strain. For instance, *Williopsis saturnus* strain DBVPG 4561 showed antimycotic properties against *Candida glabrata*, *Issatchenkia orientalis* and *P. guilliermondii* whereas strain WC91-2 displayed killer activity against *Saccharomyces* spp. W0, *Candida albicans*, *Candida tropicalis*, *Cryptococcus aureus*, *Yarrowia lipolytica* and *Lodderomyces elongisporous* (167).

Some of them occasionally display killer activity against wine strains of *S. cerevisiae* (47). For instance, *Schwanniomyces occidentalis* secretes a killer toxin lethal to *S. cerevisiae* (22). The spectrum of the killer phenotype exhibited by the filamentous fungus *Ustilago maydis* has been well characterized. Three strains of *U. maydis*, P1, P4 and P6 are reported to secrete killer toxins KP1, KP4 and KP6, respectively. These strains are immune to these toxins but other strains of *U. maydis*, are susceptible to them (35, 56, 77, 80). Recently, it has been shown that KP6 has a distinct molecular structure and mode of action from KP4 (13.97 kDa) and KP1 (32.01 kDa). KP6 is a 24.20 kDa neutral protein with α (KP6α) and β (KP6β) subunits. KP6α binds to the receptor while KP6β causes the lethal action to the targeted cell (2). The KP6 toxin exhibited the ability to inhibit the *B. bruxellensis* but *S. cerevisiae* is fully resistant to it (141). Genetically, non-*Saccharomyces*

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killer toxins are encoded by dsRNA viruses (e.g. *Hanseniaspora uvarum*, *Z bailii* and *U. maydis*) (141, 148, 157), linear dsDNA plasmids (e.g. *K. lactis*, *P. acaciae*, *P. inositovora*) (76, 147) and chromosomal DNA genes, e.g. in *P. farinosa*, *P. kluyveri*, *W. anomalus* and *W. mrakii* (147).

Table 2.3 Representation of the antagonistic activities caused by different killer toxins/hydrolases secreting non-*Saccharomyces* yeasts

Killer toxins and associated enzymatic activity	**Producing yeasts**	**Susceptible yeasts**	**Note**	**References**
Zymocin: (α,β,γ subunits) α/β shows exo-chitinase activity, γ subunit shows killer toxin activity	*Kluyveromyces lactis*	*S. cerevisiae*	Hypothesized to play some role in fungal-fungal interactions	(19)
Panomycocin shows exo-β-1,3-glucanase	*W. anomalus*	Various species of yeast (e.g. *B. cinerea*) *in vitro*	Designated as topical antifungal agent	(67)
β-1,3-glucanase	*P. anomala* YF07B	Pathogenic yeast in crab	-	(166)
β-1,3-glucanase, chitinase and protease	Twelve species of *Debaryomyces hansenii* strains	*Penicillium italicum*	Biological control of blue mould decay of Mexican lemon	(110)
β-1,3-glucanase	*P. guilliermondii*	Degrades the fungal cell walls of *B. cinerea*	Used as a post harvest bio-control yeast	(169)
Alkaline serine protease	*A. pullulans* strain PL5	*Monilinia laxa* on stone fruit and *Botrytis cinerea* and *Penicillium expansum* on pome fruit	Biological control of postharvest pathogens	(179)
β-glucanases	*W. anomalus* strains BS91, BCA15, BCU24	*S. cerevisiae* CEN.PK2-1c reference strain	-	(111)

As mentioned in section 2.3.2 studies have shown that the killer phenotypes of some of the non-*Saccharomyces* yeasts are strongly associated with yeast cell wall hydrolysing enzyme activities. For instance, non-*Saccharomyces* wine yeasts of the genera *Pichia* and *Kluyveromyces* have been shown to inhibit the growth of *Dekkera*/*Brettanomyces* spp. and their antagonistic activities are mediated through the extracellular hydrolases. For instance, PMKT2 secreted by *P. membranifaciens* has inhibitory effect against a variety of spoilage yeasts like *B. bruxellensis* and other fungi of agronomical interest. Its mode of action is mediated through β-1,6-glucanase activities. It has been reported that the toxin primarily binds to the β-1,6-D-glucan as the primary

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receptors on the cell wall (140). Table 2.3 provides a few examples of killer toxins of non-*Saccharomyces* yeasts, their origin and associated extracellular hydrolytic activities.

The killer activity of these yeasts has increasingly gained interest over the years. Some of the yeasts or their toxins are currently being used as postharvest bio-control agents and also in the food industry as bio-preservatives for controlling the development of harmful yeasts and bacteria (31, 143). Antagonistic yeasts such as *M. pulcherrima*, various species of *Candida*, *Pichia*, *Cryptococcus* and some *Saccharomyces* and *Zygosaccharomyces* have been proposed to be used as natural bio-control agents. For instance, *C. oleophila* that exhibit glucanase activity has been registered for commercial use (46). Moreover, the killer toxins of *P. membranifaciens* (PMKT2) and *T. phaffii* (KpKt) (24) have also been suggested for use as bio-control agents in wine. KpKt has already been tested under winemaking conditions (27), and its activity against *B. bruxellensis* was shown to be stimulated under winemaking condition (142). This suggests that the production of killer toxins by non-*Saccharomyces* yeasts could be important drivers of population dynamics during wine fermentation.

2.3.3.2 Killer toxins and their impact on yeast population dynamics

As mentioned in the section 2.3.2.1, *S. cerevisiae*'s killer toxins have been well characterized. Under wine making conditions however, they usually do not play a major role in selection and evolution of different yeast species because of their narrow spectrum of killer activity, mainly directed against other strains of *S. cerevisiae*. Many factors affect the expression of these killer toxins (46). The killer toxin production by *S. cerevisiae* might be a metabolically expensive process. A study showed that these killer toxins are produced by the yeast cells when dispersal is allowed under abundant nutrient sources (170). These findings actually contradict the previous hypothesis that under nutrient deficiency, the yeast cells adopt the killing strategy as the ultimate way for their survival in a highly competitive environment (122). A Later study has shown that in a mixed fermentation where both the *Saccharomyces* killer strain and sensitive strain were used, a stuck/sluggish fermentation was observed. The possible cause cited was primarily the interactions between these two strains. These interactions were dependent on the inoculum size, the amount of nitrogen present in the must initially and the time point when nitrogen was added to the media during the fermentation because of the nitrogen demand of the strains in competition (105). It has also been reported that the killer phenotypes exhibited by *S. cerevisiae* might be essential for their establishment and maintenance of dominance till the completion of the alcoholic fermentation. For instance, the effect of killer phenotype on the establishment of a cellar population was investigated over a 5-year period. The authors demonstrated that different killer positive strains of *S. cerevisiae* co-exist at different fermentative stages and the dominant phenotype varies with the year (53). These interactions between killer and sensitive cells may have a positive impact on the wine

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quality. For instance, a study has demonstrated that an interaction between 2 sensitive and 2 killer *Saccharomyces* strains could initiate the autolysis of the sensitive cells that releases the proteinaceous compounds, enriching the wine quality (159). However, the ecological benefits attributed to the killer toxin producing *S. cerevisiae* strains are not well understood.

Considering the facts that *S. cerevisiae* killer activities is not a predominating factor for driving the wine microbial population dynamics and also as not much investigations were done on the non-*Saccharomyces* killer toxins, it would be interesting to elucidate how the latter influence yeast-yeast interactions and drive the microbial population dynamics in a spontaneous/inoculated fermentation. Considering the antagonistic role played by these non-*Saccharomyces* yeasts, as mentioned above, mediated through hydrolytic enzymes/ killer toxins and potential role in microbial interactions, it is therefore necessary to search for more indigenous extracellular enzymes produced by these autochthonous wine yeasts and to identify the potential roles they play in the population dynamics.

2.4 Screening and isolation of novel enzymes/killer toxins

The wine microbiome is a rich resource of various biocatalysts which are relevant in winemaking but may also be employed in other applications such as postharvest disease control and other beverage fermentations. For many years, the production of such biocatalysts especially hydrolytic enzymes has been mostly evaluated in wine yeasts using culture dependent studies. However, these approaches were limited to a few cultivable yeast species. In the past decade, there has been tremendous growth in the use of metagenomic and high-throughput approaches to tap into the broader microbial diversity present within a given ecosystem. This has resulted in the retrieval of novel biocatalysts from different matrices and ecosystems. However, the application of these techniques in wine fermentation remains limited.

2.4.1 Metagenomics as a tool for bioprospecting

Metagenomics refers to genome analysis of microbial assemblages directly from natural environment without prior cultivation process. The word metagenomics was first coined by Handelsman (55). The direct cloning of environmental samples was first proposed by Pace (1986) but only occurred in 1991(146). This tool offers access to the unculturable microbes which could otherwise be missed when using a cultivation-based approach. Since this tool was developed, it has been employed on a number of matrices such as rice straw compost, sugarcane bagasse, gut microbiota of abalone etc. (71, 74, 176) resulting in the discovery of a wide variety of novel enzymes (endoglucanases, chitinases, cellulases, etc.). The metagenomic bioprospecting exercise usually involves the extraction of community DNA, followed by either construction of the metagenomic libraries of various insert sizes in suitable vectors or direct sequencing of the DNA.

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The extraction of environmental genetic material is one of the important steps for successful construction of a metagenomic library and also for direct sequencing. A major hurdle associated with it is the co-extraction of other compounds with the nucleic acids. In the case of grape juice/wine the polyphenols, that crosslink with the plant cell wall polysaccharides, get co-extracted with the nucleic acids and bind irreversibly to them (29, 52, 92, 132). To circumvent this problem, the cells are typically washed with Polyvinyl Pyrrolidone (PVP) (68). PVP binds to polyphenols preventing them from adhering to DNA, making them accessible to all enzymes (93). However, studies have also shown that DNA from wine sample could be extracted by using DNAeasy Blood and Tissue kit (Qiagen, Hilden, Germany) (18) and Qiagen Faecal DNA Extraction Kit (14) as well.

The extracted metagenome can be subjected to either a metagenomic library construction or direct sequencing. The metagenomic library construction depends on suitable cloning vectors and host strains. Initially, researchers used to clone small fragments in sequencing vectors (plasmids) to generate small insert libraries (<10 kb). However, these libraries reduce the likelihood to detect large gene clusters or operons and therefore increase the chance of missing important sequence information (58). To overcome this limitation, large insert libraries are constructed using cosmid, fosmid and Bacterial Artificial Chromosome (BAC) as vectors. Cosmids and Fosmids can indeed accommodate 25–35 kb (41) and 40 kb (10) fragments, respectively whereas BAC can take-up insert sizes up to 200 kb (11, 135). The three main features of these vectors can be summarized as follows: (a) they can be induced for controlled copy number genes which could allow high level gene expression for ease of detection during screenings, (b) ability to hold large inserts stably, and (c) ability to replicate in different hosts with minor modifications. Large insert size libraries have advantage over the small insert size libraries where the number of clones to be screened for positive hits is comparatively lower than when dealing with latter one. Nevertheless, the large insert libraries are technically challenging to construct. *E. coli* is always the preferred host organism for cloning and expression of any metagenome-derived genes.

Alternatively, direct sequencing of the metagenomes which does not require the construction of a clone library can be employed. The metagenomes extracted from various matrices are directly sequenced using different sequencing platforms (e.g. 454 pyrosequencing, Ion Torrent, Illumina, SOLiD). The sequencing data obtained are used for both metagenomic phylogenetic and functional profiling.

Metagenomic libraries can be subjected to two types of analysis based on the biological function of the sought proteins (sequence-independent approach) or on their genetic sequence (145) (Figure 2). Both of these approaches have their limitations and advantages which are discussed in the following sections.

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2.4.1.1 Function-based screening

Function-based analysis of metagenomic libraries involves heterologous gene expression and subsequent screening for the desired trait, usually an enzymatic activity easily detectable using direct plate assays or biochemical tests. Various enzymes such as endoglucanases, β-glucosidases, proteases, chitinases, cellulases have been retrieved from various metagenomic libraries through this process (13, 49, 106, 172, 174) (Table 2.4). Using this technique, novel genes can be detected as it is entirely independent of any sequences of known genes, unlike techniques that rely on the identification of conserved motifs in known sequences. However, there are certain limitations to the function-based analysis. It has been found that very low number (0.001% - 0.01%) of positive clones are obtained following the screening of several thousand clones (58, 59, 95). For instance in a study of the microbial metagenome extracted from sea water, only 11 chitinase-encoding genes were obtained following screening of 825,000 clones from sea water (28). This has been attributed to the fact that a substantial amount of transformed genes cannot express in a single heterologous hosts due to codon usage differences, improper or no recognition of the promoter sequences, ribosomal entry, lack of proper initiation factor, improper protein folding due to the absence of the necessary chaperons in the host cell, absence of essential cofactors, accelerated enzymatic breakdown of the gene product, inclusion body formation, gene product toxicity, and lack of the secretory signals in the host to secrete the gene product (156). Also, there are various compounds that adhere to the DNA fragments, inhibiting the enzymatic reactions required for cloning, causing potential cloning biases as reviewed earlier (133).

Table 2.4 Representation of various metagenomic libraries constructed from different environmental sources, strategies of screening and the gene retrieved

Environment	Substrate used for screening	Vector Type	Target gene	References
Vermicomposting of paper	Carboxymethylcellulose (0.25%)	Fosmid	Endo-β-1,4-glucanase	(174)
Biogas digester	Carboxymethylcellulose (0.5%), esculin hydrate (0.1%) and ferric ammonium citrate (0.25%)	Fosmid	Endo-β-1,4-glucanase, β-glucosidase	(172)
Forest soil	AZCL-casein (1g/L)	Plasmid	Alkaline Serine protease gene	(13)
Tinto river (Acidic environment)	HCl (37%, w/v)	Plasmid	Acid resistant protease genes	(49)
Bioreactor samples	2,4- Dinitrophenyl β-cellobioside	Fosmid	Cellulase	(106)

To circumvent this problem, a multiple host expression system has been adopted by certain authors in order to diversify the available expression machinery, thereby enhancing the chance of successful gene expression. For instance, a study conducted by Craig et al. (2010) (30) reported on the construction of soil metagenomic libraries in IncP1-α broad-host-range cosmid vector and their transformation into 6 proteobacterial host strains such as *Agrobacterium tumefaciens*, *Burkholderia graminis*, *Caulobacter vibrioides*, *E. coli*, *Pseudomonas putida* and *Ralstonia mettalidurans*. The functional screening of these bacterial clones based on three phenotypic traits (i.e. antibiosis, pigmentation and colony morphology) exhibited a diversified expression profiles between different hosts with a little overlap. In another study, a wide host-range cosmid was used to construct environmental libraries in *E. coli*, *Pseudomonas aeruginosa* and *Rhizobium leguminosarum*. It was observed that each of these libraries when subjected to function-based screening revealed different classes of novel alcohol dehydrogenases and tryptophan biosynthetic genes (89). This confirms the importance of using multiple hosts for screening metagenomic libraries as this increases the chance of retrieving novel genes originating from different groups of microorganisms.

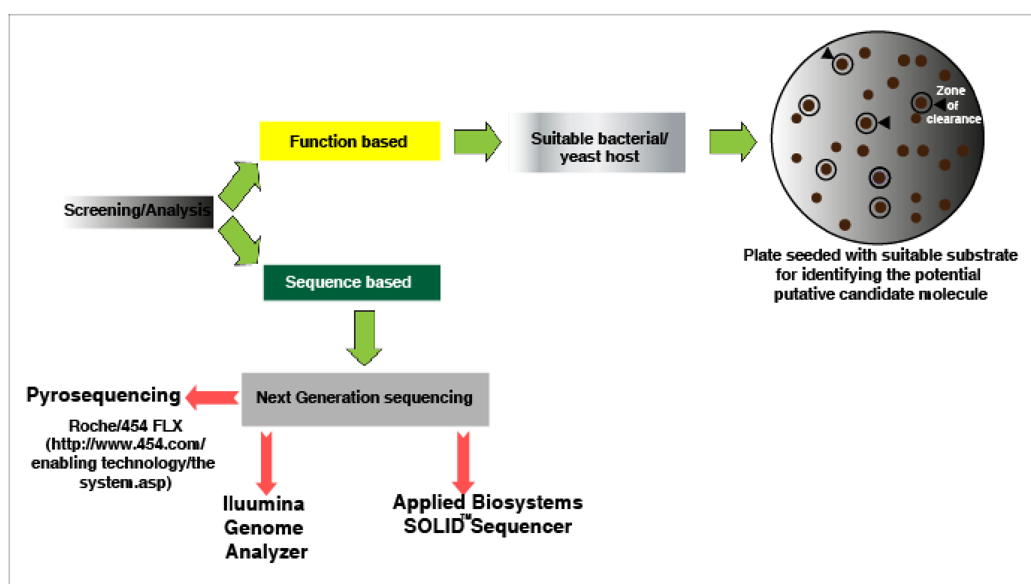

Figure 2.2 Schematic representation of the analysis adopted for the metagenomic library.

2.4.1.2 Sequence-based screening

The large insert size libraries as well as the extracted metagenomes can be subjected to Next Generation Sequencing (NGS). Direct sequencing of the metagenomes circumvents the problem associated with the cloning for a metagenomic library. Studies have shown that sequence analyses of the metagenome evaluated its functional potential and also could successfully identify and heterologously express the novel gene sequences. For instance, a study was conducted to identity

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the biomass degrading genes and genomes from the rumen of the cow. About 268 gigabases of the metagenome sequence data was generated from which 27,755 putative carbohydrate-active genes and 90 active proteins were retrieved, of which 57% has shown activity against cellulosic substrates. The average ORF length was around 542 bp and more than half of the ORF represented the full genes (60). A similar study conducted on the metagenomes of soil samples that had a prolonged exposure to chitin, identified an array of GH18 chitinase sequences through 454-pyrosequencing. Furthermore, a full length chitinase open reading frame was also isolated from the sequences and successfully cloned and expressed in *E. coli* (154).

Although direct sequencing of metagenomes bypasses the difficulties associated with the cloning of the metagenomes for library construction and heterologous expression, it does have some limitations as well (88). Firstly, it only identifies the genes that are homologous to known conserved sequences and secondly, the chance of retrieving full ORFs depends on the sequencing depth. For instance, when NGS was first implemented, a study could identify only 3 bacterial and archeal lineages from whole metagenomes of acid mines drainage (32). With the advances in the NGS techniques, although this limitation has somewhat been overcome, there are still problems related to the assembly of the reads. There are many NGS sequence reads that remain unassembled due to variation of the sizes of the environmental DNA and there is therefore a high chance of missing the potential gene of interest in those unassembled sequences. Moreover, many assembled sequence represent partial genes which cannot be processed further (32, 160). Ideally, the fragment sequence identified should be long enough so that it can encompass a complete open reading frame to retrieve the target gene as mentioned earlier (88).

As understood from the above discussion that both these function- and sequence-based techniques have their own limitations and benefits and therefore cannot replace each other. Therefore, it is recommended to implement both these techniques simultaneously to allow for extensive mining for novel gene sequences/enzymes of biotechnological relevance.

2.5 Sequencing platforms and bioinformatics tools

Metagenomics projects have been supported by the rapid development of high-throughput Next Generation Sequencing (NGS) techniques (96) that can generate huge amounts of data in a relatively shorter time and with less effort and cost in comparison to the traditional Sanger technique (164). The first commercial application of the NGS technology took place in 2004. The Roche's 454-pyrosequencing, ABI's SOLiD and Illumina's Genome Analyzer are examples of the most commonly used second generation sequencing (SGS) platforms. These techniques all rely on the preparation of sequencing libraries of clonally amplified templates in a cell free system. Emerging techniques which are collectively referred to as Third generation sequencing (TGS) techniques are being developed. These include Helicos Heliscope sequencer, Pacific Bioscience's

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single-molecule real-time sequencer and Oxford nanopore sequencer, which are single-molecule sequencing technologies that do not require DNA amplification (75).

2.5.1 Second Generation Sequencing (SGS) Techniques

The sequencing work flow of SGS platforms entails three steps viz. library preparation, clonal amplification and cyclic array sequencing. During library preparation DNA samples are randomly fragmented and platform specific adaptors are added to the flanking ends of the fragments to produce a “library”. The library is then amplified through either emulsion PCR (emPCR) or bridge PCR. In emPCR the DNA fragments are clonally amplified on beads within a water-in-oil emulsion, followed by enrichment. In contrast, during bridge amplification, the library is flowed across a solid surface onto which the fragments randomly attach. The surface also contains a dense lawn of two different primers complementary to the adaptors, therefore allowing hybridization of the single stranded DNA fragments to the primers and formation of a template bridge which is then amplified following addition of unlabelled nucleotides and enzyme. Following clonal amplification the libraries are sequenced using platform-specific sequencing and detection chemistry.

2.5.1.1 The Roche 454 sequencing technique

The Roche 454 sequencing platforms employ a sequencing-by-synthesis pyrosequencing method which uses a cascade of enzymes to produce light from a phyrophosphate molecule which is released during nucleotide incorporation. Following emPCR the beads are deposited into a PicoTiterPlate so that each of the well contains a single bead. Enzyme-containing beads are then added and a sequencing reaction is initiated. The reaction involves incorporation of a dNTP complementary to the template strand with the help of ATP sulfurylase, luciferase, luciferin DNA polymerase and adenosine 5′ phosphosulphate (APS) with concomitant release of a pyrophosphate, while the ATP from PPi is used to drive the conversion of luciferin to oxyluciferin and generates visible light. At the end of the reaction unmatched bases are degraded by the enzyme apyrase and another dNTP is added and the reaction is repeated. The imaging happens at every nucleotide incorporation step and the fluorescence intensity is directly proportional to the number of nucleotides incorporated. However, in pyrosequencing the homopolymer stretches are not properly interpreted and therefore there are insertion/deletion errors during base calling. This has a run time of 8 hours with an average read length of 250 nucleotides which are further processed by the 454 analysis software (97).

2.5.1.2 The Illumina Hi Seq2000 platform

The Ilumina Genome analyzers also adopt the technology of sequencing by synthesis. The sequencing library is generated through bridge amplification. Prior to a sequencing reaction, the

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library is spliced into single strands with the help of linearization enzyme and all the four nucleotides each containing a different cleavable fluorescent dye molecule and a removable blocking group are added simultaneously to the flow cell channels, along with DNA polymerase for incorporation into oligo-primed cluster fragments. Following incorporation of a nucleotide complementary to the single stranded template the 3'-OH end of the same nucleotide is chemically blocked to prevent further extension, and the fluorescent tag is released and the signal captured by a charge-coupled device. Following the imaging of the signals the blocking group is removed chemically to prepare each strand for the next incorporation by DNA polymerase. This series of steps continue for a specific number of cycles to obtain a length of 25-35 bases. The base calling algorithms assigns sequences and evaluates the Illumina data from each run (97).

2.5.1.3 The Applied Biosystems SOLiD system

The SOLiD™ platform uses an adapter-ligated fragment library similar to the Roche/454 emulsion PCR approach with small magnetic beads to amplify the fragments for sequencing and also involves the unique use of DNA ligase. The ligase-mediated approach involves the amplification of the DNA fragments on the surface of 1 μm magnetic beads where 8 mers are annealed by the ligase whose 4th and 5th bases are encoded by the attached fluorescent group. Each ligation step is followed by fluorescence detection, after which a regeneration step removes bases from the ligated 8mer and prepares the extended primer for another round of ligation (97).

Although only three of the most common SGS techniques are discussed above, the Ion Torrent which was launched in 2010 is becoming increasing prominent in high throughput sequencing endeavours. The platform also uses a sequencing-by-synthesis strategy and a similar chemistry to pyrosequencing, however, this sequencer does not rely on fluorescence, chemiluminiscence or enzyme cascades for sequencing signal detection. The technique uses a semiconducting chip which measures differences in pH to detect dNTP incorporation. When a nucleotide is incorporated into the DNA molecules by the polymerase a proton (H^+) is released and alters the pH. This pH change is detected by an ion sensor thus identifying the base sequence. Key features of each of these NGS techniques are highlighted in Table 2.6.

2.5.2 Third generation sequencing

Although SGS techniques have proved to be successful in many respects, these technologies all rely on multiple manipulation steps that introduce artefacts and inaccuracies in DNA measurements. Consequently, scientists have continued to search for alternative sequencing methods and a new generation of single-molecule-sequencing (SMS) techniques which are collectively termed Third generation sequencing (TGS) technologies is emerging. These

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sequencing techniques exclude the initial PCR amplification of the DNA fragments to produce the clonally amplified fragment libraries and promise longer read lengths, shorter time to result and lower overall cost. SMS techniques characteristic of TGS can be grouped into two main categories: (i) SMS synthesis by sequencing techniques, and (ii) nanopore-sequencing technologies. Recently launched TGS platforms include Helicos sequencer, PacBio and Oxford Nanopore sequencer. The Helicos Heliscope sequencer system employs the sequencing-by-synthesis methodology in which fragmented DNA molecules are melted into single strands and ploy-A tailed. The strands are attached to Helicos Flow cell surface coated with oligo-dT—50 oligonucleotides and synthesis occurs through the incorporation of fluorescently labelled Virtual Terminator nucleotides added one at a time. In contrast PacBio (Pacific Bioscience) developed a process enabling single molecule real-time sequencing. In this process DNA templates to be sequenced are loaded into zero-mode waveguides (ZMWs) which are micro-wells that are nanometres in diameter. The ZMWs contain a DNA polymerase fixed to the surface with a biotin-streptavidin linkage. The polymerase is allowed to carry out second strand DNA synthesis in the presence of γ-phosphate fluorescently labelled nucleotides. The complementary pairing of the DNA template cleaves the fluorescent dye previously linked to the terminal phosphate of the nucleotide, which is captured by an inbuilt camera as videos on a real time basis. This measures both the colour and intensity giving the information not only for the sequence but also about its structure. Finally, nanopore sequencing generally refers to processes in which single molecules of nucleic acids electrophoretically pass through nanoscale pores. DNA is threaded through a microscopic pore in a membrane and bases are identified by the way the affect ions flowing through the pore from onside of the membrane to the other. This sequencing system was first launched by Oxford Nanopore technologies.This system does not require labelling of the DNA fragments.

Although they hold much promise, TGS techniques are all still in developmental stage and have mostly been plagued by high error rates. However, these teething challenges will probably be resolved as the platforms evolve.

2.5.2.1 Pre-processing of the raw sequencing data

All of these NGS techniques produce short reads (100-700 bp). These contigs are further subjected to a basic pre-processing which is necessary for amplicon reads. This involves filtering out erroneous sequences such as the reads containing 'N', adaptors. Several algorithms such as Pyronoise, denoiser, DADA, Acacia are developed (Table 2.5). For instance, Pyronoise use a flowgram clustering method while others such as denoiser, DADA, Acacia use sequence abundance information on the denoising process (75).

Following the denoising process, the sequences are processed further to exclude the chimeric sequences (75). However, to get rid of the chimeras is always challenging as there is a high possibility that there could be a break at any point in the sequence, leading to insufficient

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taxonomic informations. Several algorithms such as Perseus, UCHIME, ChimeraSlayer, Decipher that specifically detect chimeras have been designed. Most of these tools, except ChimeraSlayer, detect chimera sequences based on the sequence frequency information with an assumption that the chimera sequences are less frequently present in a NGS dataset. To date there are no tools that detect the chimera sequences fully, only UCHIME has been reported to have the best performance (75).

Following, the pre-processing of the metagenomics data sets, the amplicons are usually subjected to sequence assembly, functional annotation/gene prediction and taxonomical binning for taxonomical classification.

Table 2.5 A few bioinformatic tools are listed which are used to process the NGS data. Also the functions and their corresponding websites are also cited. This table was taken from a review article (75).

Resources	Function	Website
Pyronoise	Denoising	http://code.google.com/p/ampliconnoise
Denoiser	Denoising	http://qiime.org
DADA	Denoising	http://sites.google.com/site/dadadeioniser
UCHIME	Chimera detection	http://www.drive5.com/uchime
/ChimeraSlayer	Chimera detection	http://microbiomeutil.sourceforge.net
UCLUST	OTU clustering	http://www.drive5.com/usearch
Mothur	All in one	http://www.mothur.org
QIIME	All in one	http://quiime.org
MEGAN	All in one	http://ab.inf.uni-tuebingen.de/software/megan

2.5.3.1 Assembly and Taxonomic binning

The assembly process involves the merging of NGS short sequences to form longer contiguous sequences. The assembly is necessary since it offers the possibility to reconstitute entire microbial genomes, retrieving all the coding regions and therefore more accurate functional annotation is possible. A variety of assemblers such as Bambus 2 (79), Genovo (87), Meta-IDBA (121), Metagenomic assembly program (MAP) (86) and Meta Velvet (115) are use for this purpose. The algorithms developed for these assemblers ensure that the contigs from different species assemble separately and decreases the chances of forming the chimeric contigs constructs, although it has been observed that the reconstruction of the metagenomes is a complex task due to the high species complexity and short length of contigs (97). The assemblers, MetaVelvet and Meta-IDBA solve this problem by separating the de bruijn graph based on k-mer coverage and separately assemble each of the sub-graph. Meta-IDBA, MetaVelvet and Ray Meta are formulated

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to assemble the short reads while MAP and Genovo were developed for long reads (75). However, there is high probability that many of the reads resulting in wrong assemblies leading to wrong identification of the microbes. Because of these challenges it has often been recommended that the metagenomic data not be assembled prior annotation, however this also makes retrieval of genes of interest from the short reads difficult as reviewed previously (37). The assembled DNA fragments are sometimes used to constitute whole scaffolds using Bambus2. This software tool avoids joining between the distantly related microorganisms and also identifies the repeats and genomic variants (79).

Taxonomical binning is the first step for the taxonomical classification of the microbial community which involves the classification of the reads or sequences. Binning can be performed before or after the assembly. However, as recommended binning is not a non-trivial undertake. Since the shotgun sequencing generates short reads and many times the sequencing coverage does not reach to that point where assembly can be performed, binning is the preferred choice. Binning is usually performed based on the difference of the G+C ratio, di-, tri- or tetranucleotide frequency or codon usage between the microorganisms. Tools such as TETRA, Phylopythia, TACOA, PCAHIER and AbundanceBin are usually employed for this purpose as reviewed earlier (37). Each of these tools has different requirements. For instance, AbundanceBin can use short reads because of their limited compositional data within them. However, binning with the short reads does possess drawbacks as the partial gene informations cause lack of alignment confidence and difficulties in predicting protein sequences (37, 75). The taxonomic classification of the metagenomes is performed by searching for similar sequences against the known databases. Several tools are used for this purpose. MEGAN is a Basic local alignment search tool (BLAST) based study of marker genes such as rRNA genes (20) and DNA polymerase genes (109) that have the lowest algorithm of assigning reads to each of the taxa (65). ML TreeTrap (165) and AMPHORA (171) use the phylogeny-based phylotyping of the marker genes for taxonomic distribution while CARMA (84) identifies those genes from the metagenomic reads that encode known proteins (environmental gene tags) and construct a phylogenetic tree classifying them in a high order taxonomy as reviewed earlier (37, 75).

2.5.3.2 Gene prediction

It is very necessary to identify genes and their functionalities in the metagenomes. Several 'gene predictors', such as MetaGeneAnnotator (116), Orphelia (62, 63), ORFome (175), FragGeneScan (131), Glimmer-MG (73), MetaGenemark (180) were designed. Each of these tools uses different programs, e.g. MetaGenemark uses codon usage-incorporated HMM whereas MetaGeneAnnotator uses a machine learning algorithm and di-codon usage information. Orphelia uses a codon usage-incorporated machine learning algorithm whereas FragGeneScan uses a sequence error model and codon usage- incorporated HMM. Glimmer-MG uses a codon usage-incorporated Markov model (75).

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2.5.3.3 Functional annotation

In order to functionally annotate the metagenomes, a homology-based approach is usually undertaken which actually involves a BLAST search against databases. COG and eggNOG are the main databases used for functional annotation. COG was constructed from 66 genomes and since it has not been updated for a long time it has a very low sensitivity. Conversely, eggNOG is more updated and being constructed based on the pre-annotation of the orthologous groups from 1,133 genomes. Although BLAST-based approach is widely used for functional annotation, its use is someway disrupted by the lack of homologous sequences in the available databases as reviewed earlier (75).

Protein family prediction is performed by using the resources such as Pfam (126) and TIGRfam databases (54). These databases are applied through the HMM-based algorithm as reviewed earlier (75). Both these databases consists of curated multiple alignments and generated HMMs. While Pfam contains a higher number of protein families (14, 831), TIGRfam database contains only 4, 284. MG-RAST use the HMM-independent database, such as FIGfams while IGM/M, METATREP, and CAMERA uses the HMM-based databases as reviewed earlier (75). FIGfams are generated from National Microbial Pathogen Data Resource (NMPDR), contains set of protein sequences that are similar along their full length and also implement similar type of functions.

Lastly, the reconstruction of the metabolic pathways are generally made by using KEGG database IMG/M, CAMERA and MG-RAST use the KEGG database and KEGG graphs, while METATREP uses PRIAM. PRIAM is based on the available enzyme database with the KEGG graph for visualization. MetaCyc and MetaPath are also used for metagenomic functional analysis as reviewed earlier. However, the possible artefacts related to these algorithms cannot be ruled out.

Metagenomic data analysis does have certain limitations in terms of low genomic coverage in comparison to single genome. Efforts are already on the way to bridge the gap by doing co-assembly with single cell genomics and combined analysis between multiple metagenomes simultaneously. In conclusion, these high-throughput sequencing platforms indeed brought a revolutionary change in the field of genome sequencing and especially for metagenomic studies from the ecological, phylogenetic and diagnostic perspectives.

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Table 2.6 Different next-generation sequencing techniques have been highlighted and being compared. This table was taken from a review article (37).

Sequencing platforms	Chemistry	Read length (bp)	Run time	Through put per run	Reads per run
454 GS FLX + (Roche)	Pyrosequencing	700	23 h	700 Mb	≈1,000,000 shotgun, ≈700,000 amplicon
Hiseq 2000/2500 (Illumina)	Reversible terminator	2 x 150	High output: ≈11 days Rapid run: ≈27 h	High output: 600 Gb Rapid run: 120 Gb	High output: 3 billion x 2 Rapid run: 600 million x 2
5500xl W SOLiD (Life technologies) Bench-top devices	Ligation	1 x 75 Frag, 2 x 50 MP	8 days	≈320 Gb	1.4 billion x 2
454 GS Junior (Roche)	Pyrosequencing	400	8 h	35 Mb	100,000 shotgun
Ion PGM (Life Technologies)	Proton detection	100 or 200	3 h	100 Mb (314 chip)	400-550 thousand (314 chip)
				1 Gb (316 chip) 2 Gb (318 chip)	2-3 million (316 chip) 4-5.5 million (318 chip)
MiSeq (Illumina)	Reversible terminator	2 x 250	27 h	8.35 Gb	6.8 million (LRGC routinely getting > 15 M)

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2.6 Conclusions and future outlooks

The wine microbial consortium encompasses a wide range of genera and species of filamentous fungi, yeasts and bacteria. All of these species interact with one another, thereby inducing a microbial population dynamics that ultimately contributes to the organoleptic properties of wine. Interactions between yeasts are of particular relevance as these microbes are responsible for alcoholic fermentation, the main process leading to wine. Competition for nutrients, tolerance to metabolites and enzymes produced by the different strains and direct physical interactions govern these interactions. Studies have also shown that certain wine microorganisms secrete extracellular hydrolytic enzymes, antimicrobial peptides/killer toxins that display antagonistic activities against other yeasts and might play some role in microbial interactions as well. However, the identification of most of these extracellular enzymes/killer toxins has been limited to the conventional cultivation-based studies and plate-based enzyme assays for a long time. Since the concept of metagenomics was introduced and successfully implemented, many new enzymes have been discovered from different ecosystems but never in the grape/must/wine ecosystem. This approach could prove to be a valuable tool to unravel the functional potential of the wine microbiome and retrieve novel biocatalysts of oenological relevance that may otherwise have been excluded through cultivation-based methods. However, as discussed above, both the function- and sequence-based screening approaches have their own limitations. Therefore, in order to circumvent these problems, both of these approaches should be applied in parallel as this would increase the chance to retrieve more novel genes of interest.

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2.6 Bibliography

1. **Adams, D. J.** 2004. Fungal cell wall chitinases and glucanases. Microbiology **150:**2029-35.
2. **Allen, A., E. Chatt, and T. J. Smith.** 2013. The atomic structure of the virally encoded antifungal protein, KP6. J Mol Bio **425:**609-21.
3. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-402.
4. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in-*situ* detection of Individual microbial-cells without cultivation. Microbiol Rev **59:**143-169.
5. **AppliedBiosytems.** 2009. Genemapper. **4.1 Foster city**.
6. **Barata, A., S. Gonzalez, M. Malfeito-Ferreira, A. Querol, and V. Loureiro.** 2008. Sour rot-damaged grapes are sources of wine spoilage yeasts. FEMS Yeast Res **8:**1008-17.
7. **Barata, A., M. Malfeito-Ferreira, and V. Loureiro.** 2012. Changes in sour rotten grape berry microbiota during ripening and wine fermentation. Int J Food Microbiol **154:**152-61.
8. **Barata, A., M. Malfeito-Ferreira, and V. Loureiro.** 2012. The microbial ecology of wine grape berries. Int J Food Microbiol **153:**243-59.
9. **Bartowsky, E. J., and P. A. Henschke.** 2008. Acetic acid bacteria spoilage of bottled red wine -- a review. Int J Food Microbiol **125:**60-70.
10. **Beja, O., E. V. Koonin, L. Aravind, L. T. Taylor, H. Seitz, J. L. Stein, D. C. Bensen, R. A. Feldman, R. V. Swanson, and E. F. DeLong.** 2002. Comparative genomic analysis of archaeal genotypic variants in a single population and in two different oceanic provinces. Appl Environ Microbiol **68:**335-345.
11. **Beja, O., M. T. Suzuki, E. V. Koonin, L. Aravind, A. Hadd, L. P. Nguyen, R. Villacorta, M. Amjadi, C. Garrigues, S. B. Jovanovich, R. A. Feldman, and E. F. DeLong.** 2000. Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. Environ Microbiol **2:**516-529.
12. **Bevan, E. A., and M. Makower.** 1963. The physiological basis of the killing-character in yeast. Procedings of International Congress Genetics. 11203.
13. **Biver, S., D. Portetelle, and M. Vandenbol.** 2013. Characterization of a new oxidant-stable serine protease isolated by fucntional metagenomics. SpringerPlus **2:**1-10.
14. **Bokulich, N. A., C. M. Joseph, G. Allen, A. K. Benson, and D. A. Mills.** 2012. Next-generation sequencing reveals significant bacterial diversity of botrytized wine. PLoS One **7:**e36357.
15. **Bokulich, N. A., J. H. Thorngate, P. M. Richardson, and D. A. Mills.** 2014. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc Natl Acad Sci U S A **111:**E139-48.

Stellenbosch University https://scholar.sun.ac.za

16. **Bostian, K. A., Q. Elliott, H. Bussey, V. Burn, A. Smith, and D. J. Tipper.** 1984. Sequence of the preprotoxin dsrna gene of type-I killer yeast - multiple processing events produce a 2-component toxin. Cell **36:**741-751.
17. **Breinig, F., D. J. Tipper, and M. J. Schmitt.** 2002. Kre1p, the plasma membrane receptor for the yeast K1 viral toxin. Cell **108:**395-405.
18. **Brezna, B., K. Zenisova, K. Chovanova, V. Chebenova, L. Krakova, T. Kuchta, and D. Pangallo.** 2010. Evaluation of fungal and yeast diversity in Slovakian wine-related microbial communities. Antonie Van Leeuwenhoek **98:**519-29.
19. **Butler, A. R., R. W. Odonnell, V. J. Martin, G. W. Gooday, and M. J. R. Stark.** 1991. *Kluyveromyces lactis* Toxin Has an Essential Chitinase Activity. Eur J Biochem **199:**483-488.
20. **Chakravorty, S., D. Helb, M. Burday, N. Connell, and D. Alland.** 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Meth **69:**330-339.
21. **Charoenchai, C., G. H. Fleet, P. A. Henschke, and B. E. N. Todd.** 1997. Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. Aust J Grape Wine Res **3:**2-8.
22. **Chen, W. B., Y. F. Han, S. C. Jong, and S. C. Chang.** 2000. Isolation, purification, and characterization of a killer protein from *Schwanniomyces occidentalis*. Appl Environ Microbiol **66:**5348-52.
23. **Chovanova, K., L. Krakova, K. Zenisova, V. Turcovska, B. Brezna, T. Kuchta, and D. Pangallo.** 2011. Selection and identification of autochthonous yeasts in Slovakian wine samples using a rapid and reliable three-step approach. Lett Appl Microbiol **53:**231-7.
24. **Ciani, M., and F. Fatichenti.** 2001. Killer toxin of *Kluyveromyces phaffii* DBVPG 6076 as a biopreservative agent to control apiculate wine Yeasts. Appl Environ Microbiol **67:**3058-63.
25. **Cocolin, L., S. Campolongo, V. Alessandria, O. Dolci, and K. Rantsiou.** 2010. Culture independent analyses and wine fermentation: an overview of achievements 10 years after first application. Ann Microbiol **61:**17-23.
26. **Comitini, F., and M. Ciani.** 2009. The zymocidial activity of Tetrapisispora phaffii in the control of *Hanseniapora uvarum* during the early satges of winemaking. Letters in Applied Microbiology**:**1 - 7.
27. **Comitini, F., N. Di Pietro, L. Zacchi, I. Mannazzu, and M. Ciani.** 2004. *Kluyveromyces phaffii* killer toxin active against wine spoilage yeasts: purification and characterization. Microbiol **150:**2535-41.
28. **Cottrell, M. T., J. A. Moore, and D. L. Kirchman.** 1999. Chitinases from uncultured marine microorganisms. Appl Environ Microbiol **65:**2553-2557.
29. **Couch, J. A., and P. J. Fritz.** 1990. Isolation of DNA from plants high in polyphenolics. Plant Mol Bio Rep **8:**8-12.

Stellenbosch University https://scholar.sun.ac.za

30. **Craig, J. W., F. Y. Chang, J. H. Kim, S. C. Obiajulu, and S. F. Brady.** 2010. Expanding small-molecule functional metagenomics through parallel screening of broad-host-range cosmid environmental DNA libraries in diverse proteobacteria. Appl Environ Microbiol **76:**1633-41.
31. **da Silva Portes, C., A. V. de Oliveira, P. Simer, A. M. Lunkes, and A. R. Coelho.** 2013. Role of Killer factors in the inhibitory activity of biocontrol yeasts against *Penicillium expansum* and *Aspergillus ochraceus*. Braz Arch Biol Technol **56:**619-627.
32. **Dalevi, D., N. N. Ivanova, K. Mavromatis, S. D. Hooper, E. Szeto, P. Hugenholtz, N. C. Kyrpides, and V. M. Markowitz.** 2008. Annotation of metagenome short reads using proxygenes. Bioinformatics **24:**i7-13.
33. **David, V., S. Terrat, K. Herzine, O. Claisse, S. Rousseaux, R. Tourdot-Marechal, I. Masneuf-Pomarede, L. Ranjard, and H. Alexandre.** 2014. Highthroughput sequencing of amplicons for monitoring yeast biodiversity in must and during alcoholic fermentation. J Ind Microbiol Biotechnol **41:**811-21.
34. **Davis, C. R., D. Wibowo, R. Eschenbruch, T. H. Lee, and G. H. Fleet.** 1985. Practical Implications of Malolactic Fermentation - a Review. Am J Enol Vitic **36:**290-301.
35. **Day, P. R. A., S.I.** 1973. The killer system in *Ustilago maydis* - heterokaryon transfer and loss of determinants. Phytopathology **63:**1017-1018.
36. **de la Cruz, J., J. A. Pintor-Toro, T. Benitez, A. Llobell, and L. C. Romero.** 1995. A novel endo-beta-1,3-glucanase, BGN13.1, involved in the mycoparasitism of *Trichoderma harzianum*. J Bacteriol **177:**6937-45.
37. **Di Bella, J. M., Y. Bao, G. B. Gloor, J. P. Burton, and G. Reid.** 2013. High throughput sequencing methods and analysis for microbiome research. J Microbiol Methods **95:**401-14.
38. **Drysdale, G. S., and G. H. Fleet.** 1989. The growth and survival of acetic-acid bacteria in wines at different concentrations of oxygen. Am J Enol Vitic **40:**99-105.
39. **Du Toit, W. J., and I. S. Pretorius.** 2002. The occurence, control and estoric effect of acetic acid bacteria in winemaking. Ann Microbiol **52:**155-179.
40. **Egli, C. M., W. D. Edinger, C. M. Mitrakul, and T. Henick-Kling.** 1998. Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. J Appl Microbiol **85:**779-89.
41. **Entcheva, P., W. Liebl, A. Johann, T. Hartsch, and W. R. Streit.** 2001. Direct cloning from enrichment cultures, a reliable strategy for isolation of complete operons and genes from microbial consortia. Applied and Environmental Microbiology **67:**89-99.
42. **Esteve-Zarzoso, B., C. Belloch, F. Uruburu, and A. Querol.** 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int J Syst Bacteriol **49 Pt 1:**329-37.

Stellenbosch University https://scholar.sun.ac.za

43. **Esteve-Zarzoso, B., P. Manzanares, D. Ramon, and A. Querol.** 1998. The role of non-*Saccharomyces* yeasts in industrial winemaking. Int Microbiol **1:**143-8.
44. **Fischer, M. M., and E. Triplett.** 1999. Automated approach for ribosomal intergenic analysis of microbial diversity and its application to freshwater bacterial communities Appl Environ Microbiol **65:**4630-4636.
45. **Fischer, S. G., and L. S. Lerman.** 1979. Length-independent separation of DNA restriction fragments in 2-dimensional gel-electrophoresis. Cell **16:**191-200.
46. **Fleet, G. H.** 2003. Yeast interactions and wine flavour. Int J Food Microbiol **86:**11-22.
47. **Fleet, G. H., and G. M. Heard.** 1993. Yeasts - growth during ferementation. Wine Microbiology and Biotechnology**:**27-54.
48. **Fleet, G. H., Prakittchaiwattana, C., Beh, A.L., Heard, G.M.** 2002. The yeast ecology of wine grapes. Biodiversity and Biotechnology of wine yeasts**:**1-17.
49. **Guazzaroni, M. E., V. Morgante, S. Mirete, and J. E. Gonzalez-Pastor.** 2013. Novel acid resistance genes from the metagenome of the Tinto River, an extremely acidic environment. Environ Microbiol **15:**1088-102.
50. **Guillamon, J. M., and A. Mas.** 2010. Acetic Acid Bacteria. Biology of Microorganisms on Grapes, in must and in wine**:**31-46.
51. **Guillamon, J. M., J. Sabate, E. Barrio, J. Cano, and A. Querol.** 1998. Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. Arch Microbiol **169:**387-92.
52. **Guillemaut, P., and L. Maréchal-Drouard.** 1992. Isolation of plant DNA: A fast inexpensive and reliable method. Plant Mol Biol Rep **10:**60-65.
53. **Gutiérez, A. R., S. Epifanio, P. Garijo, R. López, and P. Santamaría.** 2001. Killer yeasts: Incidence in the ecology of spontaneous fermentation. Am J Enol Vitic **52**.
54. **Haft, D. H., J. D. Selengut, and O. White.** 2003. The TIGRFAMs database of protein families. Nucleic Acids Res **31:**371-3.
55. **Handelsman, J.** 2004. Metagenomics: application of genomics to uncultured microorganisms. Microbiol Mol Biol Rev **68:**669-85.
56. **Hankin, L., and J. E. Puhalla.** 1971. Nature of a factor causing interstrain lethality in *Ustilago maydis*. Phytopathology **61:**50-53.
57. **Henick-Kling, T., W. Edinger, P. Daniel, and P. Monk.** 1998. Selective effects of Sulphur dioxide and yeast started culture addition on indeginous yeast populations and sensory characteristics of wine. J Appl Microbiol **84:**269-273.
58. **Henne, A., R. Daniel, R. A. Schmitz, and G. Gottschalk.** 1999. Construction of environmental DNA libraries in *Escherichia coli* and screening for the presence of genes conferring utilization of 4-hydroxybutyrate. Appl Environ Microbiol **65:**3901-7.

Stellenbosch University https://scholar.sun.ac.za

59. **Henne, A., R. A. Schmitz, M. Bomeke, G. Gottschalk, and R. Daniel.** 2000. Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on Escherichia coli. Appl Environ Microbiol **66:**3113-3116.
60. **Hess, M., A. Sczyrba, R. Egan, T. W. Kim, H. Chokhawala, G. Schroth, S. Luo, D. S. Clark, F. Chen, T. Zhang, R. I. Mackie, L. A. Pennacchio, S. G. Tringe, A. Visel, T. Woyke, Z. Wang, and E. M. Rubin.** 2011. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. Science **331:**463-7.
61. **Hewson, I., and J. A. Fuhrman.** 2004. Richness and diversity of bacterioplankton species along an estuarine gradient in Moreton Bay, Australia. Appl Environ Microbiol **70:**3425-3433.
62. **Hoff, K. J., T. Lingner, P. Meinicke, and M. Tech.** 2009. Orphelia: predicting genes in metagenomic sequencing reads. Nucleic Acids Res **37:**W101-5.
63. **Hoff, K. J., M. Tech, T. Lingner, R. Daniel, B. Morgenstern, and P. Meinicke.** 2008. Gene prediction in metagenomic fragments: a large scale machine learning approach. BMC Bioinformatics **9:**217.
64. **Hugenholtz, P., B. M. Goebel, and N. R. Pace.** 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol **180:**4765-4774.
65. **Husson, D. H., A. F. Auch, J. Qi, and S. C. Schuster.** 2007. MEGAN analysis of metagenomic data. Genome Res **17:**377-386.
66. **Izgu, F., and D. Altinbay.** 2004. Isolation and characterization of the K5-type yeast killer protein and its homology with an exo-beta-1,3-glucanase. Biosci Biotechnol Biochem **68:**685-93.
67. **Izgü, F., D. Atinbay, and A. K. Türeli.** 2007. *In vitro* activity of panomycocin, a novel exo-ß-1,3-glucanase isolated from *Pichia anomala* NCYC 434, against dermatophytes. Mycoses **50:**31-34.
68. **Jara, C., E. Mateo, J. M. Guillamon, M. J. Torija, and A. Mas.** 2008. Analysis of several methods for the extraction of high quality DNA from acetic acid bacteria in wine and vinegar for characterization by PCR-based methods. Int J of Food Microbiol **128:**336-41.
69. **Jolly, N., O. Augustyn, and I. S. Pretorius.** 2006. The role and use of non-*Saccharomyces* yeasts in wine production. S Afr J Enol Vitic **27:**15-39.
70. **Jolly, N. P., C. Varela, and I. S. Pretorius.** 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res **14:**215-37.
71. **Kanokratana, P., L. Eurwilaichitr, K. Pootanakit, and V. Champreda.** 2014. Identification of glycosyl hydrolases from a metagenomic library of microflora in sugarcane bagasse collection site and their cooperative action on cellulose degradation. J Biosci Bioen**:**1-8.
72. **Kell, D. B., A. S. Kaprelyants, D. H. Weichart, C. R. Harwood, and M. R. Barer.** 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Antonie Van Leeuwenhoek **73:**169-187.

Stellenbosch University https://scholar.sun.ac.za

73. **Kelley, D. R., B. Liu, A. L. Delcher, M. Pop, and S. L. Salzberg.** 2012. Gene prediction with Glimmer for metagenomic sequences augmented by classification and clustering. Nucleic Acids Research **40:**e9.
74. **Kim, D., S. N. Kim, K. S. Baik, S. C. Park, C. H. Lim, J. O. Kim, T. S. Shin, M. J. Oh, and C. N. Seong.** 2011. Screening and characterization of a cellulase gene from the gut microflora of abalone using metagenomic library. J Microbiol **49:**141-5.
75. **Kim, M., K. H. Lee, S. W. Yoon, B. S. Kim, J. Chun, and H. Yi.** 2013. Analytical tools and databases for metagenomics in the next-generation sequencing era. Genomics Inform **11:**102-13.
76. **Klassen, R., and F. Meinhardt.** 2003. Structural and functional analysis of the killer element pPin1-3 from *Pichia inositovora*. Mol Genet Genomics **270:**190-9.
77. **Koltin, Y., and P. R. Day.** 1975. Specificity of *Ustilago maydis* killer proteins. Appl Microbiol **30:**694-696.
78. **König, H., and J. Fröhlich.** 2010. Lactic Acid Bacteria. Biology of Microorganisms on Grapes, in must and in wine**:**3-29.
79. **Koren, S., T. J. Treangen, and M. Pop.** 2011. Bambus 2: scaffolding metagenomes. Bioinformatics **27:**2964-71.
80. **Kotlin, Y.** 1988. The killer system of *Ustilago maydis*: secreted polypeptides encoded by viruses. In Viruses of Fungi and Simple Eukaryotes.
81. **Kovacs, A., K. Yacoby, and U. Gophna.** 2010. A systematic assessment of automated ribosomal intergenic spacer analysis (ARISA) as a tool for estimating bacterial richness. Res Microbiol **161:**192-7.
82. **Kowalchuk, G. A., J. Stephen, W. DeBoer, J. I. Prosser, T. M. Embley, and J. W. Woldendorp.** 1997. Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl Environ Microbiol **63:**1489-1497.
83. **Kraková, L., K. Chovanová, K. Zenisová, V. Turkovská, B. Brezná, T. Kuchta, and D. Pangallo.** 2012. Yeast diversity investigation of wine-related samples from two different Slovakian wine-producing areas through a multistep procedure. Food Sci Technol **46:**406-411.
84. **Krause, L., N. N. Diaz, A. Goesmann, S. Kelley, T. W. Nattkemper, F. Rohwer, R. A. Edwards, and J. Stoye.** 2008. Phylogenetic classification of short environmental DNA fragments. Nucleic Acids Res **36:**2230-9.
85. **Kurtzman, C. P., and C. J. Robnett.** 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. J Clin Microbiol **35:**1216-23.

Stellenbosch University https://scholar.sun.ac.za

86. **Lai, B., R. Ding, Y. Li, L. Duan, and H. Zhu.** 2012. A de novo metagenomic assembly program for shotgun DNA reads. Bioinformatics **28:**1455-62.
87. **Laserson, J., V. Jojic, and D. Koller.** 2011. Genovo: de novo assembly for metagenomes. J Comput Biol **18:**429-43.
88. **Li, L. L., S. R. McCorkle, S. Monchy, S. Taghavi, and D. Van der Lilie.** 2009. Bioprospecting metagenomes: glycosyl hydrolases for converting biomass Biotech Biofules **2:**1-11.
89. **Li, Y., M. Wexler, D. J. Richardson, P. L. Bond, and A. W. Johnston.** 2005. Screening a wide host-range, waste-water metagenomic library in tryptophan auxotrophs of *Rhizobium leguminosarum* and of *Escherichia coli* reveals different classes of cloned trp genes. Environ Microbiol **7:**1927-36.
90. **Liu, G. L., Z. Chi, G. Y. Wang, Z. P. Wang, Y. Li, and Z. M. Chi.** 2013. Yeast Killer toxins, molecular mechanisms of their action and application Crit Rev Biotechnol**:1-13.**
91. **Liu, J., Y. Sui, M. Wisniewski, S. Droby, and Y. Liu.** 2013. Review: Utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit. Int J Food Microbiol **167:**153-60.
92. **Lodhi, M. A., G. N. Ye, N. F. Weeden, and B. I. Reisch.** 1994. A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. Plant Mol Bio Rep **12:**6-13.
93. **Loomis, W. D.** 1974. Biochemistry of lower terpenoids in plants. Chemistry in New Zealand **38:**129-129.
94. **Magliani, W., S. Conti, M. Gerloni, D. Bertolotti, and L. Polonelli.** 1997. Yeast Killer Systems. Clin Microbiol Rev **10:**369 - 400.
95. **Majernik, A., G. Gottschalk, and R. Daniel.** 2001. Screening of environmental DNA libraries for the presence of genes conferring Na+(Li+)/H+ antiporter activity on *Escherichia coli*: Characterization of the recovered genes and the corresponding gene products. J Bacteriol **183:**6645-6653.
96. **Mardis, E. R.** 2006. Anticipating the 1,000 dollar genome. Gen Biol **7:**112.
97. **Mardis, E. R.** 2008. Next-Generation DNA sequencing methods. Annu Rev Genom Human Genet **9:**387 - 402.
98. **Margesin, R., and V. Miteva.** 2011. Diversity and ecology of psychrophilic microorganisms. Res Microbiol **162:**346-61.
99. **Marquina, D., A. Santos, and J. M. Peinado.** 2002. Biology of killer yeasts. Int Microbiol **5:**65-71.
100. **Marquina, D. A., J. Barroso, A. Santos, and J. M. Peinado.** 2001. Production and characteristics of *Debaryomyces hansenii* killer toxin. Microbiol Res **156:**387-391.
101. **Martinac, B., H. Zhu, A. Kubalski, X. L. Zhou, M. Culbertson, H. Bussey, and C. Kung.** 1990. Yeast K1 killer toxin forms ion channels in sensitive yeast spheroplasts and in artificial liposomes. PNAS **87:**6228-32.

Stellenbosch University https://scholar.sun.ac.za

102. **Masih, E. I., S. Slezack-Deschaumes, I. Marmaras, E. A. Barka, G. Vernet, C. Charpentier, A. Adholeya, and B. Paul.** 2001. Characterisation of the yeast *Pichia membranifaciens* and its possible use in the biological control of *Botrytis cinerea*, causing the grey mould disease of grapevine. FEMS Microbiol Lett **202:**227-32.
103. **Mateo, E., M. J. Torija, A. Mas, and E. J. Bartowsky.** 2014. Acetic acid bacteria isolated from grapes of South Australian vineyards. Int J Food Microbiol **178:**98-106.
104. **Maturano, Y. P., L. A. Rodriguez Assaf, M. E. Toro, M. C. Nally, M. Vallejo, L. I. Castellanos de Figueroa, M. Combina, and F. Vazquez.** 2012. Multi-enzyme production by pure and mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts during wine fermentation. Int J Food Microbiol **155:**43-50.
105. **Medina, K., F. M. Carrau, O. Gioia, and N. Bracesco.** 1997. Nitrogen availability of grape juice limits killer yeast growth and fermentation activity during mixed-culture fermentation with sensitive commercial yeast strains. Appl Environ Microbiol **63:**2821-5.
106. **Mewis, K., Z. Armstrong, Y. C. Song, S. A. Baldwin, S. G. Withers, and S. J. Hallam.** 2013. Biomining active cellulases from a mining bioremediation system. J Biotechnol **167:**462-71.
107. **Millet, V., and A. Lonvaud-Funel.** 2000. The viable but non-culturable state of wine micro-organisms during storage. Lett Appl Microbiol **30:**136-41.
108. **Mills, D. A., E. A. Johannsen, and L. Cocolin.** 2002. Yeast diversity and persistence in botrytis-affected wine fermentations. Applied and Environmental Microbiology **68:**4884-4893.
109. **Monier, A., J. M. Claverie, and H. Ogata.** 2008. Taxonomic distribution of large DNA viruses in the sea. Gen Biol **9:**R106.1-R106.15.
110. **Montiel, H. G. H., C. P. L. Corron, S. Vero, M. G. L. Aburto, J. L. Ochoa, and F. A. Valle.** 2010. Characterisation of yeast *Debaryomyces hansenii* for the biological control of blue mold decay of Mexican lemon. J Food **8:**49-56.
111. **Muccilli, S., S. Wemhoff, C. Restuccia, and F. Meinhardt.** 2013. Exoglucanase-encoding genes from three *Wickerhamomyces anomalus* killer strains isolated from olive brine. Yeast **30:**33-43.
112. **Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich.** 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol **51 Pt 1:**263-73.
113. **Muyzer, G., and E. C. de Waal.** 1994. Determination of the genetic diversity of microbial communities using DGGE analysis of PCR amplified 16S rRNA. NATO ASI series **G35:**207-214.
114. **Myers, R. M., S. G. Fischer, L. S. Lerman, and T. Maniatis.** 1985. Nearly all single base substitutions in DNA fragments joined to a GC-Clamp can be detected by denaturing gradient gel-electrophoresis. Nucleic Acids Res **13:**3131-3145.

Stellenbosch University https://scholar.sun.ac.za

115. **Namiki, T., T. Hachiya, H. Tanaka, and Y. Sakakibara.** 2012. MetaVelvet: an extension of Velvet assembler to de novo metagenome assembly from short sequence reads. Nucleic Acids Res **40:**e155.

116. **Noguchi, H., J. Park, and T. Takagi.** 2006. MetaGene: prokaryotic gene finding from environmental genome shotgun sequences. Nucleic Acids Res **34:**5623-5630.

117. **Ortin, A. B. B., P. E.J.;, N. B. Valverde, H. M. L. Roca, J. M. R. Garcia, and E. G. Plaza.** 2013. Effect of wine maceration enzymes on the extraction of grape seed Proanthocyanidins. Food Biporocess Technol **6:**2207-2212.

118. **Pace, N. R.** 1997. A molecular view of microbial diversity and the biosphere. Science **276:**734-740.

119. **Parish, M. E., and D. E. Caroll.** 1985. Indigenous yeasts associated with Muscadine (*Vitis routundifolia*) grapes and must. Am J Enol Vitic **36:**165 - 169.

120. **Pasteur, L.** 1866. Nouvelles expériences pour démontrer que le germe de la levure qui fait le vin provint de l´ exterieur des grains de raisin. Compte-Rendu de l´-Académie des Sciences Paris 75**:**781-793.

121. **Peng, Y., H. C. Leung, S. M. Yiu, and F. Y. Chin.** 2011. Meta-IDBA: a de Novo assembler for metagenomic data. Bioinformatics **27:**i94-101.

122. **Pintar, J., and W. T. Starmer.** 2003. The costs and benefits of killer toxin production by the yeast *Pichia kluyveri*. Antonie Van Leeuwenhoek **83:**89-97.

123. **Pinto, C., D. Pinho, S. Sousa, M. Pinheiro, C. Egas, and C. G. A.** 2014. Unravelling the diversity of grapevine microbiome. PLoS One **9:**e85622.

124. **Pozo, M. J., J. M. Baek, J. M. Garcia, and C. M. Kenerley.** 2004. Functional analysis of tvsp1, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*. Fungal Genet Biol **41:**336-48.

125. **Prakitchaiwattana, C. J., G. H. Fleet, and G. M. Heard.** 2004. Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. FEMS Yeast Res **4:**865-877.

126. **Punta, M., P. C. Coggill, R. Y. Eberhardt, J. Mistry, J. Tate, C. Boursnell, N. Pang, K. Forslund, G. Ceric, J. Clements, A. Heger, L. Holm, E. L. Sonnhammer, S. R. Eddy, A. Bateman, and R. D. Finn.** 2012. The Pfam protein families database. Nucleic Acids Res **40:**D290-301.

127. **Raskin, L., D. D. Zheng, M. E. Griffin, P. G. Stroot, and P. Misra.** 1995. Characterization of microbial communities in anaerobic bioreactors using molecular probes. Antonie Van Leeuwenhoek **68:**297-308.

128. **Renouf, V., O. Claisse, and A. Lonvaud-Funel.** 2007. Inventory and monitoring of wine microbial consortia. Appl Microbiol Biotechnol **75:**149-64.

Stellenbosch University https://scholar.sun.ac.za

129. **Renouf, V., O. Claisse, and A. Lonvaud-Funel.** 2005. Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. Aus J Grape Wine Res **11:**316-327.

130. **Renouf, V., P. Strehaiano, and A. Lonvaud-Funel.** 2007. Yeast and bacterial analysis of grape, wine and cellar equipments by PCR-DGGE. Journal International des Sciences de la Vigne et du Vin **41:**51-61.

131. **Rho, M., H. Tang, and Y. Ye.** 2010. FragGeneScan: predicting genes in short and error-prone reads. Nucleic Acids Research **38:**e191.

132. **Richards, E., M. Reichardt, and S. Rogers.** 1994. Preparation of genomic DNA from plant tissue. Current protocols in molecular biology**:**2.3.1-2.3.7.

133. **Riesenfeld, C. S., P. D. Schloss, and J. Handelsman.** 2004. Metagenomics: genomic analysis of microbial communities. Annu Rev Genet **38:**525-52.

134. **Riesner, D., Henco, K. & Steger, G.** 1991. Temperature-gradient gel electrophoresis: a method for the analysis of conformational transitions and mutations in nucleic acids and proteins. In: Advances in Electrophoresis **4:**169-250.

135. **Rondon, M. R., P. R. August, A. D. Bettermann, S. F. Brady, T. H. Grossman, M. R. Liles, K. A. Loiacono, B. A. Lynch, I. A. MacNeil, C. Minor, C. L. Tiong, M. Gilman, M. S. Osburne, J. Clardy, J. Handelsman, and R. M. Goodman.** 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl Environ Microbiol **66:**2541-7.

136. **Rosenbaum, V., and D. Riesner.** 1987. Temperature gradient gel electrophoresis - Thermodynamic analysis of nucleic acids and proteins in purified form and in cellular extracts. Biophys Chem **26:**235-246.

137. **Roszak, D. B., and R. R. Colwell.** 1987. Survival Strategies of Bacteria in the Natural-Environment. Microbiol Rev **51:**365-379.

138. **Ruiz, P., S. Sesena, P. M. Izquierdo, and M. L. Palop.** 2010. Bacterial biodiversity and dynamics during malolactic fermentation of Tempranillo wines as determined by a culture-independent method (PCR-DGGE). Appl Microbiol Biotechnol **86:**1555-62.

139. **Saeki, A., M. Taniguchi, K. Matsushita, H. Toyama, G. Theeragool, N. Lotong, and O. Adachi.** 1997. Microbiological aspects of acetate oxidation by acetic acid bacteria, unfavorable phenomena in vinegar fermentation. Biosci Biotechnol Biochem **61:**317-323.

140. **Santos, A., D. Marquina, J. A. Leal, and J. M. Peinado.** 2000. (1 -> 6)-beta-D-glucan as cell wall receptor for *Pichia membranifaciens* killer toxin. Appl Environ Microbiol **66:**1809-1813.

141. **Santos, A., E. Navascues, E. Bravo, and D. Marquina.** 2011. *Ustilago maydis* killer toxin as a new tool for the biocontrol of the wine spoilage yeast *Brettanomyces bruxellensis*. Int J Food Microbiol **145:**147-54.

Stellenbosch University https://scholar.sun.ac.za

142. **Santos, A., M. San Mauro, E. Bravo, and D. Marquina.** 2009. PMKT2, a new killer toxin from *Pichia membranifaciens*, and its promising biotechnological properties for control of the spoilage yeast *Brettanomyces bruxellensis*. Microbiology **155:**624-34.

143. **Santos, A., A. Sanchez, and D. Marquina.** 2004. Yeasts as biological agents to control *Botrytis cinerea*. Microbiol Res **159:**331-8.

144. **Sanz, L., M. Montero, J. Redondo, A. Llobell, and E. Monte.** 2005. Expression of an alpha-1,3-glucanase during mycoparasitic interaction of *Trichoderma asperellum*. FEBS J **272:**493-9.

145. **Schloss, P. D., and J. Handelsman.** 2003. Biotechnological prospects from metagenomics. Current Opinion Biotechnol **14:**303-10.

146. **Schmidt, T. M., E. F. DeLong, and N. R. Pace.** 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J Bacteriol **173:**4371-8.

147. **Schmitt, M. J., and F. Breinig.** 2002. The viral killer system in yeast: from molecular biology to application. FEMS Microbiol Rev **26:**257-276.

148. **Schmitt, M. J., and F. Neuhausen.** 1994. Killer toxin-secreting double-stranded RNA mycoviruses in the yeasts *Hanseniaspora uvarum* and *Zygosaccharomyces bailii*. J Virol **68:**1765-72.

149. **Setati, M. E., D. Jacobson, U. C. Andong, and F. Bauer.** 2012. The vineyard yeast microbiome, a mixed model microbial map. PLoS One **7:**e52609.

150. **Soden, A., I. L. Francis, H. Oakey, and P. A. Henscheke.** 2000. Effects of co-fermentation with *Candida Stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. Aust j Grape wine res **6:**21-30.

151. **Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl Environ Microbiol **54:**1079-1084.

152. **Starmer, W. T., P. F. Ganter, V. Aberdeen, M. A. Lachance, and H. J. Phaff.** 1987. The ecological role of killer yeasts in natural communities of yeasts. Can J Microbiol **33:**783-796.

153. **Stewart, E. J.** 2012. Growing unculturable bacteria. Journal of Bacteriology **194:**4151-60.

154. **Stoveken, J., R. Singh, S. Kolkenbrock, M. Zakrzewski, D. Wibberg, F. G. Eikmeyer, A. Puhler, A. Schluter, and B. M. Moerschbacher.** 2014. Successful heterologous expression of a novel chitinase identified by sequence analyses of the metagenome from a chitin-enriched soil sample. J Biotechnol.

155. **Strauss, M. L. A., N. P. Jolly, M. G. Lambrechts, and P. van Rensburg.** 2001. Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. J Appl Microbiol **91:**182-190.

156. **Streit, W. R., and R. A. Schmitz.** 2004. Metagenomics--the key to the uncultured microbes. Current Opinion in Microbiology **7:**492-8.

Stellenbosch University https://scholar.sun.ac.za

157. **Tao, J., I. Ginsberg, N. Banerjee, W. Held, Y. Koltin, and J. A. Bruenn.** 1990. *Ustilago maydis* KP6 killer toxin: structure, expression in *Saccharomyces cerevisiae*, and relationship to other cellular toxins. Mol Cell Biol **10:**1373-81.

158. **Tatusova, T., S. Ciufo, B. Fedorov, K. O'Neill, and I. Tolstoy.** 2014. RefSeq microbial genomes database: new representation and annotation strategy. Nucleic Acids Res **42:**D553-9.

159. **Todd, B. E. N., G. H. Fleet, and P. A. Henschke.** 2000. Promotion of autolysis through the interaction of killer and sensitive yeasts: Potential application in sparkling wine production. American Journal of Enology and Viticulture **51:**65-72.

160. **Tyson, G. W., J. Chapman, P. Hugenholtz, E. E. Allen, R. J. Ram, P. M. Richardson, V. V. Solovyev, E. M. Rubin, D. S. Rokhsar, and J. F. Banfield.** 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature **428:**37-43.

161. **Tzelepis, G. D., P. Melin, J. Stenlid, D. F. Jensen, and M. Karlsson.** 2014. Functional analysis of the C-II subgroup killer toxin-like chitinases in the filamentous ascomycete *Aspergillus nidulans*. Fungal Genet Biol **64:**58-66.

162. **Vahedi, M., M. Nasrolahei, M. Sharif, and A. M. Mirabi.** 2013. Bacteriological study of raw and unexpired pasteurized cow's milk collected at the dairy farms and super markets in Sari city in 2011. J Prev Med Hyg **54:**120-3.

163. **van Rensburg, P., and I. S. Pretorius.** 2000. Enzymes in Wine making: Harnessing natural catalysts for efficient biotransformations - A Review. S Afr J Enol Vitic **21:**52-73.

164. **Venter, J. C., K. Remington, J. F. Heidelberg, A. L. Halpern, D. Rusch, J. A. Eisen, D. Wu, I. Paulsen, K. E. Nelson, W. Nelson, D. E. Fouts, S. Levy, A. H. Knap, M. W. Lomas, K. Nealson, O. White, J. Peterson, J. Hoffman, R. Parsons, H. Baden-Tillson, C. Pfannkoch, Y. H. Rogers, and H. O. Smith.** 2004. Environmental genome shotgun sequencing of the Sargasso Sea. Science **304:**66-74.

165. **Von Mering, C., P. Hugenholtz, J. Raes, S. G. Tringe, T. Doerks, L. J. Jensen, N. Ward, and P. Bork.** 2007. Quantitative phylogenetic assessment of microbial communities in diverse environments. Science **315:**1126-30.

166. **Wang, X., Z. Chi, L. Yue, J. Li, M. Li, and L. Wu.** 2007. A marine killer yeast against the pathogenic yeast strain in crab (*Portunus trituberculatus*) and an optimization of the toxin production. Microbiol Res **162:**77-85.

167. **Wang, X. X., Z. Chi, Y. Peng, X. H. Wang, S. G. Ru, and Z. M. Chi.** 2012. Purification, characterization and gene cloning of the killer toxin produced by the marine-derived yeast *Williopsis saturnus* WC91-2. Microbiol Res **167:**558-63.

168. **Wickner, R. B.** 1986. Double-stranded RNA replication in yeast: the killer system. Annu Rev Microbiol **55:**373-393.

Stellenbosch University https://scholar.sun.ac.za

169. **Wisniewski, M., C. Biles, S. Droby, R. McLaughlin, C. Wilson, and E. Chalutz.** 1991. Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*. I Characterisation of attachment to *Botrytis cinera*. Physiol Mol Plant Path **39:**245-258.

170. **Wloch-Salamon, D. M., D. Gerla, R. F. Hoekstra, and J. A. de Visser.** 2008. Effect of dispersal and nutrient availability on the competitive ability of toxin-producing yeast. Proc Biol Sci **275:**535-41.

171. **Wu, M., and J. A. Eisen.** 2008. A simple, fast, and accurate method of phylogenomic inference. Gen Biol **9:**R151.

172. **Yan, X., A. L. Geng, J. Zhang, Y. J. Wei, L. Zhang, C. L. Qian, Q. F. Wang, S. Y. Wang, and Z. H. Zhou.** 2013. Discovery of (hemi-) cellulase genes in a metagenomic library from a biogas digester using 454 pyrosequencing. Appl Microbiol Biotechnol **97:**8173-8182.

173. **Yarrow, D.** 2011. Methods for the isolation, maintainance and identification of yeasts. The yeasts A Taxonomic study **11:**77-105.

174. **Yasir, M., H. Khan, S. S. Azam, A. Telke, S. W. Kim, and Y. R. Chung.** 2013. Cloning and functional characterization of endo-beta-1,4-glucanase gene from metagenomic library of vermicompost. J Microbiol **51:**329-335.

175. **Ye, Y., and H. Tang.** 2009. An ORFome assembly approach to metagenomics sequences analysis. J Bioinform Comput Biol **7:**455-71.

176. **Yeh, Y. F., S. C. Chang, H. W. Kuo, C. G. Tong, S. M. Yu, and T. H. Ho.** 2013. A metagenomic approach for the identification and cloning of an endoglucanase from rice straw compost. Gene **519:**360-6.

177. **Young, W. T., and M. Yagiu.** 1978. A comparison of the killer character in different yeasts and its classification. Antonie Van Leeuwenhoek **44:**59-77.

178. **Zenisová, K., K. Chovanová, C. V. Turcovská, Z. Godálová, L. Kraková, T. Kuchta, D. Pangallo, and B. Brezná.** 2014. Mapping of wine yeast and fungal diversity in the small Carpathian wine-producing region (Slovakia): evaluation of phenotypic, genotypic and culture-independent approaches. Ann Microbiol.

179. **Zhang, D., D. Spadaro, S. Valente, A. Garibaldi, and M. L. Gullino.** 2012. Cloning, characterization, expression and antifungal activity of an alkaline serine protease of *Aureobasidium pullulans* PL5 involved in the biological control of postharvest pathogens. Int J Food Microbiol **153:**453-64.

180. **Zhu, W., A. Lomsadze, and M. Borodovsky.** 2010. Ab initio gene identification in metagenomic sequences. Nucleic Acids Research **38:**e132.

Stellenbosch University https://scholar.sun.ac.za

Chapter 3

RESEARCH RESULTS I

Evaluating the use of ARISA to investigate microbial diversity in wine environment

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CHAPTER 3

Evaluating the use of ARISA to investigate microbial diversity in wine environment

Abstract

This study investigated the microbial diversity present in grape juice and in the early stage of alcoholic fermentation. The grapes were obtained from a South African biodynamic vineyard in 3 consecutive vintages (2012, 2013 and 2014). Culture-dependent and -independent approaches were used to investigate yeast and bacterial diversity. For the culture-independent approach, Automated Ribosomal Intergenic Spacer Analysis (ARISA) was employed. Using basic microbiological analyses 4, 12 and 15 yeast species were obtained in 2012, 2013 and 2014, respectively. In contrast, ARISA profiles revealed 13 fungal peaks for the year 2013 and 14 for 2012 and 2014. Out of 14 peaks in 2012, 2 peaks were identified as probable corresponding fungal Operational Taxonomical Units (OTUs) and 6 peaks were identified for years 2013 and 2014. The bacterial ARISA revealed 10, 7 and 12 peaks for these 3 years, respectively. Furthermore, the same technique was used to assess the evolution of the fungal community in the first 3 days of fermentation of the 2013 grape must. The data show that the yeast population diversity declined rapidly and revealed 12, 10 and 6 peaks for days 1, 2 and 3, respectively. The study demonstrated the suitability of ARISA for studying microbial diversity and dynamics in grape must and during wine fermentation.

3.1 Introduction

Fermenting grape must harbors a wide range of microorganisms comprising several species of yeasts and bacteria derived from the vineyard and winery equipment and surfaces (Prakichaiwattana et al. 2004; Renouf et al. 2007; Ocón et al. 2010; Jolly et al. 2014). These diverse microbial populations and their potential interplay in the different stages of alcoholic fermentation constitute a complex biological process that contributes largely to the final chemical composition of wine and therefore to its sensory properties (Fleet 2003). The diversity within the wine microbial population also evolves throughout the wine-making process (Fleet et al. 2003; Renouf et al. 2007; Jolly et al. 2014). The early phase of spontaneous alcoholic fermentation is dominated by non-*Saccharomyces* yeasts of the genera *Hanseniaspora*/*Kloeckera*, *Rhodotorula*, *Pichia*, *Candida*, *Metschnikowia*, *Debaryomyces*, *Issatchenkia*, *Kluyveromyces* and *Starmerella*. Among these, *Hanseniaspora uvarum* usually has the highest abundance followed by different *Candida* spp. By the middle phase of alcoholic fermentation, *Saccharomyces cerevisiae* outnumbers the non-*Saccharomyces* yeasts and remains the dominant yeast species until the end of fermentation (Renouf et al. 2007; Bezzera-Bussoli et al. 2012). The bacterial population in wine is dominated by lactic acid and acetic acid bacteria (LAB and AAB). The LAB population

responsible for wine malolactic fermentation mainly comprises *Oenococcus oeni* and *Lactobacillus plantarum*. Other LAB such as *Leuconostoc mesenteroides, Pediocococcus* spp. and most of the other *Lactobacillus* spp. are present in low levels (König and Fröhlich 2009). Acetic acid bacteria mainly of the genera *Acetobacter*, *Acidomonas*, *Gluconobacter* and *Gluconoacetobacter* are responsible for post fermentation spoilage of wine (Bartowsky and Henschke 2008). Minor populations of other bacteria such as *Chryseobacterium*, *Methylobacterium*, *Sphingomonas*, *Arcobacter*, *Naxibacter*, *Ralstonia*, *Frigoribacterium*, *Pseudomonas*, *Zymobacter* and *Acinetobacter* have also been reported to be present in must (Bokulich et al. 2012).

This knowledge of the microbial diversity associated with the grape and wine environments, as described above, has been progressively established over the past one-and-a-half century through an array of techniques ranging from microscopic observation to the molecular biology techniques. Traditional culture-dependent methods used in conjunction with PCR-based methods such as RFLP and RAPD for species and strain identification have generated valuable information on the ecology of grapes and fermenting must. However, these methods can be laborious, time-consuming and to some extent unreliable. Consequently, culture-independent methods such as PCR-DGGE, qRT-PCR and FISH have increasingly been employed as tools for monitoring microbial dynamics during wine fermentation. While these methods are more sensitive and provide more reliable information on species diversity and richness, they also have limitations. For instance, q-PCR and FISH mostly rely on species specific primers or probes and therefore only provide information on selected species, while PCR-DGGE is not quantitative, cannot detect certain yeasts at concentrations below 10^3 cfu/mL and requires additional steps for identification of the various bands (Cocolin et al. 2000; Prakichaiiwattana et al. 2004; Renouf et al. 2007). Recently, T-RFLP has been evaluated as a tool to assess the composition and species dynamics of yeast and bacteria during wine fermentation. This method allows for high-throughput data processing and semi-quantitative estimation of species rich and abundance in microbial samples and is also suited for microbial communities with low to intermediate richness (i.e. ≤ 50 taxa) (Ivey and Phister, 2011). Despite these advantages, the use of T-RFLP in wine fermentation ecology might be limited by poor resolution of yeast species. For instance, Sun and Liu (2014) reported that 15 wine yeast species generated the same TRF profiles using *Hae*III-TRFs and/or *Hinf*I-TRFs, thus necessitating the use of more enzymes. Such challenges can make this method more time-consuming as more enzymes would be required to get a better estimation of species richness. In contrast, ARISA uses the natural variability of the ITS region to compare microbial communities among samples without the additional restriction digestion analyses, thus making it less time-consuming. It has been successfully used in a number of ecological studies (Fisher and Triplet 1999; Green et al. 2004; Torzilli et al. 2006; Slabbert et al.2010b). The focus of these studies was however limited to the yeast diversity and not to the entire wine microbial consortium. The current study employed ARISA in conjunction with culture-dependent approaches to evaluate microbial

population fluctuation (for both yeasts and bacteria) over vintages in grape must. In addition, the yeast population dynamics was investigated in the early stages of fermentation.

3.2 Materials and methods

3.2.1 Collection of wine must

Samples of red grape must (*Vitis vinifera* cv. Cabernet Sauvignon) from the vintages 2012, 2013 and 2014 were collected from the Reyneke biodynamic vineyard (33°57´ 39.33´´ S 18° 45´13.46´´ E elev 183m), Stellenbosch, South Africa. The 2012 must samples were collected immediately after crushing from the commercial wine cellar, while in 2013 and 2014 grape samples were picked from the vineyard and transported in sterile zipper storage bags to our laboratory and crushed under aseptic conditions with sterilized utensils. Additionally in 2013, spontaneous fermentation was performed on the must and samples were withdrawn during the first 3 days to analyze the yeast dynamics.

3.2.2 Yeasts and bacterial enumeration and isolation

Decimal dilutions of the samples were made in 0.9% (w/v) NaCl solution and 100 µL of both the undiluted and diluted samples were plated on different culture media for isolation of yeasts. The Wallerstein Laboratory (WL) Nutrient agar (Sigma Aldrich, Steinheim, Germany) supplemented with 34 mg/L chloramphenicol (Sigma Aldrich) and 200 mg/L biphenyl (Riedel-de Haen AG, Seelze, Germany) to prevent the growth of bacteria and moulds respectively was used for the isolation of yeasts. The MRS (Biolab, Merck, South Africa) media supplemented with Kanamycin sulphate (Roche, Germany) (25 mg/L) to prevent acetic acid bacteria and GYC (5% Glucose, 1% yeast extract, 3% calcium carbonate) (Dey Ley et al. 1984) media supplemented with Streptomycin sulphate (25 mg/L) (Sigma Aldrich, Germany) to inhibit gram positive bacteria were used to cultivate the lactic acid bacteria (LAB) and acetic acid bacteria respectively. One hundred milligram per liter Delvocid (DSM, The Netherlands) was added in the both MRS and GYC media to avoid the growth of the yeast (Dey Ley and Swings, 1984). The plates were incubated till the growth was observed at 30°C and 37°C for yeast and bacteria respectively before enumeration. Morphologically distinguishable colonies were selected and further streaked out onto the WL agar media to obtain pure cultures. Each of the pure yeast isolates was grown in 5 mL YPD (Merck, Biolabs, Modderfontein, South Africa) broth and an aliquot was stored in 20% (v/v) glycerol at -80°C.

3.2.3 Molecular identification of the yeast isolates

Yeast genomic DNA (gDNA) extraction was performed according to the protocol of Hoffman (2003). PCR amplification of the ITS1-5.8S-ITS2 rRNA region was performed using primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) (White et al. 1990) in a 2720 Gene Thermal cycler (Applied Biosystems, Life Technologies, Johannesburg,

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South Africa). The 50 µL PCR reaction contained 100-200 ng of template DNA, 0.25 µM of each of the primers, 1 µM of deoxynucleotides, 1 mM of $MgCl_2$ along with 1U of Ex Taq™ polymerase (TaKaRa Bio Inc., Olsu, Shiga, Japan) in Ex Taq polymerase buffer (1x). The thermal cycling parameters were set with an initial denaturation at 94°C for 2 min 30 s, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension for 40 s at 72°C. A final extension was performed for 10 min at 72°C. The amplified PCR products were individually digested with restriction endonucleases *Hae*III, *Hinf*I and *Cfo*I in three separate reactions and the digested fragments were resolved on a 1% agarose gel containing ethidium bromide. Then restriction fragment sizes estimated against a 100 bp DNA ladder and the banding profiles were used to categorize the yeast isolates into different groups. The PCR products of two representatives from each of the groups were sent for sequencing at the Central analytical Facility (CAF), Stellenbosch University. The nucleotide sequences obtained from each of the isolates were compared using the BLAST (Basic local alignment search tool) algorithm with the available sequences in GenBank at National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/genebank/index.html (Altschul et al. 1997). Sequences exhibiting more than 98% identity to the previously deposited sequences were identified as such. Isolates that could not be identified from the ITS-5.8S rRNA region were further analyzed by sequencing of the domains 1 and 2 of the large ribosomal subunit (26/28S rDNA). The NLI (5´-GCATATCAATAAGCGGAGGAAAAG-3´) and NL4 (5´-GGTCCGTGTTTCAAGACGG-3´) primers (Bezerra-Bussoli et al. 2013) were employed to amplify the said domains with an identical PCR reaction as mentioned above for the amplification of the ITS1-5.8S-ITS2 rRNA region. The thermal cycle parameters were set with an initial denaturation at 94°C for 1 min, followed by 36 cycles of denaturation at 94°C for 1min, annealing at 58°C for 1 min, extension at 72°C for 1.5 min and a final 5 min extension at 72°C (Bezerra-Bussoli et al. 2013).The yeast species relative abundance was calculated as the percent of each of the isolates present in the sample (retrieved by colony counts) to the total population of the isolates.

3.2.4 DNA extraction from the fermenting wine must

Twenty milliliters of the fermenting grape must sample, which were collected daily, were centrifuged at 5,000 *g* for 10 min. The pellet was washed 3 times with EDTA-PVP solution (0.15 M NaCl, 0.1 M EDTA, 2% (w/v) PVP) (Jara et al. 2008) and subsequently 3 times with TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). The DNA was then extracted according to the protocol described by Wilson (1997) with the following modifications at the cell lysis step: along with proteinase K and SDS, 200 µL glass beads (diameter 0.65 mm), 20 µL lysozyme solution (10 mg/mL) were added in the TE buffer and this was followed by 3 min vortexing and an incubation at 37°C for 50 min.

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3.2.5 Fungal and bacterial Automated Ribosomal Intergenic Spacer Analysis (F-ARISA and B-ARISA)

The fungal community was evaluated by amplification of the ITS1-5.8S rRNA-ITS2 region of the metagenomic DNA using a caboxy-fluorescein labeled forward primer (ITS1-6FAM) and ITS4 while the bacterial community was analyzed by amplifying the ITS region (i.e. located between the 16S and 23S rRNA genes) with the 6FAM-ITSF (5´-GTCGTAACAAGGTAGCCGTA-3´) and ITS-Reub (5´-GCCAAGGCATCCACC-3´) primers (Cardinale et al. 2004). PCR amplification was done in triplicates using the same conditions described for isolation of yeast isolates. The labeled PCR-products were separated by capillary electrophoresis on ABI 3010xl Genetic analyzer (Applied Biosystems) at the Central Analytical Facility, Stellenbosch University. Both the labeled fungal and bacterial PCR-amplicons were resolved with ROX 1.1 size standards which varied from 75 bp – 1121 bp (Slabbert et al. 2010a). The raw data were converted to electropherograms and further analyzed in Genemapper 4.1 (Applied Biosystems). Only the fragments whose size was larger than 0.5% (>50 fluorescence units) of the total fluorescence were considered for analysis. A bin size of 3 bp below 700 bp and 5 bp above 700 bp was employed to minimize the inaccuracies in the ARISA analysis (Slabbert et al. 2010b). The average abundance of each of the individual peaks was calculated and represented as a percentage of the total number of peak heights displayed in the sample.

3.2.6 Diversity analysis

The fungal, yeast and bacterial species richness and diversity for each of the year were calculated using the Menhinick's index (I) - and the Shannon-Weinner index (H) (Danilov and Ekelund 2001), respectively.

3.3 Results

The current study employed ARISA to evaluate the diversity of yeast and bacteria in Cabernet sauvignon grape juice over a period of 3 consecutive years. In parallel, a culture-based approach was followed to monitor the diversity and dynamics of the yeast population in grape juice and during fermentation.

3.3.1 Yeast community composition

The total yeast population concentrations in the 2012, 2013 and 2014 fresh grape must were 3 x 10^6 CFU/mL, 1 x 10^6 CFU/mL and 1.56 x 10^6 CFU/mL, respectively. Based on visual differences in colony characteristics, a total of 130, 84 and 275 yeast colonies were isolated from 2012, 2013 and 2014 samples respectively. In parallel, ARISA analysis of the fungal communities was performed using the total DNA extracted directly from the grape juice samples. The electropherograms generated peaks ranging from 100 bp and 900 bp (data not shown).

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Following the amplification of the fungal ITS regions, ARISA revealed 14 peaks in the 2012 sample, the 579 bp peak being the most abundant and accounting for 26%, followed by peaks 546 bp, 749 bp, 799 bp and 842 bp which accounted for 9 - 17% of the total fungal population. Peak 629 bp and 652 bp were present as 5.05 and 5.54%, respectively while the remainder of the peaks represented less than 2% of the total fungal population (Figure 3.1A) In contrast, only 4 yeast species were obtained through cultivation: *Lodderomyces elongisporus* was identified as the dominant species (33%), followed by *Rhodotorula mucilaginosa* (26.67%) *Saccharomyces cerevisiae* (23.33%) and *Candida spandovensis* (17%) (Figure 3.1B).

During the 2013 harvest, 12 yeast species were identified in comparison to 13 peaks revealed by ARISA. *Starmerella bacillaris* (synonym *Candida zemplinina*) was found to be the dominant yeast species (21.88%) followed by *Hanseniaspora uvarum* (19.79%) and *Metchnikowia pulcherrima* (18.75%). Minor yeast species including *Cryptococcus bhutanensis*, *Aureobasidium pullulans*, *Issatchenkia terricola*, *Candida parapsilosis*, *Lachancea thermotolerans* were only present at lower abundance (Figure 3.1B). ARISA revealed that the 592 bp peak was the most dominant (30.17%), followed by the 561 bp peak with an abundance of 24.41%. Conversely, peak 685 bp exhibited the lowest (0.66%) abundance. Four peaks (553 bp, 582 bp, 586 bp and 611 bp) represented between 5 - 9% of the total peaks present in the grape juice while the remainder of the peaks were below 3.5% (Figure 3.1A).

In 2014, 15 yeast species were isolated and identified from grape juice. Similar to 2013, *Candida* spp. dominated the yeast population with *S. bacillaris* as the dominant species (50.32%) followed by *Candida apicola* (11.60%). *Rhodotorula glutinis*, *H. uvarum*, *A. pullulans*, *Kazachstania aerobia* and *Zygosacchoromyces bisporus* accounted for 3-8% of the population, while the rest of the yeast species were below 3% (Figure 3.1B). Fourteen ARISA-peaks were observed for the 2014 grape juice sample with 553 bp as the dominant peak (17.94%) followed by peak 472 bp, 561 bp, 586 bp, 592 bp and 763 bp with an abundance-range of 8%-14% while peak 696 bp was the least abundant at 0.60% (Figure 3.1A).

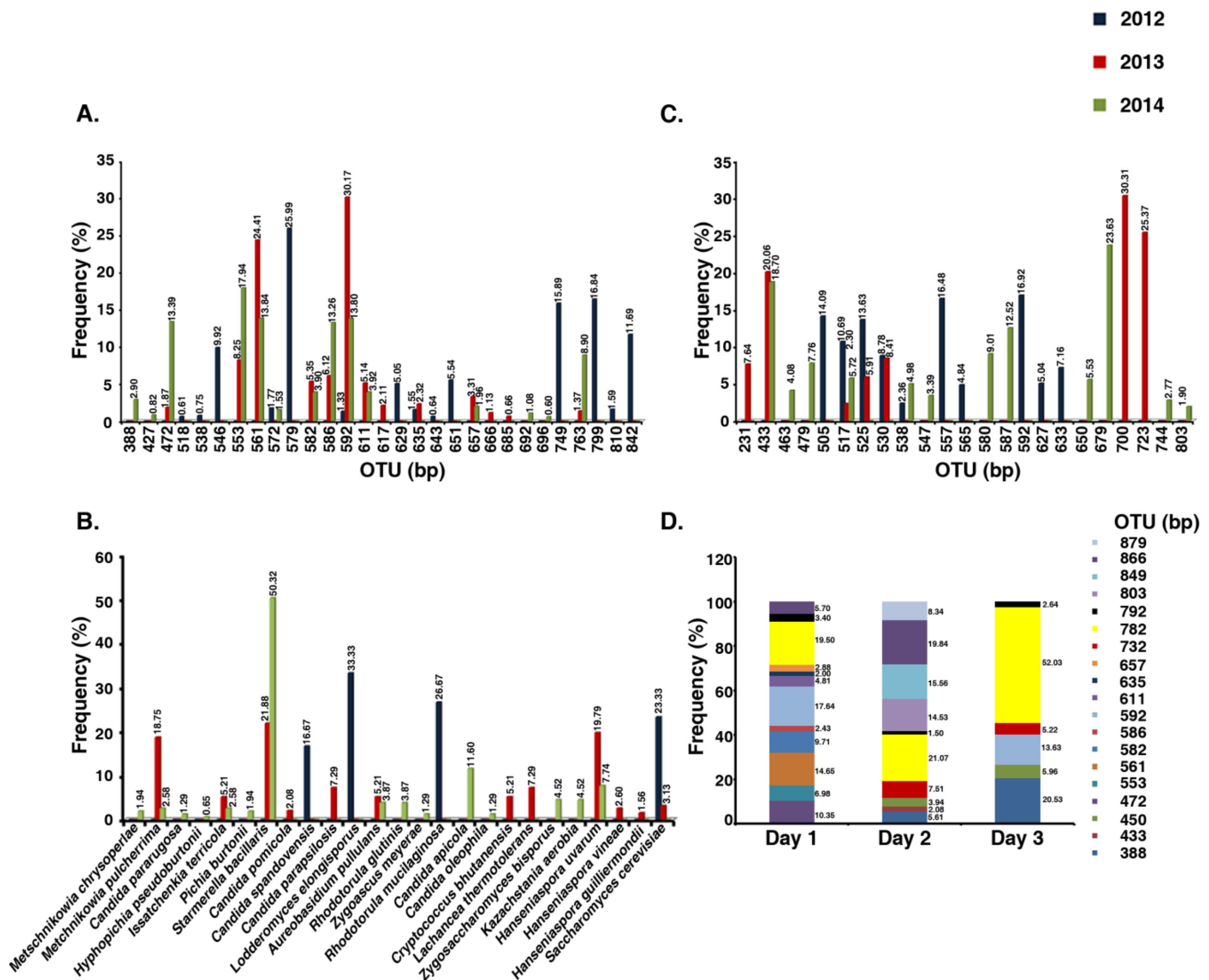

Figure 3.1 Representation of the fungal, yeast and bacterial diversity. The histograms exhibit the (A) fungal diversity by ARISA, (B) yeast diversity by PCR-RFLP and (C) bacterial diversity by ARISA of the grape juice sample for the year 2012, 2013 and 2014 respectively. The values on the top of the vertical bars represent the relative peak sizes/species abundance of the sample. (D) The stacked columns represent the fungal population dynamics on the onset of fermentation for the 2013 grape juice sample. The days 1, 2 and 3 represents 3 consecutive days of fermentation after the day 0. Each vertical rectangle of the stacked columns represents the relative fungal peak sizes abundance (values shown immediate next to the vertical rectangles).

3.3.2 Bacterial community analysis

The MRS and the GYC medium plates that were employed to identify the lactic acid bacteria and acetic acid bacteria respectively showed extremely low colony counts (< 30 colonies) even with the undiluted samples. Therefore it was decided not to consider them for further analysis and the bacterial community composition was only analyzed using ARISA over the 3-year period. Ten peaks were identified in the 2012 grape juice sample where peak 592 bp was the most abundant (16.92%) followed by 557 bp, 525 bp, 505 bp, 517 bp, 530 bp and 633 bp which showed an abundance of 16.48%, 13.63%, 14.09%, 10.69%, 8.78% and 7.16% respectively. The remainder of the peaks exhibited abundance below 5.50 % (Figure 3.1C). In contrast 7 peaks were identified in the 2013 grape juice sample. The peaks 700 bp (30.31%), 723 bp (25.37%) and 433 bp (20.06%)

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were the most abundant whereas the 517 bp was least abundant (2.30%) although in 2012 it accounted for > 10% of the total peaks (Figure 3.1C).

The 2014 grape juice sample displayed 12 peaks, dominated by 679 bp at 23.63% whereas the 803 bp fragment has the lowest abundance (1.90%). Similar to 2013, 433 bp was present in high levels (18.70%). Other dominant peaks were 479 bp, 580 bp and 587 bp which accounted for 7% - 13% of the total peaks (Figure 3.1C). The 2012 fungal and bacterial populations exhibited the highest species richness (Menhinick's index; I) whereas the 2014 represents the highest species diversities (Shannon Weinner index; H) for both the populations. The cultivation based study showed the highest yeast species richness (I) and diversities (H) for 2014 and 2013 respectively. Both the species richness and diversity indexes showed a gradual reduction from day 1 to day 3 of the 2013 fermenting samples (Table 3.1).

3.3.3 Population dynamics study of the fungal community

The use of ARISA as a tool to monitor yeast population dynamics was tested by monitoring the fungal population in the first 3 consecutive days of spontaneous fermentation in 2013. Our data revealed a decline in diversity from 13 peaks in the initial must to 12, 10 and 6 the next consecutive days. The 592 bp peak which was dominant (30.17%) declined rapidly while peak 782 bp, 791 bp and 866 bp which were not detected on the day 0 increased in abundance and remained present throughout the fermentation. (Figure 3.1D). Similarly, peaks 450 bp and 732 bp only appeared from day 2 of fermentation (Figure 3.1D). The objective of the current study was to investigate the microbial consortium in the fermenting wine must over three consecutive years employing both cultivation-dependent and -independent approaches. The yeast population dynamics was also monitored over the first few days of fermentation in one specific year in order to assess the rate at which the yeast population changes.

Table 3.1 Ecological diversity indices demonstrating the fungal, cultivable yeast and bacterial diversity from the years 2012, 2013 and 2014.

Years	Investigations		Techniques used	Species richness (Menhinick's index; I)	Species diversity (Shannon Weinner index; H)
2012	Fungal population		ARISA	0.13	2.10
			Cultivation-based	0.73	1.36
	Bacterial population		ARISA	0.17	2.17
2013	Fungal population		ARISA	0.08	1.97
			Cultivation-based	0.87	2.16
	Bacterial population		ARISA	0.04	1.69
	Fungal population dynamics	Day 1	ARISA	0.05	2.25
		Day 2	ARISA	0.05	2.05
		Day 3	ARISA	0.04	1.35

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Table 3.1 (cont.)

2014	Fungal population	ARISA	0.07	2.36
		Cultivation-based	1.21	1.87
	Bacterial population	ARISA	0.10	2.22

The yeast load (about 10^6 CFU/mL) of the grape juice obtained in our study was similar to those reported previously (Cocolin et al. 2000). Cultivation-based analysis showed that the yeast community comprised *S. cerevisiae* and non-*Saccharomyces* yeasts including *I. terricola*, *P. burtonii*, *H. uvarum*, *H. viniae*, *H. guilliermondii*, *C. apicola*, *C. oleophila*, *S. bacillaris*, *C. pomiciola*, *C. parapsilosis*, *C. pararugosa*, *C. spandovensis*, *C. bhutanensis*, *M. chrysoperlae*, *M. pulcherrima*, *A. pullulans*, *R. glutinis*, *R. mucilaginosa*, *Hyphophichia pseudoburtonii*, *L. elongisporus*, *Zygoascus meyerae*, *L. thermotolerans*, *Z. bisporus* and *K. aerobia*. Some of these species such as *C. parapsilosis* and *A. pullulans* have already been shown to be part of the microbiota in this vineyard (Setati et al. 2012). Overall, the yeast diversity in the grape musts evaluated in the current study included typical wine yeasts as reviewed by Jolly et al. (2014).

3.4 Discussion

Our study revealed similar yeast diversity between the culture-based study and the ARISA for 3 consecutive years (2012, 2013 and 2014). However, exact correlation between the isolates and the peaks could not be established. The 2013 and 2014 samples produced similar ARISA profiles with 9 peaks of fungal ITS regions shared between the two years. An attempt was made to correlate the sizes of the peaks with those of the known ITS sequences of the yeast isolates (Table 3.2) with the assumption that it could possibly provide us with some information on the yeast species present in the grape juice and fermenting must sample. The 388-bp peak (closely related to *M. pulcherrima*, abundance frequency 2-19%) identified from 2013 and 2014 samples was also detected at a comparable population frequency (2-21%) on plates for both years (Figure 3.1A, 3.1B, 3.1D and Table 3.2).

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Table 3.2 Tentative identification of fungal ARISA peaks through possible correlations between fungal ARISA peak sizes and yeast isolate´s ITS-5.8S rRNA-ITS2 PCR amplicons. The symbol (-) implies to undetectable level.

Yeasts isolates	ITS1-5.8S rRNA-ITS2 PCR amplicons (bp)	Peak sizes derived from ARISA (bp)
Metschnikowia chrysoperlae	375	-
Metschnikowia pulcherrima	390	388
Candida pararugosa	414	-
Hyphopichia pseudoburtonii	416	-
Issatchenkia terricola	421	427
Pichia burtonii	444	-
Starmerella bacillaris	475	472
Candida pomicola	480	-
Candida spandovensis	480	-
Candida parapsilosis	522	-
Lodderomyces elongisporous	550	553
Auerobasidium pullulans	580	582
Rhodotorula glutinis	606	-
Zygoascus meyerae	609	611
Rhodotorula mucilaginosa	610	611
Candida apicola	458	450
Candida oleophila	628	629
Cryptococcus bhutanensis	630	635
Lachancea thermotolerans	675	-
Zygosaccharomyces bisporus	741	-
Kazachstania aerobia	751	749
Hanseniaspora uvarum	747	749
Hanseniaspora vineae	738	732
Hanseniaspora guilliermondii	749	749
Saccharomyces cerevisiae	840	842

The 427-bp and 582-bp peaks (possibly *I. terricola,* and *A. pullulans,* respectively) also showed similar abundance levels between the ARISA and culture-based method. The 472-bp peak (possibly *S. bacillaris*) identified in both the 2013 and 2014 grape juice sample was also reflected in the culture-based studies. However, large discrepancies in its population abundance were observed between ARISA and PCR-RFLP for both years. In contrast, peak 611-bp could represent several yeast species which have ITS regions of similar sizes for instance *Z. meyerae* and *Rh. mucilaginosa* (Table 3.2). Similar discrepancies were observed regarding the presence or absence of *H. vineae* and *S. cerevisiae* as well as their relative abundance in the ARISA profiles compared to culture-based analysis. No tentative identification could be established for the 553-bp, 586-bp, 592-bp, 657-bp and 763-bp peak sizes. These peaks could represent filamentous fungi or other yeasts that could not be isolated. Such discrepancies between the culture-independent and culture-dependent analyses can be expected since both approaches are biased. Failure to retrieve certain yeast isolates could be due to the rapid growth of the other competing yeasts and similarities between colony morphologies resulting in poor isolation. ARISA like other PCR-based methods could be affected by DNA extraction efficiencies in mixed populations Brezna et al. (2010) reported similar challenges relating to ambiguous identification of some peaks as well as overlapping peaks preventing confident taxonomic assignment of the peaks. Ultimately, proper

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taxonomic identification of the peak sizes will require the establishment of a database consisting of cultivable grape and wine fungal ARISA profiles.

The current study also evaluated the yeast dynamics at the onset of fermentation as the behavior of the yeast population at this stage is an important determinant of fermentation tempo and ARISA. Our data show that some peaks which were initially detected in must disappear while new ones appear. This is consistent with the tumultuous nature of the onset of fermentation especially in spontaneous fermentation. Previous studies have mainly focused on yeast dynamics in three phases and show that by the middle of fermentation the yeast population stabilizes and is characterized by a clear dominance of weakly fermentative yeasts such as *Hanseniaspora* spp. and strongly fermentative yeasts especially *S. cerevisiae* (Brezna et al. 2010; Krakova et al. 2012; Ženišová et al. 2014).

Each year revealed a unique bacterial ARISA profile. Common wine bacterial species whose genomes have been sequenced were selected to generate an ITS database that was used to tentatively identify the peaks. *In silico* analysis of the genomes of *Oenococcus oeni*, *Lactobacillus brevis*, *Lactobaccillus plantarum*, *Pediococcus pentosaceus*, *Lactococcus lactis*, *Enterobacter cloacae*, *Leuconostoc mesenteroides*, *Lactobacillus salivarius, Enterococcus faecium* and *Gluconobacter oxydans* genomes showed that the size of their ITS regions varies from 200-700 bp (Table 3.3).

Table 3.3 Tentative identification of fungal ARISA peaks through possible correlations between bacterial ARISA-peak sizes and bacterial ITS region retrieved by *in silico* analysis. The symbol (-) implies to undetectable level.

Bacteria	ITS amplicons (bp)	Peak's size derived from ARISA (bp)
Lactobaccillus plantarum	206	-
Pediococcus pentosaceus	229	231
Lactococcus lactis	305	-
Enterobacter cloacae	341	-
Leuconostoc mesenteroides	376	-
Lactobacillus salivarius	409	-
Enterococcus faecium	430	433
Oenococcus oeni	471	479
Lactobacillus brevis	504	505
Gluconobacter oxydans	739	744

The ARISA of the 2014 grape juice sample exhibited peak sizes of 433 bp (18.70% abundance), 479 bp (7.76%) and 744 bp (2.77%) which were close to the size of the *E. faecium*, *O. oeni* and *G. oxydans* ITS region, respectively. The 433- bp peak (20.06%) was also present in the year 2013. Also, the 2012 and 2013 ARISA exhibited peak sizes of 505 bp (14.09%) and 231 bp (7.64%), respectively. These could be related to *L. brevis* (504 bp) and *P. pentosaceus* (229 bp) ITS regions. It was observed that a 517-bp peak with variable abundance was consistently present in all the 3 years of sampling but could not be clearly identified. No probable correlation could be

established, *in silico*, with the remaining peak sizes since many of the common wine eubacterial genomes have not been sequenced.

Large discrepancies were noted between the 3 vintages studied. Fluctuations in the microbial communities associated with the grape berry surface and present in grape must are common and can be attributed to various factors including berry ripeness levels, berry damage and climatic conditions. In addition, the detection of the different microorganisms in ARISA may be influenced not only by the relative abundance levels, cell lysis and DNA extraction efficiency (Rastogi and Saini 2011). Furthermore, the population dynamics studied with different culture independent techniques like DGGE (Renouf et al. 2007) and FISH (Xufre et al. 2006) have shown similar trends like our study.

Our current data confirm that ARISA can be employed as a tool to monitor microbial community structures in grape juice and their dynamics throughout fermentation. The data reveal similar trends as shown with other methods such as DGGE and FISH, with a rapid decline of the yeast population in the first few days of fermentation. However, since ARISA is semi-automated it allows for quantitative estimation of the relative abundance of different peaks and with the support of a robust database it can also allow for taxonomic identification of the peaks. This method is relatively fast, less time consuming and is more sensitive than DGGE. Future studies could also focus on retrieving the ITS nucleotide sequences from ARISA by eluting out each DNA fragments from the capillaries to allow direct identification from ARISA spectra when necessary.

3.5 References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402

Bartowsky EJ, Henschke PA (2008) Acetic acid bacteria spoilage of bottled red wine - a review. Int J Food Microbiol 125:60-70

Bezerra-Bussoli C, Baffi MA, Gomes E, Da-Silva R (2013) Yeast diversity isolated from Grape musts during spontaneous fermentation from a Brazilian winery. Curr Microbiol 67: 356-361

Bokulich NA, Joseph CM, Allen G, Benson AK, Mills DA (2012) Next-generation sequencing reveals significant bacterial diversity of botrytized wine. PLoS One 7:e36357

Brezna B, Zenisova K, Chovanova K, Chebenova V, Krakova L, Kuchta T, Pangallo D (2010) Evaluation of fungal and yeast diversity in Slovakian wine-related microbial communities. Antonie van Leeuwenhoek 98:519-529

Cardinale M, Brusetti L, Quatrini P, Borin S, Puglia AM, Rizzi A, Zanardini E, Sorlini C, Corselli C, Daffonchio D (2004) Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. Appl Environ Microbiol 70:6147-6156

Chovanova K, Krakova L, Zenisova V, Turcovska V, Brezna B, Kuchta T, Pangallo D. (2011)

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Selection and identification of autochthonous yeasts in Slovakian wine samples using a rapid and reliable three-step approach. Lett Appl Microbiol 53:231-237

Cocolin L, Bisson LF, Mills DA (2000) Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiol Lett 189:81-87

Danilov RA, Ekelund NG (2001) Comparative studies on the usefullness of seven ecological indices for the marine coastal monitoring close to the shore on the Swedish east coast. Environ Monit Assess 66:265-279

De Ley J, Gossele F, Swings, J (1984) Genus I Acetobacter. In: Holt (ed) Bergey's Manual of systametic bacteriology 1, Williams and Wilkins Co, Baltimore, MD1, pp 1268-274

De Ley J, Swings J (1984) Genus II Gluconobacter. In: JG Holt (ed) Bergey's Manual of systematic bacteriology Williams and Wilkins Co, Baltimore, MD1, pp 275-278

Fisher MM, Triplett EW (1999) Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Appl Environ Microbiol 65:4630-4636

Fleet GH (2003) Yeast interactions and wine flavour. Int J Food Microbiol 86:11-22

Green JL, Holmes AJ, Westoby M, Oliver I, Briscoe D, Dangerfield M, Gillings M, Beattie AJ (2004) Spatial scaling of microbial eukaryote diversity. Nature 432:747-750

Hoffman S (2003) Rapid isolation of yeast chromosomal DNA. In: Ausubel FM, Brent R, Kingstone RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Current protocols in molecular biology John Wiley and Sons, New Jersey, pp 13.11.2-13.11.4

Ivey ML, Phister TG 2011 Detection and identification of microorganisms in wine: A review of molecular techniques. J Ind Microbiol Biotechnol 38:1619-1634

Jara C, Mateo E, Guillamon JM, Torija MJ, Mas A (2008) Analysis of several methods for the extraction of high quality DNA from acetic acid bacteria in wine and vinegar for characterization by PCR-based methods. Int J Food Microbiol 128:336-341

Jolly NP, Varela C, Pretorius IS (2014) Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res 14:215-237

König H, Fröhlich J (2009) Lactic Acid Bacteria. In: König H, Unden G, Fröhlich J (eds) Biology of Microorganisms on Grapes, in must and in wine, Springer-Verlag, Berlin Heidelberg, pp 3-29

Krakova L, Chovanova K, Zenisova K, Turcovska V, Brezna B, Kuchta T, Pangallo D (2012) Yeast diversity investigation of wine-related samples from two different Slovakian wine-producing areas through a multistep procedure. LWT-Food Science and Technology 46:406-411

Ocón E, Gutierrez AR, Garijo P, López R, Santamaría P (2010) Presence of non-*Saccharomyces* yeasts in cellar equipment and grape juice during harvest time. Food Microbiol 27:123-127

Prakichaiwattana CJ, Fleet GH, Heard GM (2004) Application and evaluation of denaturing

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gradient gel electrophoresis to analyse the yeast ecology of wine grapes. FEMS Yeast Res 4:865-867

Rastogi G, Saini KR (2009) Molecular techniques to assess microbial community structure, function and dynamics in the environment In: Ahmad I, Ahmad F, Pichtel J (eds) Microbes and Microbial Technology: Agricultural and Environmental Applications, Springer, New york, pp. 29-57

Renouf V, Claisse O, Lonvaud-Funel A (2007) Inventory and monitoring of wine microbial consortia. Appl Microbiol Biotechnol75:149-164

Setati ME, Jacobson D, Andong UC, Bauer F (2012) The vineyard yeast microbiome, a mixed model microbial map. PLoS One 7:e52609

Slabbert E, van Heerden CJ, Jacobs K (2010a) Optimisation of Automated Ribosomal Intergenic Spacer Analysis for the Estimation of Microbial Diversity in Fynbos Soil. S Afr J Sci 106:52-55

Slabbert E, Kongor RY, Esler KJ, Jacobs K (2010b) Microbial diversity and community structure in Fynbos soil. Mol Ecol 19:1031-1041

Sun Y, Liu Y (2014) Investigating of yeast species in wine fermentation using terminal restriction fragment length polymorphism method. Food Microbiol 38:201-207

Torzilli AP, Sikaroodi M, Chalkley D, Gillevet PM (2006) A comparison of fungal communities from four salt marsh plants using automated ribosomal intergenic spacer analysis (ARISA). Mycologia 98:690-698

Wilson K, (1997) Genomic DNA extraction using the modified CTAB method. In: Ausubel FM, Brent R, Kingstone RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Current protocols in Molecular Biology, John Willey and Sons, New Jersey, pp 2.4.1-2.4.5

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungi ribosomal RNA genesfor phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) A guide to methods and applications, Academic press, New York, pp 315-322

Xufre A, Albergaria H, Inácio J, Martins-Spencer I, Gírio F (2006) Application of fluorescence in situ hybridization (FISH) to the analysis of yeast population dynamics in winery and laboratory grape must fermentations. Int J Food Microbiol 108:376-384

Zenisova K, Chovanova K, Chebenova-Turcovska V, Godalova Z, Krakova L, Kuchta T, Pangallo D, Brezna B (2014) Mapping of wine yeast and fungal diversity in the small Carpathian wine-producing region (Slovakia): evaluation of phenotypic, genotypic and culture-independent approaches. Ann Microbiol. doi: 10.1007/s13213-014-0827-x

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Chapter 4

RESEARCH RESULTS II

Phenotypic and genetic screening for extracellular hydrolytic enzymes and antifungal activity in selected non-*Saccharomyces* wine yeasts

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CHAPTER 4

Phenotypic and genetic screening for extracellular hydrolytic enzymes and antifungal activity in selected non-*Saccharomyces* wine yeasts

Abstract

Eleven yeast strains isolated from Cabernet sauvignon grape juice during the 2011, 2012 and 2014 vintages were tested for their hydrolytic and antifungal activities. Cellulase activity and β-1,3-1,6-glucanase activity was observed in 7 of the 12 yeast strains tested. These activities could only be detected in the absence of glucose in the media. *Metschnikowia chrysoperlae* was the only species which displayed β-glucosidase activity, while chitinase activity was detected in *M. chrysoperlae*, *Pichia burtonii*, *Hyphopichia pseudoburtonii*, *Candida oleophila*, *Rhodotorula glutinis* and *Candida glabrata*. The partial sequence of putative chitinase genes were retrieved from *M. chrysoperlae*, *P. burtonii*, *H. pseudoburtonii* and *C. glabrata* following PCR amplification using degenerate primers. Thereafter, longer gene sequences (although still incomplete) were obtained by inverse PCR. The full chitinase gene sequences could not be retrieved. *Pseudozyma fusiformata* was the only yeast that exhibited a strong growth inhibitory activity against certain strains of the wine spoilage yeasts *Brettanomyces bruxellensis* and *Brettanomyces anomalus*, while *M. chrysoperlae* only exhibited a weak activity against *Pichia kluyveri*, *Schizosaccharomyces pombe* and *Saccharomycodes ludwigii*. Our study has shown that the growth inhibitory activity of *P. fusiformata* is not due to viral dsRNA and could be due to presence of non-proteinaceous compounds. However, further investigation is required to confirm these findings. Our results nevertheless contribute to our knowledge on wine non-*Saccharomyces* yeasts that have never been characterised for their extracellular hydrolase activities of oenological interest in the past.

4.1 Introduction

Grape berries harbour a diverse microbiome comprising yeasts, filamentous fungi and bacteria that exist either as epiphytic or endophytic communities. These microorganisms play a critical role in grape health during berry development and have a direct influence on grape quality at harvest and subsequently on wine fermentation (8). Throughout the grape ripening and wine fermentation process, microbial interactions occur between yeasts and filamentous fungi, between yeasts of different species/strains and between yeasts and bacteria (7, 17). The yeasts-filamentous fungi interactions commonly occur on the berry surface where the microorganisms compete for limited nutrients, while yeasts-yeasts and yeasts-bacteria interactions predominantly occur in grape must and during fermentation (17). Several studies have demonstrated that some grape-associated yeast-like fungi and yeasts produce cell wall-hydrolyzing enzymes and antimicrobial peptides (14). The production of cell wall-degrading enzymes such as exo-glucanases and chitinases is a well known mechanism of action in mycoparasitic fungi such as *Trichoderma* spp. and has been shown

to be pivotal in the interactions occurring among antagonistic yeasts and phytopathogenic fungi (1). For instance, *Pichia guilliermondii* has been shown to attach to the hyphae of the fungal pathogen *Botrytis cinerea*, secrete extracellular β-1,3-glucanase that degrade the cell wall of the hyphae, thereby inhibiting the development of the filamentous fungus (38). Recently, *Metschnikowia pulcherrima* UMY15 strain isolated from a Turkish vineyard, was similarly shown to exhibit an inhibitory effect on the spore germination and hyphae formation of most of the species of *Penicillium* and *Aspergillus* (37).

During wine fermentation, interactions within the wine microbial consortium have been attributed to an array of factors such as high ethanol concentration, accumulation of toxic metabolites (e.g. medium and small chain fatty acids), depletion of certain nutrients, physical interactions and secretion of killer toxins and anti-microbial peptides (17). In recent years, the latter compounds have gained considerable amount of interest because of their potential role in microbial interactions within the fermenting must, especially between the *Saccharomyces* and non-*Saccharomyces* yeasts where killer phenotypes could offer an advantages to the killer toxin-producing strain over the sensitive cells in such a competitive environment (23). Several investigations on non-*Saccharomyces* yeast killer toxins have suggested that the killer activity in some yeasts may be associated with cell wall-degrading enzymes especially exo-glucanases. For instance, KpKt, the killer toxin secreted by *Tetrapisispora phaffii* was shown to be active against *Hanseniaspora*/*Kloeckera* (12) and the activity was mediated through glucanases that disrupted cell wall integrity (13). In addition, fragments of the amino acid sequence of Panomycin, a K5-type yeast killer protein from the culture supernatant of *Wickerhamomyces anomalus* (formerly known as *Pichia anomala*) strain K which exhibits anti-*Candida* activity was found to be 100% identical to an exo-β-1,3-glucanase (19), while the trimeric zymocin killer toxin (γβα) secreted by *Kluyveromyces lactis* was shown to exhibit exo-chitinase activity (9). It is believed that the α subunit, by means of exo-chitinase activity degrades the cell wall chitin layer which facilitates the diffusion of the toxic γ subunit. Finally, the partially purified chitinase protein from *M. pulcherrima* strain MACH1 was shown to inhibit the growth of *B. cinerea* mycelium (31). However, as seen above, most of the studies tend to focus on common wine yeasts, especially those that persist longer in wine fermentation and secrete enzymes such as proteases, β-glucosidases, glucanases, cellulases, xylanases and sulphite reductase.

The aim of the study was to determine whether certain poorly documented autochthonous yeasts isolated from the broader wine environment display any hydrolytic and/or antagonistic activity against typically frequent/dominant fermentative yeasts. The yeasts included *Pichia burtonii*, *Hyphopichia pseudoburtonii*, *Kazachstania aerobia*, *Candida apicola*, *Candida oleophila*, *Zygosaccharomyces bisporus* and *Pseudozyma fusiformata*. Some of these yeasts have been shown to possess antifungal activity and to have the potential to control plant pathogens. For instance, a study has shown *C. oleophila* possess antagonistic effect against *Penicillium digitatum* which is a postharvest pathogen for citrus fruit (6, 15). Similarly, in another study, *P. burtonii* was

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shown to decrease barley seed infection and ochratoxin A production by *Penicillium verrucosum* (2). However, the potential impact of these yeasts on grapevine pathogens has never been tested and no genetic information is available concerning the proteins responsible for the antifungal activity. The current study therefore attempted to retrieve the sequences of the genes encoding chitinases, enzymes that have previously been associated with antifungal activity.

4.2 Materials and methods

All the yeasts investigated in this study were isolated during the vintages 2011, 2012 and 2014 (Table 4.1). These yeast isolates were selected based on their low occurrence in wine and our limited knowledge regarding their extracellular hydrolytic activities and killer phenotypes. The isolation and identification of some of these yeasts was described in Chapter 3.

4.2.1 Screening of yeast isolates for enzymatic activities

Each of the yeast isolates were grown in the Yeast Peptone Dextrose (YPD) broth medium (Biolab, MERCK, Modderfontein, South Africa) at 30°C overnight except for *Pseudozyma fusiformata* which required 2 days. The grown yeast isolates were spot-plated on each of the YPD-agar plates containing different substrates. The substrates chitin, carboxy-methyl cellulose (CMC), laminarin, arbutin, polygalacturonic acid (PGA) and skimmed milk were employed to screen for chitinase, β-1,4-glucanases, β-1,3-1,6-glucanases, β-glucosidases, pectinases and acid-proteases, respectively. The pH of all the media was adjusted to 4.7 with HCl (1M). The 1% (w/v) CMC, 0.1% (w/v) laminarin, 0.5% (w/v) arbutin, 1.25% (w/v) PGA and 1.14% (w/v) skimmed milk-containing media were prepared in accordance to the protocols previously (11, 34). The 0.45% (w/v) chitin medium was prepared as per the protocol described by earlier described (3). All the protocols were followed with minor modifications i.e. the YNB (Yeast Nitrogen Base, Difco) medium was substituted by YPD since the yeast isolated did not grow on the YNB media with ammonium sulphate and amino acids. In addition, glucanase activity was also screened on YP-agar containing 0.5% (w/v) yeast extract, 1% (w/v) peptone and 0.2% (w/v) CMC or 0.2% (w/v) laminarin. The pH of the media adjusted to pH 4 with HCl (1M) and the medium was mixed with bacteriological agar to the final concentration of 2% (w/v) after autoclaving. The plates were incubated at 25°C for a period of 3 days for the enzyme activities to be observed. The Chitinase and β-glucosidase activities were identified as purple and brown pigmentation around the yeast colony, respectively. The glucanase activities on CMC and laminarin plates were observed by staining the plates with 0.1% (w/v) Congo red, followed by de-staining with 1 M NaCl till a clear zone around the colony was observed (Teather and Wood, 1982). Both the pectinase and acid protease activities were detected as a clear zone around the yeast colony. All the substrates selected for screening were purchased from Sigma-Aldrich (St Louis, MO).

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Table 4.1 Production of extracellular hydrolases by selected non-*Saccharomyces* wine yeasts.

Yeast isolates	**Year isolated**	**Enzymatic activities**			
		Chitinase	β-Glucosidase	β-1,4-cellulase	β-1,3-1,6-glucanase
***Pichia burtonii* IWBT Y951**	2014	+	-	+	+
***Metschnikowia chrysoperlae* IWBT Y955**	2014	+	+	+	+
***Rhodotorula glutinis* IWBT Y958**	2014	+	-	+	+
***Pseudozyma fusiformata* IWBT Y850**	2011	-	-	+	+
***Candida glabrata* IWBT Y800**	2012	+	-	-	-
***Hyphopichia pseudoburtonii* IWBT Y964**	2014	+	-	+	+
***Candida oleophila* IWBT Y964**	2014	+	-	+	+
***Candida apicola* IWBT Y957**	2014	-	-	-	-
***Zygosaccharomyces bisporus* IWBT Y959**	2014	-	-	-	-
***Zygoascus meyerae* IWBT Y960**	2014	-	-	+	+
***Kazachstania aerobia* IWBT Y965**	2014	ND	ND	-	-
***Candida pararugosa* IWBT Y961**	2014	-	-	-	+/-

'+', '–','+/-' and 'ND' denotes 'activity', 'no activity', 'weak activity' and 'Not Determined' respectively

4.2.2 Screening of the yeast isolates for killer activity

All the yeast isolates were screened for their killer toxin activity using the protocol previously described (28). All the sensitive yeast strains were grown initially in a 5 mL YPD medium at 30°C for 48 h. Furthermore, 50 mL YPD medium were inoculated with 50 µL of the 5 mL pre-culture and incubated with shaking at 30°C. Commercially available white grape juice (WGJ) (pH adjusted to 4.5 using 2 N NaOH) mixed with bacteriological agar and sensitive cells with a final concentration of 10^6 cfu/mL was employed for the screening. All the yeast isolates were spot-plated (10 µL) on each of the WGJ-agar-sensitive cells plate. Killer activity was tested against common wine spoilage yeasts *Brettanomyces bruxellensis* AWRI 1499, *B. bruxellensis* IWBT Y117, *B. bruxellensis* IWBT Y121, *Brettanomyces anomalus* IWBT Y105, *B. anomalus* IWBT Y132, *Schizosaccharomyces pombe* ATCC 24844, *Saccharomycodes ludwigii* ARC Y0154 as well as non-spoilage yeasts including *Pichia kluyveri* Viniflora® FrootZen™ (Hersholm, Denmark), *Torulaspora delbrueckii* Biodiva 291 (Lallemand SAS, Toulouse, France), *Saccharomyces cerevisiae* Uvaferm 228 (Lallemand) and *Lachancea thermotolerans* IWBT Y1240.

4.2.3 Cloning of the partial chitinase candidate genes

Previously published degenerate primers (CHI forward and CHI reverse) (Table 4.2) (31) were used to retrieve partial sequences (≈ 566 bp) of chitinase-encoding genes from *Metschnikowia chrysoperlae*, *Pichia burtonii*, *Hyphopichia pseudoburtonii*, *Candida oleophila, Rhodotorula glutinis*

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and *Candida glabrata*. *Saccharomyces cerevisiae* was used as a positive control for the PCR reaction. A 50-µl PCR reaction containing 100-200 ng of DNA, 1 µM of each primers, 1 mM of dNTPs, 2 mM of $MgCl_2$ and 1U of Ex Taq™ polymerase (TaKaRa Bio Inc., Otsu Shiga, Japan) in ExTaq polymerase buffer (1x) was set up. The thermal cycling parameters were set at an initial denaturation temperature of 94°C for 5 min, followed by 30 cycles of denaturation for 95°C for 1 min with an annealing temperature ranging from 48°C–58°C with an interval of 2°C and an extension of 72°C for 2 min. The final extension was set at 72°C for 7 min. The PCR-products were resolved on a 0.7% agarose gel containing ethidium bromide against O'Gene Ruler 1kb (Thermo Scientific, Waltham, MA). Following the separation of the PCR products on the agarose gel, the DNA bands of the sample were observed on the UV-transilluminator (Syngene, Synoptics Ltd, Cambridge, UK) and the bands which were approximately 566 bp were excised. Thereafter, DNA was extracted with the QIAquick® Gel extraction kit (Qiagen, Whitehead, Scientific (Pty) Ltd, Brackenfell, South Africa). The DNA fragments were cloned into the pGEM®-T Easy vector (Promega, Wisconsin) and transformed into *E. coli* DH5α. Positive transformants were selected on Luria Broth agar supplemented with 100 µg/mL ampicillin, 40 µg/mL X-gal (5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside) and 0.1 mM IPTG (Isopropyl β-D-1-thiogalactopyronoside). Positive colonies were grown in 5 mL LB broth containing 100 µg/mL ampicillin and plasmid DNA was extracted from the overnight cultures using the Gen Elute™ Plasmid Miniprep kit (Sigma Aldrich). Presence of the insert was confirmed by restriction digestion with *Eco*RI (Roche, Mannheim, Germany) and the plasmids were sequenced at the Central analytical Facility (CAF), Stellenbosch University. The resulting sequences were compared to those in Genbank at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genome) (36) using the Basic local alignment search tool (BLAST) algorithm (4). Sequences exhibiting identity to the chitinase genes were considered for further analysis.

Table 4.2 List of primers used in this work. All the primers were designed in this study except CHI forward and CHI reverse which were previously designed (31).

Primers	Sequences	Purpose of use
CHI forward CHI reverse	5´-ATCATGRTITAYTGGGGICRAA-3´ 5´-AGCARTARTARTTRTTRTARAAYTG-3'	Amplification of partial chitinase gene
CG-IFw CG-IRv	5'-CAATAATGGTAAAGGTTATGCCGCTCTAGTATCAAAAC-3' 5'-TGAGAACATTGTAGCAAGCCATCTGGAAATG-3'	Inverse PCR to retrieve full chitinase gene of *C. glabrata*
MC-IFw MC-IRv	5'-TGGCAGTACTTGATGTCATCAGAAATGTCACG-3' 5'-CTCAGGAATTGAACCGCTTGCACG-3'	Inverse PCR to retrieve full chitinase gene of *M. chrysoperlae*
PB-IFw PB-IRv	5´-AGCACCACCCATTGATAATAAGACCGCTTTA-3´ 5´-CGGTTACGTTGCTTTAGCCAATGGTTTAAGA-3´	Inverse PCR to retrieve full chitinase gene of *P. burtonii*

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Table 4.2 (cont.)

HP-IFw HP-IRv	5´-AACAACGATCAAACCGGTTACGTTGCAT-3´ 5´-CAAGTTTCAGTCCAACATTGGTTAGAGAAATTAATGGT-3´	Inverse PCR of *H. pseudoburtonii* chitinase gene
CG-NFw CG-NRv	5'-ACTGTTCAGCAATGGCTCTAAACA-3' 5´-GAAGGATAGGAGAAAAATGTCTGCTT-3´	Nested PCR of *C. glabrata* chitinase gene
MC-NFw MC-NRv	5´-GTTGAGCTTCATCTTGGGGAA-3´ 5´-CCCCGACCAAGCGCT-3´	Nested PCR of *M. chrysoperlae* chitinase gene
PB-NFw PB-NRv	5´-GTCCAACATTGATTAGAAAAATTAATGG-3´ 5´-CAATGTGTTTAACCAGATGCCA-3´	Nested PCR of *P. burtonii* chitinase gene
HP-NFw HP-NRv	5´-CTCAATTCTCTGAAGATTCTTCCAAAT-3´ 5´-CAGAGTCAGAAGCACAGTAGGTACTTAAT-3´	Nested PCR of *H. pseudoburtonii* chitinase gene

4.2.4 Retrieval of the full chitinase encoding gene sequences

Inverse-PCR was employed to retrieve the full sequences of the chitinase-encoding genes. After the first PCR reaction, a nested PCR was performed to further amplify the expected DNA fragments. The inverse (CG-IFw, CG-IRv, PB-IFw, PB-IRv, HP-IFw, HP-IRv, MC-IFw, MC-IRv) and nested PCR primers (CG-NFw, CG-NRv, PB-NFw, PB-NRv, HP-NFw, HP-NRv, MC-NFw, MC-NRv) (Table 4.2) were designed on the partial sequences of the chitinase genes obtained as described above. Two hundred nanograms of genomic DNA were subjected to digestion with different endonucleases. Fifty microliter digestion reactions were set up with 10 U of restriction enzymes in respective digestion buffers (1x). *Eco*RI and *Hpa*I were used for the digestion of the genomic DNA of all yeast isolates. Additionally, the genomic DNA of *M. chrysoperlae*, *C. glabrata* and *H. pseudoburtonii* were also digested with *Eco*RV and that of *C. glabrata* and *P. burtonii* with *Xba*I and *Bam*HI. Finally the genomic DNA of *M. chrysoperlae* and *H. pseudoburtonii* were digested with *Dra*I and *Bam*HI, respectively. All the restriction digestions were carried out at 37°C for 2 h and inactivated by incubation at 65°C for 20 min. After restriction digestions, the fragments were ligated overnight at 16°C as follows. Fifty microliters of each of the digestion reactions were used in a 100-µL ligation reaction that contained 10 U T4 DNA ligase (5U/µL) (Promega) and T4 DNA ligase buffer (1X). Two microliters of each of the ligation reactions were used as template for the 50-µL inverse-PCR reaction. Each of the reactions contained forward and reverse primers (0.45 µM each), dNTPs (0.80 mM) and 1 µL Elongase® Enzyme mix (Invitrogen™, Carlsbad, CA) in Buffer A (0.1x) and Buffer B (0.1x). The thermocycling parameters were set at an initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 64°C for 20 s and an extension at 68°C for 5 min. The final extension was done at 68°C for 5 min. Two microliters of each of the inverse-PCR products were used for nested-PCR reactions. The

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nested PCR contained the same constituents as described for the inverse PCR, however, new primer sets (forward- and reverse-nested) (Table 4.2) were used. The PCR parameters were set at 94°C initial denaturation for 2 min followed by 30 cycles of 94°C denaturation for 20 sec, 57°C annealing for 20 sec and 68°C extension for 5 min and a final 5 min extension at 68°C. The PCR products were further resolved on 0.7% agarose gel against O'Gene Ruler 1 Kb (Thermo Scientific). The band whose size exceeded 2 Kb were excised from the gel and sequenced with the nested primers at the CAF, Stellenbosch University. An attempt was made to reconstitute the whole gene from the sequences obtained. These were then compared with the pre-deposited sequences using that BLAST algorithm at NCBI. Furthermore, the amino acids sequences were deduced from all the partial chitinase gene sequences by *in silico* translation using ExPASy (http://web.expasy.org/translate/) (5).

4.2.5 Sequence alignment and phylogenetic analysis

The chitinase sequences were obtained from the Protein data bank (NCBI) and were aligned with the *in silico* translated peptide sequences by MUSCLE with default parameters. These chitinase sequences were selected mostly from the basidiomycetous and ascomycetous yeasts that harbour the grape and the fermenting must. The phylogenetic tree was constructed using MEGA6 by Neighbour-Joining method and with bootstrap test of 1000 replicates (35) (Figure 4.2).

4.2.6 dsRNA isolation and lipase activity screening

The total RNA was extracted from *P. fusiformata* and *S. cerevisiae* (positive control) according to the protocol described in Schmitt et al. (1990) (32). The lipase activity plate assay was performed on the YPD-agar media plate containing Tween 80 and methyl Red (30). Cultures on *P. fusiformata* were prepared by inoculating single colonies in YPD broth and incubating for 2 days at 30°C. Ten microliters of the actively growing cultures were spotted on the plate and incubated at 30°C until the zone of clearance was observed.

4.3 Results

4.3.1 Enzymatic and killer activity of the yeast isolates

Selected yeasts isolated from Cabernet sauvignon grape berries and must were screened for various hydrolytic enzyme activities as well as killer activity against other wine yeasts. None of the yeast isolates exhibited acid protease, glucanase (in the presence of glucose) and pectinase activity. In contrast, *M. chrysoperlae* displayed both β-glucosidase and chitinase activities while *P. burtonii*, *H. pseudoburtonii*, *C. oleophila*, *R. glutinis* and *C. glabrata* only displayed chitinase activity. Strong β-glucanase activity was exhibited by *P. burtonii*, *H. pseudoburtonii*, *R. glutinis*, *M. chrysoperlae*, *C. oleophila*, *P. fusiformata* and *Z. meyerae* on CMC and laminarin agar devoid of

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glucose whereas a weak glucanase activity was exhibited by *C. pararugosa* (Table 4.1). *Zygosaccharomyces bisporus*, *Kazachstania aerobia* and *Candida apicola* showed poor growth on both substrates and no activity was observed.

P. fusiformata exhibited strong antifungal against the sensitive strains of *B. bruxellensis* AWRI 1499 and *B. anomalus* IWBT Y105 while *M. chrysoperlae* displayed a weak activity against *S. pombe* ATCC 24844, *P. kluyverri* and *S. ludwigii* ARC Y0154. The other yeast isolates did not show any antifungal activity. In an attempt to identify the genetic origin of the antifungal activity of *P. fusiformata*, total RNA was extracted from a 48-h culture (Sup. Figure 4.1A). Unlike for 2 killer *S. cerevisiae* killer strains used as positive controls, no dsRNA could be visualized for *P. fusiformata*.

4.3.2 Isolation of putative chitinase-encoding genes from the chitinase positive yeasts

PCR using the degenerate primers CHI forward and CHI reverse was carried out on the genomic DNA of the isolates displaying chitinase activity in an attempt to retrieve the genes responsible for this enzymatic activity. Multiple amplicons were obtained for *M. chrysoperlae*, *P. burtonii*, *H. pseudoburtonii*, *C. oleophila* and *C. glabrata*. The bands in the vicinity of 600 bp were excised as a size of 566 bp was expected for *S. cerevisiae* which was used as a positive control (Figure 4.1A and B). After cloning and sequencing the amplicons from *M. chrysoperlae* revealed 96% identity to the *M. pulcherrima MACH1* encoding a chitinase gene (GenBank Accession number: EU153550), while the *C. glabrata* fragment showed 70% identity to the *CTS1-1* gene encoding an endochitinase (GenBank Accession number: NM001182173) of *S. cerevisiae*. The fragments from *P. burtonii* and *H. pseudoburtonii* displayed 69% identity to MYA-3404 chitinase I precursor mRNA (GenBank Accession number: EU418795) of *C. tropicalis* and 70% identity to chitinase *CHT2* gene (GenBank Accession number: AY445050) of *C. albicans*, respectively.

Based on these partial sequences, inverse PCR was employed in an attempt to retrieve the full gene sequences. PCR products ranging from 1 to 6 kb were obtained. The fragment whose sizes ranged from 2 to 2.5 kb were excised for *P. burtonii*, *H. pseudoburtonii* and *M. chrysoperlae* while a fragment of 5.8 kb was excised for *C. glabrata* (Figure 4.1C). The most intense bands of the expected approximate sizes (> 1 kb), as visible on the gel, were chosen. This is due to the fact that the size of most of the chitinase genes is around 1 kb. For instance, chitinase DNA sequences of *M. pulcherrima* MACH1 (ADV90769.1), *M. fruticola* AP47 (ADV90768.1) and *C. dubliniensis* CD36 (XP002418160.1) retrieved from Genbank were 1077 bp, 1095 bp and1164 bp, respectively. From the sequences we could extend the partial sequences obtained above, but unfortunately not the full ORFs: The sequence obtained for *M. chrysoperlae*, showed 97% identity, covering 80% of both *M. fruticola* (Accession number: ADV90768) and *M. pulcherrima* chitinase proteins (Accession number: ADV90769), while that of *P. burtonii* showed 49% identity with 49% coverage to a hypothetical protein CPAR2_502140 (Accession number: CCE43989, similar to *C. albicans* CHT2,

GPI linked chitinase) from *C. parapsilosis* and *H. pseudoburtonii* exhibited 55% identity with 43% coverage to hypothetical protein DEHA2D00924p from *D. hansenii* CBS 767 (Accession number: XP458510; similar to *S. cerevisiae* YLR286C Cts1 Endochitinase). The evolutionary relatedness as depicted in the dendrogram revealed that the partial chitinase protein sequence from *M. chrysoperlae* falls in the same clade with *M. pulcherrima* and *M. fruticola* while both *P. burtonii* and *H. pseudoburtonii* clustered together (Figure 4.2). Unlike the previous sequences, the nested-PCR product obtained from *C. glabrata* did not match with any known chitinase gene sequences.

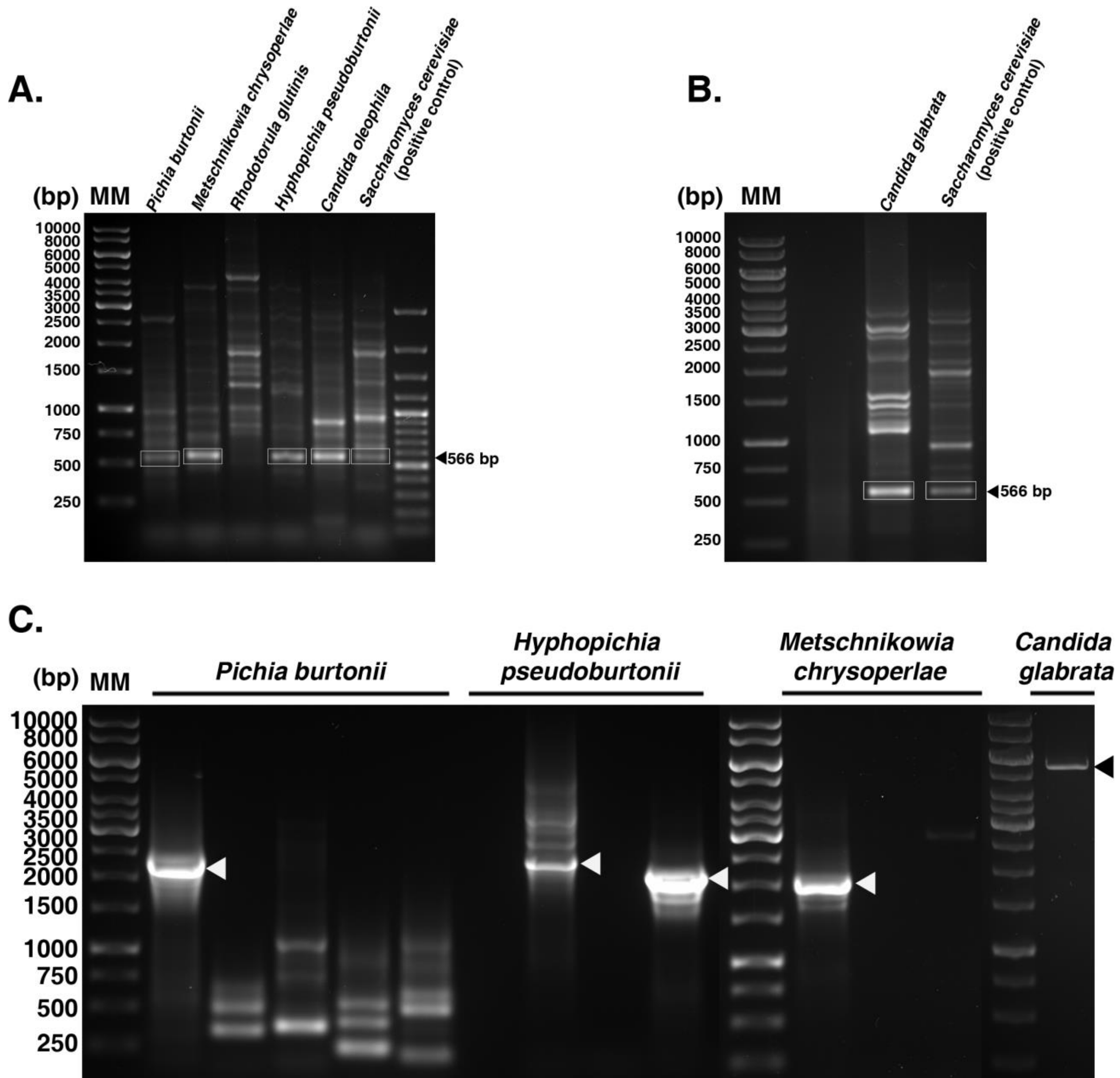

Figure 4.1 PCR with degenerate primers, inverse and nested PCRs of the chitinase fragments (A-B) The chitinase gene fragments from *P. burtonii*, *M. chrysoperlae*, *R. glutinis*, *H. pseudoburtonii*, *C. oleophila* and *C. glabrata* were PCR-amplified using the degenerate primers. The black arrow indicating the size (bp) of the chitinase fragments from *Saccharomyces cerevisiae*, used as a positive control. The white outlined rectangles show the fragments excised. (C) The nested-PCR products of *P. burtonii*, *H. pseudoburtonii*, *M. chrysoperlae* and *C. glabrata*. The white and black arrow heads indicate the chitinase fragments extracted from the agarose gel.

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4.4 Discussion

Wine is the product of complex microbial interactions between bacteria, yeasts and filamentous fungi. In fact, these interactions play a critical role in contributing to the sensory properties of wine as they influence population dynamics and the production of certain metabolites (17). Among the key players that drive these interactions, extracellular hydrolytic enzymes and antimicrobial peptides have drawn a considerable amount of attention in recent years. ..

Our study revealed two indigenous yeast isolates belonging to the species *P. fusiformata* and *M. chrysoperlae* that exhibit killer activity against the common wine spoilage yeasts of *B. bruxellensis* and *B. anomalus*. According to our results, *P. fusiformata*'s inhibitory activity under the conditions tested the inhibition is unlikely to be due to the presence of a dsRNA virus, as no dsRNA was visualised (Sup. Figure 4.1). This observation could be correlated with an earlier study in which, *Pseudozyma tsukubaensis*, another member of this genus, was also reported to be devoid of extra-chromosomal genetic elements although identified as a secretor of extracellular mycocin (18). In fact, the antifungal activity of several *Pseudozyma* spp. has been attributed to the production of biosurfactants rather than killer toxin production. *P. fusiformata* has indeed been shown secrete glycolipids (mainly ustilagic acid) that cause cell membrane disruption of the sensitive cells of yeasts, yeast-like and filamentous fungi (21, 22). Interestingly, *Saccharomyces cerevisiae* proved to be more tolerant to this glycolipid compared to other yeasts (21). Our study has not shown that the inhibitory effect of *P. fusiformata* is due to the glycolipids or similar molecules. We have shown lipase activity of *P. fusiformata* (Sup. Figure 4.1). However, we cannot attribute the antagonistic activity of *P. fusiformata* against wine spoilage microorganisms to its lipase activity (Sup. Figure 4.1). Further investigation is required to unravel the nature of the compound responsible for the antagonistic effect of *P. fusiformata* in our study.

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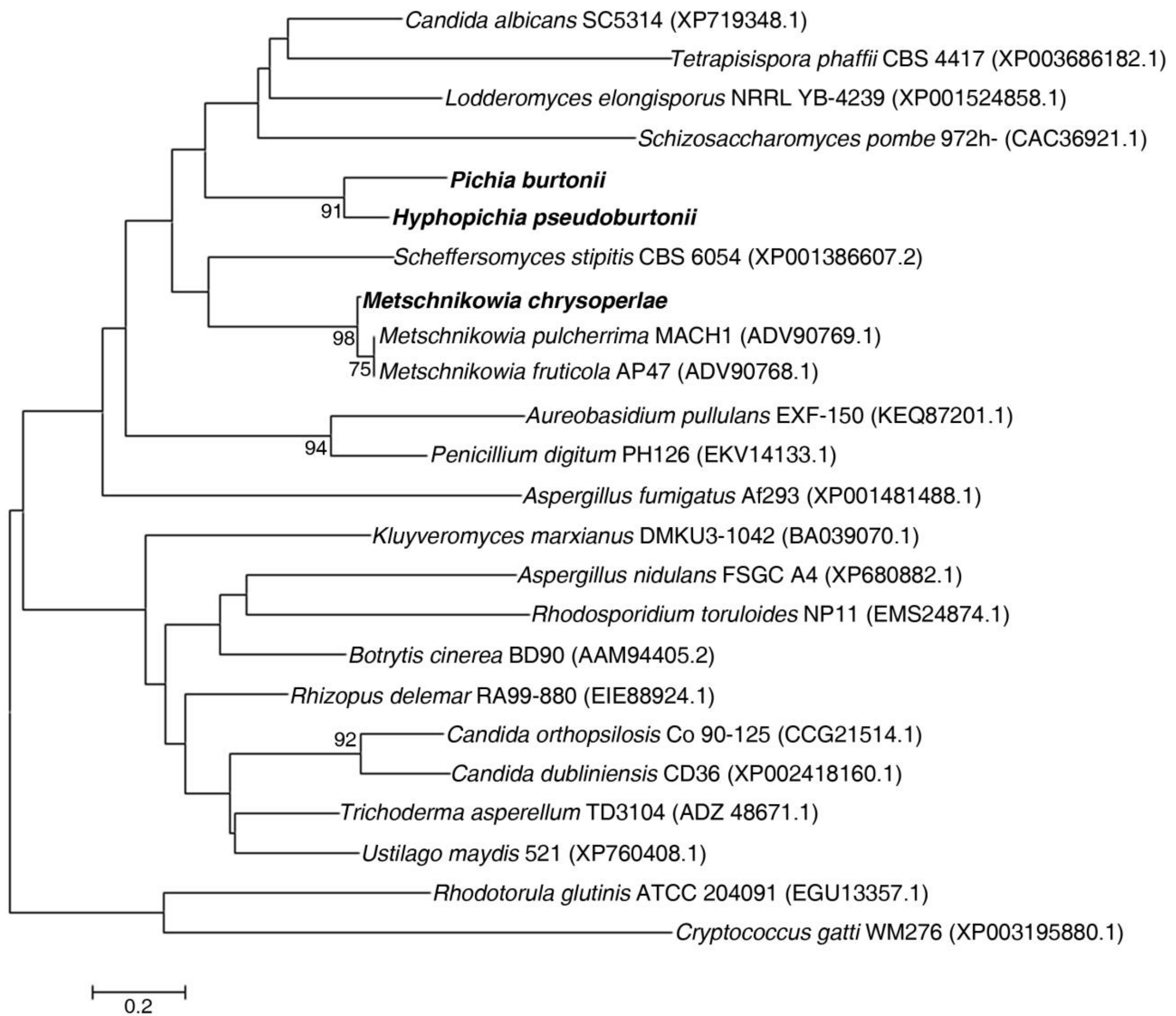

Figure 4.2 Phylogenetic relatedness among chitinases derived from different non-*Saccharomyces* yeasts. All the deduced amino acid sequences were aligned using the MEGA6 platform (MUSCLE). The optimal branch length is 12.07994547. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The values in parenthesis represent the protein accession numbers. The partial chitinase protein sequence sequences of *P. burtonii*, *H. pseudoburtonii* and *M. chrysoperlae* retrieved in this study are highlighted in bold letters in the phylogenetic tree.

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Our study also revealed a weak killer activity in *M. chrysoperlae* against *B. bruxellensis* AWRI 1499, *B. anomalus* IWBT Y105, *P. Kluyveri* Viniflora® FrootZen™ and *S. ludwigii* ARC Y0154 (data not shown). This killer phenotype of *M. chrysoperlae* could tentatively be attributed to the secretion of pulcherrimin which depletes the iron concentration of the surrounding medium, rendering it unsuitable for the growth of other microorganisms (33). The antifungal activity of other species of *Metschnikowia*, such as *M. pulcherrima* UMY15 strain against *B. cinerea in vitro* and *in vivo* on apples has indeed previously been reported and attributed to the secretion of pulcherrimin and therefore it has been proposed for a biocontrol agent (37)

Our study has demonstrated the chitinase activities by *M. chrysoperlae*, *P. burtonii*, *H. pseudoburtonii* and *C. oleophila.* A gene from *M. chrysoperlae* displaying high homology with chitinase-encoding genes of *M. pulcherrima* and *M. fruticola* was isolated. Moreover, the chitinase gene fragments (≈2-2.5 kb) isolated from *P. burtonii* and *H. pseudoburtonii* also showed homology to chitinase proteins from other yeast species (Sup. Table 4.1). Our study further indicated that both the chitinase genes from *P. burtonii* and *H. pseudoburtonii* are approximately 5 kb (Sup. Table 4.1). No previous reports showed any chitinase activities in *Pichia burtonii* and also in *H. pseudoburtonii* ,Also, a study has shown *Pichia guilliermondii* to exhibit exo-β-1,3-glucanase and chitinase activities in liquid media (38). In our study, the full sequence of the chitinase genes of *M. chrysoperlae*, *P. burtonii*, *H. pseudoburtonii* could not be retrieved and further work is required to achieve this and assess the potential relevance of their corresponding enzymes in yeast-yeast and yeast-mould interactions. Extracellular chitinase activity was also observed in *C. oleophila* and this correlates with a previous report (6) in which authors detected chitinase, exo-β-1,3-glucanase and protease activities in this species. Furthermore, we also observed the chitinase activity in *R. glutinis*. This activity has also been reported in other species of *Rhodotorula* (39). Endo- and exo-glucanase activity was observed in *P. burtonii*, *H. pseudoburtonii*, *C. oleophila*, *P. fusiformata*, *R. glutinis*, *M. chrysoperlae* and *Z. meyerae* on CMC and laminarin in the absence of glucose while on YPD in the presence of 2% (w/v) glucose these activities could not be detected, probably due to the repression of the extracellular glucanase activity by glucose as reported before (20, 26). Our findings confirm the previous study for the extracellular glucanase activities of *C. oleophila* (6) and endo- β-1,4-glucanase activity of *R. glutinis* (29). There are no previous reports on the extracellular glucanase activity for *M. chrysoperlae*, *P. fusiformata*, *Z. meyerae*, *P. burtonii*, and *H. pseudoburtonii*. However, as reported previously other species of *Pichia* such as *Pichia guilliermondii* (38), *Pichia membranifaciens* (26), *Pichia anomala* (20) have been shown to display extracellular glucanase activities. In the current study, extracellular β-glucosidase activity was only observed in *M. chrysoperlae*. A previous study also reported β-glucosidase activity in *Metschnikowia chrysoperlae* (16). Moreover, studies have also indicated that extracellular exo-glucanase activity in certain yeasts play a significant role in antifungal activity. For instance, *C. oleophila* has been reported to show antagonistic activity against *Penicillium*

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digitatum mediated through lytic enzymes (6) Also, the extracellular β-1,3-glucanase activity of *R. glutinis* has shown antagonism against *Botrytis cinerea* and *Penicillium expansum* (25). However, the yeast isolates of our study, although exhibiting extracellular glucanase activities, do not show any antagonistic effect against the wine spoilage yeasts tested. We can therefore hypothesise either that the spoilage yeasts tested are resistance to these exo-glucanases or that these specific exo-glucanases do not display antifungal activity.
Further characterization of both extracellular glucanase activities and β-glucosidase activity is required to investigate their potential impact on wine.

In summary, our study revealed extracellular hydrolytic enzyme activities in autochthonous yeast isolates from different vintages; some of which were not previously shown to exhibit these activities. We could also successfully retrieve partial chitinase gene sequences from these yeast species. Retrieving the full gene sequences is however necessary to further characterise these chitinases. However, the enzymatic screening system used in our study has certain limitations (24) as is common with plate screening assays since laboratory conditions used are not always equally conducive for expression of extracellular enzyme-encoding genes or do not promote enzyme activity. For instance, as indicated in a previous study, the secretion of chitinase depends on the environmental factors (10), and therefore it can be speculated that although all yeasts possess chitinases for cell wall remodelling (1), not all chitinases are expressed or active under the conditions tested. Hence, we cannot rule out the possibility that certain activities/yeast strains exhibiting these activities were missed in our screening. Future study should include a thorough molecular screening of these yeast isolates whenever possible (e.g. when degenerate primers can be designed). We also showed *P. fusiformata* to possess inhibitory activity against *B. bruxellensis* AWRI 1499 and *B. anomalus* IWBT Y105 under the conditions tested. Our results suggest that this inhibition cannot be attributed to viral encoded dsRNA. Based on literature, there is a strong possibility that this activity be mediated by ustilagic acid or other glycolipids but this needs to be confirmed. Future studies should also focus on identifying the physiological and genetic determinants of this activity.

4.5 References

1. **Adams, D. J.** 2004. Fungal cell wall chitinases and glucanases. Microbiology **150:**2029-35.
2. **Adel Druvefors, U., and J. Schnurer.** 2005. Mold-inhibitory activity of different yeast species during airtight storage of wheat grain. FEMS Yeast Res **5:**373-8.
3. **Agrawal, T., and A. S. Kotasthane.** 2012. Chitinolytic assay of indigenous isolates collected from different geographical locations of Chhattisgarh in Central India. SpringerPlus **1:**73.
4. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-402.

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5. **Artimo, P., M. Jonnalagedda, K. Arnold, D. Baratin, G. Csardi, E. de Castro, S. Duvaud, V. Flegel, A. Fortier, E. Gasteiger, A. Grosdidier, C. Hernandez, V. Ioannidis, D. Kuznetsov, R. Liechti, S. Moretti, K. Mostaguir, N. Redaschi, G. Rossier, I. Xenarios, and H. Stockinger.** 2012. ExPASy: SIB bioinformatics resource portal. Nucleic Acids Res **40:**W597-603.
6. **Bar-Shimon, M., H. Yehuda, L. Cohen, B. Weiss, A. Kobeshnikov, A. Daus, M. Goldway, M. Wisniewski, and S. Droby.** 2004. Characterization of extracellular lytic enzymes produced by the yeast biocontrol agent *Candida oleophila*. Curr Genet **45:**140-8.
7. **Barata, A., M. Malfeito-Ferreira, and V. Loureiro.** 2012. Changes in sour rotten grape berry microbiota during ripening and wine fermentation. Int J Food Microbiol **154:**152-61.
8. **Barata, A., M. Malfeito-Ferreira, and V. Loureiro.** 2012. The microbial ecology of wine grape berries. Int J Food Microbiol **153:**243-59.
9. **Butler, A. R., R. W. Odonnell, V. J. Martin, G. W. Gooday, and M. J. R. Stark.** 1991. *Kluyveromyces lactis* Toxin Has an Essential Chitinase Activity. Eur J Biochem **199:**483-488.
10. **Carrasco, M., J. M. Rozas, S. Barahona, J. Alcaino, V. Cifuentes, and M. Baeza.** 2012. Diversity and extracellular enzymatic activities of yeasts isolated from King George Island, the sub-Antarctic region. BMC Microbiol **12:**251.
11. **Charoenchai, C., G. H. Fleet, P. A. Henschke, and B. E. N. Todd.** 1997. Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. Aust J Grape Wine Res **3:**2-8.
12. **Ciani, M., and F. Fatichenti.** 2001. Killer toxin of *Kluyveromyces phaffii* DBVPG 6076 as a biopreservative agent to control apiculate wine Yeasts. Appl Environ Microbiol **67:**3058-63.
13. **Comitini, F., I. Mannazzu, and M. Ciani.** 2009. *Tetrapisispora phaffii* killer toxin is a highly specific beta-glucanase that disrupts the integrity of the yeast cell wall. Microb Cell Fact **8:**55.
14. **Dicks, L. M. T., S. Todorov, and A. Endo.** 2009. Microbial interactions. Biology of Microorganisms on Grapes, in must and in wine**:**335-347.
15. **Droby, S., V. Vinokur, B. Weiss, L. Cohen, A. Daus, E. E. Goldschmidt, and R. Porat.** 2002. Induction of Resistance to Penicillium digitatum in Grapefruit by the Yeast Biocontrol Agent *Candida oleophila*. Phytopathology **92:**393-9.
16. **Fernandez, M., J. F. Ubeda, and A. I. Briones.** 2000. Typing of non-*Saccharomyces* yeasts with enzymatic activities of interest in wine-making. Int J Food Microbiol **59:**29-36.
17. **Fleet, G. H.** 2003. Yeast interactions and wine flavour. Int J Food Microbiol **86:**11-22.

Stellenbosch University https://scholar.sun.ac.za

18. **Golubev, W. I., I. Pfeiffer, and E. W. Golubeva.** 2006. Mycocin production in *Pseudozyma tsukubaensis*. Mycopathologia **162:**313-6.
19. **Izgu, F., and D. Altinbay.** 2004. Isolation and characterization of the K5-type yeast killer protein and its homology with an exo-beta-1,3-glucanase. Biosci Biotechnol Biochem **68:**685-93.
20. **Jijakli, M. H., and P. Lepoivre.** 1998. Characterization of an Exo-beta-1,3-Glucanase Produced by *Pichia anomala* Strain K, Antagonist of *Botrytis cinerea* on Apples. Phytopathology **88:**335-43.
21. **Kulakovskaya, T. V., E. V. Kulakovskaya, and W. I. Golubev.** 2003. ATP leakage from yeast cells treated by extracellular glycolipids of *Pseudozyma fusiformata*. FEMS Yeast Res **3:**401-4.
22. **Kulakovskaya, T. V., A. S. Shashkov, E. V. Kulakovskaya, and W. I. Golubev.** 2005. Ustilagic acid secretion by *Pseudozyma fusiformata* strains. FEMS Yeast Res **5:**919-23.
23. **Liu, G. L., Z. Chi, G. Y. Wang, Z. P. Wang, Y. Li, and Z. M. Chi.** 2013. Yeast Killer toxins, molecular mechanisms of their action and application Crit Rev Biotechnol**:**1-13.
24. **Lorenz, P., K. Liebeton, F. Niehaus, and J. Eck.** 2002. Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space. Curr Opin Biotechnol **13:**572-7.
25. **Marusich, M. F., B. H. Robinson, J. Taanman, S. J. Kim, R. Schillace, J. L. Smith, R. A. Capaldi, R. Castoria, F. deCurtis, J. Lima, and V. de Cicco.** 1997. Beta-1,3-glucanase activity of two saprophytic yeasts and possible mode of action as biocontrol agents against postharvest diseases. Postharvest Biol Technol **12:**293-300.
26. **Masih, E. I., and B. Paul.** 2002. Secretion of beta-1,3-glucanases by the yeast *Pichia membranifaciens* and its possible role in the biocontrol of *Botrytis cinerea* causing grey mold disease of the grapevine. Curr Microbiol **44:**391-5.
27. **Maturano, Y. P., L. A. Rodriguez Assaf, M. E. Toro, M. C. Nally, M. Vallejo, L. I. Castellanos de Figueroa, M. Combina, and F. Vazquez.** 2012. Multi-enzyme production by pure and mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts during wine fermentation. Int J Food Microbiol **155:**43-50.
28. **Mehlomakulu, N. N., M. E. Setati, and B. Divol.** 2014. Characterization of novel killer toxins secreted by wine-related non-*Saccharomyces* yeasts and their action on *Brettanomyces* spp. Int J Food Microbiol **188C:**83-91.
29. **Oikawa, T., Y. Tsukagawa, M. Chino, and K. Soda.** 2001. Increased transglycosylation activity of *Rhodotorula glutinis* endo-beta-glucanase in media containing organic solvent. Biosci Biotechnol Biochem **65:**1889-92.
30. **Samad, M. Y. A., C. N. A. Razak, A. B. Slleh, W. M. Z. W. Yunus, K. Ampon, and M. Basri.** 1989. A plate assay for primary screening of lipase activity. J Microbiol Meth **9:**51-56.

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31. **Saravanakumar, D., D. Spadaro, A. Garibaldi, and M. Gullino.** 2009. Detection of enzymatic activity and partial sequence of a chitinase gene in *Metschnikowia pulcherrima* strain MACH1 used as post-harvest biocontrol agent. Eur J Plant Pathol **123:**183-193.
32. **Schmitt, M. E., T. A. Brown, and B. L. Trumpower.** 1990. A Rapid and Simple Method for Preparation of Rna from *Saccharomyces cerevisiae*. Nucleic Acids Res **18:**3091-3092.
33. **Sipiczki, M.** 2006. *Metschnikowia* strains isolated from botrytized grapes antagonize fungal and bacterial growth by iron depletion. Appl Environ Microbiol **72:**6716-24.
34. **Strauss, M. L. A., N. P. Jolly, M. G. Lambrechts, and P. van Rensburg.** 2001. Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. J Appl Microbiol **91:**182-190.
35. **Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar.** 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol **30:**2725-9.
36. **Tatusova, T., S. Ciufo, B. Fedorov, K. O'Neill, and I. Tolstoy.** 2014. RefSeq microbial genomes database: new representation and annotation strategy. Nucleic Acids Res **42:**D553-9.
37. **Turkel, S., M. Korukluoglu, and M. Yavuz.** 2014. Biocontrol Activity of the Local Strain of *Metschnikowia pulcherrima* on Different Postharvest Pathogens. Biotechnol Res Int **2014:**397167.
38. **Wisniewski, M., C. Biles, S. Droby, R. McLaughlin, C. Wilson, and E. Chalutz.** 1991. Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*. I Characterisation of attachment to *Botrytis cinera*. Physiol Mol Plant Path **39:**245-258.
39. **Zhang, H., Z. Liu, B. Xu, K. Chen, Q. Yang, and Q. Zhang.** 2013. Burdock fructooligosaccharide enhances biocontrol of *Rhodotorula mucilaginosa* to postharvest decay of peaches. Carbohydr Polym **98:**366-71.

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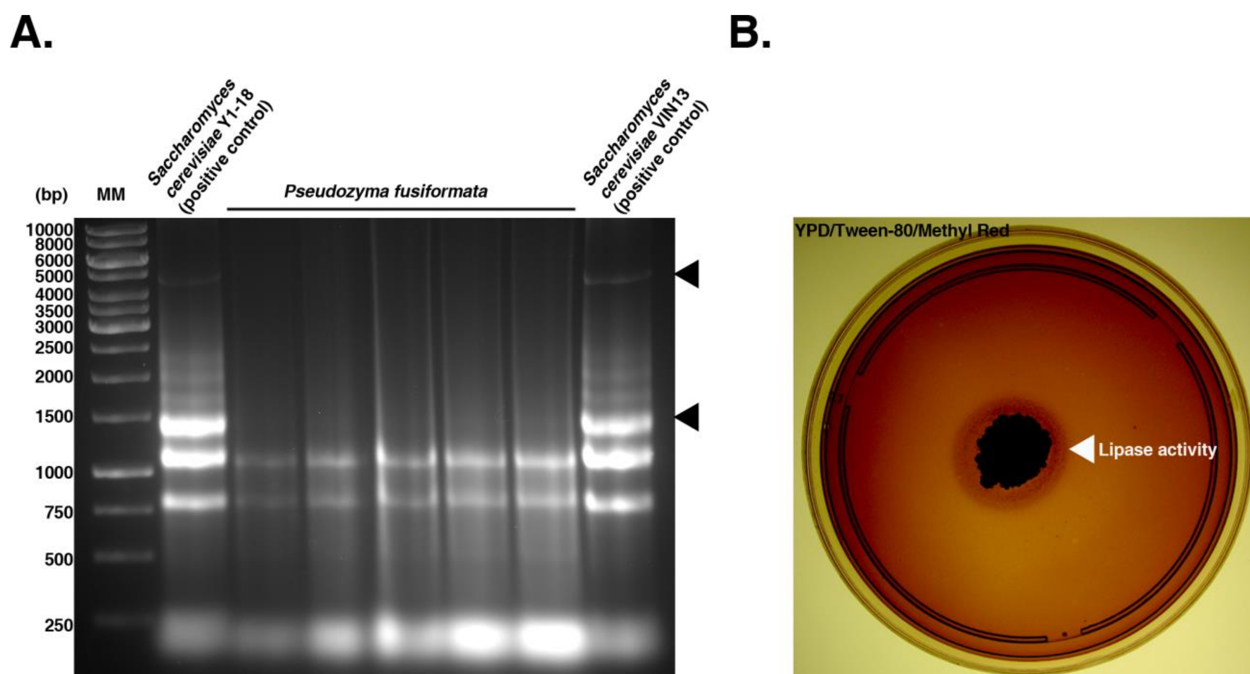

Sup. Figure 4.1 *P. fusiformata* viral dsRNA subtypes and lipase assay **.** (A) Total RNA extracted from *P. fusiformata*. The middle lanes displaying the absence of dsRNA from *P. fusiformata* compared to the flanking lanes exhibiting the dsRNA (black arrow heads) from *S. cerevisiae* Y1-18 and *S. cerevisiae* VIN-13 (positive controls). (B) The *P. fusiformata* exhibiting the lipase activities (white arrow head) on the Tween-80/Methyl-Red/YPD-Agar media plate.

Sup. Table 4.1 Showing the length of the partial chintinase sequence of the yeast isolates with identity and coverage

Yeast isolates	Length (bp)	Closest relative	Identity (%)	Coverage (%)
Metschnikowia chrysoperlae	1,168	*Metschnikowia pulcherrima, Metschnikowia fruticola*	97	80
Pichia burtonii	2,425	*Candida parapsilosis*	49	49
Hyphopichia pseudoburtonii	2,189	*Debaryomyces hansenii*	55	43

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Chapter 5

RESEARCH RESULTS III

Functional metagenomic mining reveals novel hydrolytic enzymes from Cabernet Sauvignon grape juice

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CHAPTER 5

Functional metagenomic analysis reveals a diversity of fungal hydrolases from Cabernet Sauvignon grape juice

Abstract

In the current study, two strategies were employed to evaluate the functional potential of the Cabernet sauvignon grape must microbiome. A direct sequencing approach as well as construction and functional screening of a fosmid library were used to retrieve genes encoding glucanases, glucosidases and chitinases. A fosmid library harbouring 25-50 kb DNA fragments with a titre of 6.5 x 10^5 cfu/mL was prepared in *Escherichia coli* and screened through enzyme activity plate assays. From the screening, 11 β-glucosidase and 22 chitinase positive clones were retrieved and 2 clones were further sequenced and the contigs obtained were assembled. BgluFos-G10 revealed 11 potential ORFs that showed homology to hypothetical proteins belonging to a gene cluster (ranging from CLUG_01947 to CLUG_01962) from *Clavispora lusitaniae* ATCC 42720, with two of the ORFs, ORF3 and ORF4, displaying homology to glycosyl hydrolase family 16 proteins with some exhibiting glucosidase activity. In contrast, the chitinase gene harboured by ChiFos-C21 could not be retrieved following contig assembly, although neighbouring genes were identified on scaffold. Additionally, whole metagenome Roche GS-FLX 454-pyrosequecing and analysis of sequences derived from yeasts and filamentous fungi, revealed 26 DNA fragments (350 bp – 750 bp) that exhibited sequence identity (40-90%) to chitinases, β-glucosidases, glucanases and aspartic proteases. Future work should include the retrieval of the full gene sequences. Combinedly, our results confirm the grape juice is a rich reservoir of diverse hydrolases of fungal origin of relevance for the winemaking process. However, the actual impact of these enzymes on wine quality is yet to be elucidated.

5.1 Introduction

The grape and wine ecosystem is a complex environment that encompasses different species of filamentous fungi, yeasts and bacteria (14). The epiphytic filamentous fungi mainly comprise species of the genera *Aspergillus*, *Botrytis*, *Alternaria*, *Penicillium* and *Cladosporium,* (11) while the endophytes consist of *Alternaria* spp., *Epicoccum nigrum*, *Leptosphaerulina chartarum*, *Aureobasidium pullulans*, *Botryosphaeria* spp., etc. (31) which are present in grapevine at various concentrations depending on the developmental stages of the grape. It has been reported that the frequency of filamentous fungi varies from 10^3 – 10^6 cfu/g berry (10, 11). The yeast population comprises both basidiomycetous and ascomycetous species. The

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basidiomycetous yeasts, such as *Cryptococcus* spp., *Rhodotorula* spp., *Sporobolomyces* spp. and the yeast-like fungus *A. pullulans* are predominantly present on intact unripe grape berries. In contrast, ascomycetous yeasts such as *Candida* spp., *Hanseniaspora* spp., *Metschnikowia* spp. and *Pichia* spp. mostly occur on ripe berries with the highly fermentative yeasts such as *Zygoascus hellenicus, Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* are also favoured (4, 14, 25). The principal wine yeast *Saccharomyces cerevisiae* is rarely detected on undamaged grapes (4). The unripe berries typically harbour a yeast population of 10^3 cfu/g berry while ripe berries may contain 10^4-10^6 cfu/g berry (15, 32). This population may increase to 10^8 cfu/g berry for damaged grapes (4, 16).

The most predominant bacterial populations are lactic acid bacteria (LAB) and acetic acid bacteria (AAB). Many LAB have been isolated from the grape surface, namely *Lactobacillus hilgardii*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Oenococcus oeni*, *Leuconostoc mesenteroides, Pediococcus damnosus* and *Pediococcus parvulus* (4). Few acetic acid bacteria, e.g. *Gluconobacter* spp., *Acetobacter* spp. have also been reported from intact as well as damaged grapes with 10^2-10^3 cfu/g and 10^5- 10^6 cfu/g berries, respectively (3). Other bacterial species such as *Enterobacter* spp., *Bacillus* spp., *Burkholderia* spp., *Serratia* spp. and *Staphylococcus* spp. have also been occasionally reported to be present on the surface of grape berries in lower levels (4).

The microorganisms present in grape must carry out a myriad of biochemical processes that ultimately contribute to the final composition of wine. Although filamentous fungi do not participate in the wine fermentation, they have been reported to possess enzymes of oenological interest such as pectinases and glucanases (46). Among the yeasts, the non-*Saccharomyces* species are known to produce an array of extracellular hydrolytic enzymes as previously reviewed (18). For instance, *Hanseniaspora* spp., *Debaryomyces* spp., *Candida* spp., *Pichia* spp. and *Torulaspora* spp. have been reported to produce extracellular hydrolytic enzymes like glucosidases, pectinases and proteases (5, 7, 26, 40). These extracellular enzymes catalyse different types of reactions in must and during fermentation. For instance, glycosidases hydrolyse the non-volatile precursors from grapes releasing certain volatile compounds (e.g. monoterpenes), thereby improving the wine flavour and aroma (7). Pectinases (polygalacturonase) facilitate the juice extraction from grapes by lowering the viscosity of the grape juice, improving wine clarification and facilitating filtration (46). Some of the non-*Saccharomyces* yeast species are reported to exhibit proteolytic activities. These enzymes are mainly involved in hydrolysis of proteins and they have been proposed as additives to prevent protein haze formation, although their application is not yet effective (17, 22, 33).

Most studies of these enzymes have been conducted on the individual microbial isolates (7, 40). However, these approaches only allow accessibility to enzymes from a few selected

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organisms derived from the cultivable microbiota (24) thus making metagenomic approaches more desirable to increase the chances of retrieving enzymes derived from other microorganisms that might be excluded through culture-dependent methods. Metagenomics techniques have been implemented and indeed proved successful in retrieving novel microbial enzymes such as chitinases, dehydrogenases, proteases, oxygenases, lipases, nitralases, esterases, amylases, xylanase and polyketide synthases both from extreme and non-extreme environments (39, 48). However, these approaches have never been employed to mine the wine microbiome.

The focus of the present study was to identify novel yeast genes encoding hydrolytic enzymes of oenological interest within the total DNA extracted from grape juice. Two approaches were used: (1) construction of metagenomic fosmid library followed by a function-based screening through expression in *Escherichia coli* and enzymatic plate assays, and (2) direct DNA sequencing and sequence-based screening for enzymes of oenological relevance.

5.2 Materials and Methods

5.2.1 Sample collection and fosmid library construction

Sample collection and DNA extraction was carried out according to the protocol described in Chapter 3. Two hundred and fifty micrograms of DNA were resolved on a 0.8% low melting agarose gel by Pulsed-Field Gel Electrophoresis (PFGE) (CHEF MAPPER, Biorad, Richmond, CA). The electrophoresis was programmed at an angle of 120° with a voltage gradient of 6 V/s for a period of 8 h 30 min. The initial switch time was set at 0.05 s and the final switch time at 1 s with a linear (0) ramping factor. The temperature of the TAE buffer was maintained at 14°C throughout the runtime. Thereafter, 25-50 kb DNA fragments were electro-eluted from the agarose gel and 20 µg DNA was used to construct a fosmid library using the CopyControl^{TM}Fosmid library production kit (EPICENTER, Madison, WI). The fosmid clones were selected on Luria Bertani (LB)-Chloramphenicol (12.5 µg/mL) plates. Fifteen fosmid clones were randomly chosen for restriction digestion with *Bam*HI (Roche, Germany), to investigate the insert size. Subsequently, all the fosmid clones were scraped from the media plate, resuspended in LB medium and stored in 20% glycerol at -80°C for future use.

5.2.2 Functional screening of the Fosmid library

The stored FOSMID library was thawed on ice. One hundred microliters were inoculated in 100 mL LB- brroth supplemented with 12.5 µg/mL chloramphenicol and incubated at 37°C overnight. The culture was diluted five-folds in a buffer (per litre: 8.5 g NaCl ; 0.3 g KH_2PO_4; 0.6 g Na_2HPO_4; 0.2 g $MgSO_4$; 0.1 g Gelatin) (8) and spread plated on LB agar supplemented with chloramphenicol and either 1% (w/v) carboxy-methyl cellulose (CMC), 0.1% (w/v) laminarin, 0.5% (w/v) arbutin, 1.25 (w/v) polygalacturonic acid (PGA), 1.14% (w/v) skimmed milk and 0.45% (w/v)

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chitin to screen for glucanases, β-glucosidases, pectinases, acid proteases and chitinases respectively. The pH of all the media was adjusted to pH 4.7 with HCl (1N). The plates were incubated at 30°C until the enzyme activities were visualized. All the substrates selected for screening were purchased from Sigma-Aldrich (St Louis, MO). The fosmid clones that showed enzymatic activities were streaked 2 consecutive times on the respective substrate media plate for confirmation of their activity. All clones displaying β-glucosidases and chitinase activities were designated with a prefix `G´ and `C´ respectively followed by their corresponding number (Arabic numerals) throughout the chapter. EPI300 *E. coli* cells were used as negative control in both the cases while *Lactobacillus brevis* (28) and chitinase (*Vitis vinifera* class IV CHI4D) pJET clone has been used as positive control for glucosidase and chitinase assay respectively. In the case of chitinase assay, the DH5α *E. coli* cells were also included as a negative control to exclude the possibility of the background chitinase activity of the pJET clone. All the clones were subjected to end sequencing on the ABI 3730xl DNA analyzer (Applied Biosystems, Johannesburg, South Africa) using the pCC1/pEpiFOS Forward Sequencing Primer (5'-GGATGTGCTGCAAGGCGATTAAGTTGG-3') and pCC1/pEpiFOS RP-2 Reverse Sequencing Primer (5'-TACGCCAAGCTATTTAGGTGAGA-3') (EPICENTER) to evaluate the microbial origin of their DNA inserts. Fosmids of interest were further sequenced using the Ion Torrent Proton Semiconductor Sequencer (Applied Biosystems) at the Central Analytical Facility, Stellenbosch University, Stellenbosch, South Africa.

5.2.3 Metagenomic contigs assembling

The contig sequences of selected fosmid clones obtained from the Ion Torrent sequencing were assembled using the DNA Dragon–DNA sequence Contig Assembler Software (http://www.dna-dragon.com/) (www.sequentixde). Each of the assembled contigs were compared with sequences available on the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genome) (43) using the Basic Local Alignment Search Tool X (BLASTX) algorithm (2) algorithm. In order to construct the physical map of the fosmid insert, the contigs were matched manually and arranged accordingly.

5.2.4 Whole metagenomic DNA sequencing

Approximately 500 ng genomic DNA derived from the same grape must was subjected to direct shot gun sequencing using the Roche GS-FLX 454 sequencing platform at Inqaba Biotec (Pretoria, South Africa). The DNA template was used to construct 2 independent libraries following the protocol from the GS FLX Rapid Library Preparation kit (Roche Applied Science). The emulsion PCR was optimised to 1:1 bead to DNA ratio using the GS FLX Titanium SV emPCR kit (Lib-L; Roche Applied Science). The GS FLX Titanium LV emPCR kit (Lib-L, Roche Applied Science) was

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used for the final emulsion PCR for sequencing. The library was sequenced using the Roche GS-FLX 454 (Roche Applied Science, Manheim, Germany). The 454 data were subjected to an overall (sequence functional and phylogentic assignments) diversity analysis using the Metagenome Rapid Annotation Subsystems Technology (MG-RAST) (http://metagenomics.anl.gov/) (27). Initially, the Raw 454 sff files were subjected for Quality control (QC) analysis that involved quality filtering, length filtering and de-replication. The taxonomic allocation of the metagenome data was done by comparing with the M5RNA database available on the MG-RAST. The rRNA reads were clustered at 97% identity with a minimum length of 100 bp. Organism and functional identification were performed using a BLAT [Basic Local Alignment Search Tool (BLAST)-like alignment tool](19) search where the Max e-Value Cut-off was set to 10^8, the minimum % Identity cut-off to 60% and a minimum Alignment length of 50 bp. The relative abundance of each gene or species was determined by the total number of individual hits by the total number of hits. The functional annotation of the grape must genes were done by means of the Clusters of Orthologous Groups of proteins database (COG) (41, 42).

5.3 Results

5.3.1 Grape juice metagenomic fosmid library and function-based screening

The grape juice metagenomic DNA was extracted. Using this DNA, a large insert size metagenomic fosmid library was prepared in *E. coli* with a titre of 6.75 x 10^5 cfu/mL. The restriction digestion of 30 randomly chosen metagenomic fosmid clones confirmed that the insert sizes ranged from 25 to 50 kb. The fragment sizes 45-50 kb, 40-45 kb, 30-40 kb and <30kb represented 33.33%, 20%, 30% and 6.66% of the total clones tested, respectively. Furthermore, the restriction digestion of the FOSMID clones revealed that 13.33% of the restriction banding profile looked similar.

The library was subjected to a functional based screening (expression in *E. coli* and enzymatic plate assays) for the following hydrolytic activities: chitinases, β-glucosidases, β-1,4-, β-1,3- and β-1,6-glucanases, acid proteases and pectinases. Among the 2032 and 2310 colonies screened for β-glucosidases and chitinases, 11 and 22 were positive, respectively. No positive candidates were identified for β-1,4-, β-1,3- and β-1,6-glucanases, pectinases and acid-proteases (Table 5.1). The end-sequencing, of all 33 positive candidates for β-glucosidase and chitinase activities suggested that the inserts of these fosmids originated from ascomycetous yeasts (48.48%), bacterial (9.09%), plants (*Streptophyta*) (9.09%) fungal (6.06%) and unknown sources (6.06%). (Sup. Table 5.2). Among the ascomycetes, 68.75% showed homology to *S. cerevisiae* while 31.25% were from non-*Saccharomyces* origin with homologies to DNA from *Scheffersomyces stipitis*, *Candida tenuis*, *Candida pseudolambica*, *Kluyveromyces lodderae* and *Kazachstania naganishi*. Two clones of interest designated BgluFos-G10 and ChiFos-C21

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harbouring a yeast derived glucosidase and chitinase, respectively, were selected for further analysis. Both clones were selected because the end sequencing showed a homology to DNA fragments of non-*Saccharomyces* yeasts (Supplementary Table 5.2) and could therefore be novel as very few non-*Saccharomyces* yeasts present in the wine environment have had their genome sequenced Supplementary Table 5.4).

Table 5.1 Clones showing enzymatic activities from the Fosmid library screening ('-' denotes no enzymatic activities observed)

Enzyme activity	Substrate	Number of colonies screened	Positive hits
β-1,4-Glucanase	Carboxy Methyl Cellulose	2190	-
β-1,3-Glucanases, β-1,6-Glucanases	Laminarin	2316	-
β-glucosidase	Arbutin	2032	11
Chitinase	Chitin	2310	22
Pectinase	Polygalacturonic acid	2203	-
Acid -protease	Skimmed milk	2530	-

5.3.2 Genetic analysis of selected clones

The β-glucosidase and chitinase activities of the *E. coli* mutants harbouring BgluFos-G10 and ChiFos-C21 respectively were further confirmed by re-screening on the same agar plates (as mentioned in the Materials and Methods section) (Figure 5.1).

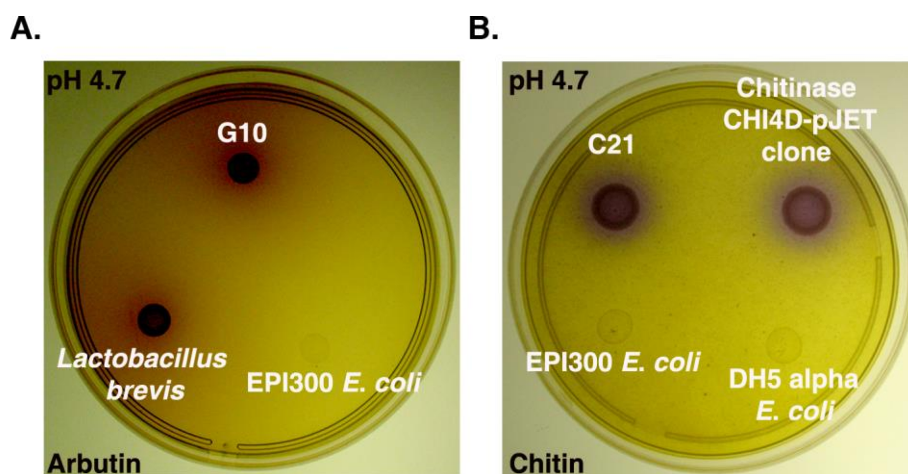

Figure 5.1. *E. coli* FOSMID clones exhibiting enzymatic activity. (A) The clone BgluFos-G10 showing the β-glucosidase activity on LB-Arbutin-Chloramphenicol agar. *Lactobacillus brevis* was used as a positive control (B) ChiFos-C21 fosmid clone showing chitinase activity on LB-chitin-Chloramphenicol agar. The chitinase (*Vitis vinifera* class IV chitinase CHI4D) pJET clone was used as a positive control. In both cases EPI300 *E. coli* cells were used as negative control. In case of the chitinase activity DH5α *E. coli* cells was also included as a negative control. The pH of both the media was adjusted to 4.7.

The sequence analysis of G10 and C21 fosmid clone revealed an insert size of ≈28.82 kb and ≈27.32 kb respectively. The G10 fosmid clones revealed 11 potential open reading frames (ORFs) (Figure 5.2). All these ORFs showed highest similarity to a gene clusters from *Clavispora lusitaniae* ATCC 42720 ranging from CLUG_01947 to CLUG_01962. ORF3 (825 bp) and ORF4 (1222 bp) exhibited 71% identity (100% coverage, E value 3^{e-107}) to CLUG_01950 (Genbank Accession number: XM002618446) and 73% identity (88% coverage, E value 3^{e-172})) to CLUG_01951 (Genbank Accession number: XM002618446), respectively. Further BLAST searches of these hypothetical proteins revealed that both of these genes encode enzymes belonging to the gylcosyl-hydrolase 16 super family. ORF3 encodes a protein that shows 70% identity to Kre6 β-glucan synthesis associated protein from *Lodderomyces elongisporus* NRRL YB-4239 (Accession number: XP001526217.1) and ORF4 encodes a protein with 60% identity (Accession number: EPY52784.1) to a glucosidase protein from *Schizosaccharomyces cryophilus* OY26.

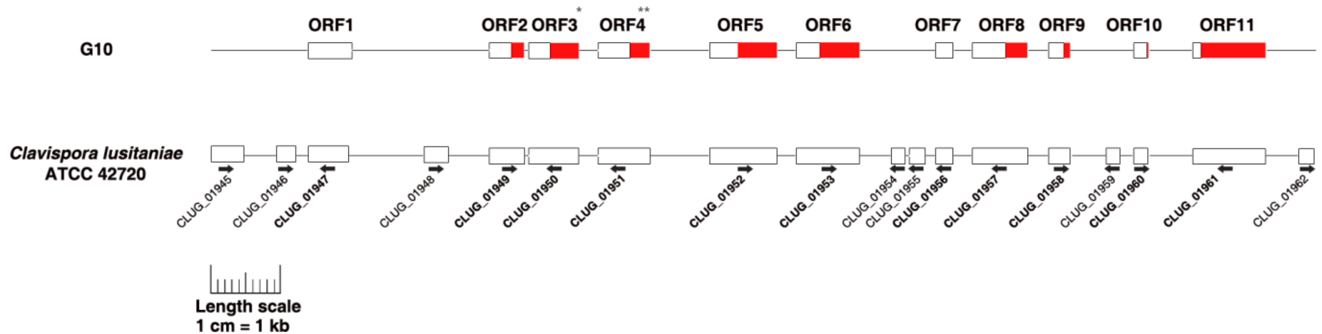

Figure 5.2. Schematic representation of the arrangement of the **BgluFos-**G10 potential ORFs with the corresponding genes of *Clavispora lusitaniae* ATCC 42720. (A) The rectangles represent the potential ORFs of the BgluFos-G10 clone. The red extension boxes represent the missing gene fragment based on the corresponding genes in *C. lusitaniae* ATCC 42720. The asterisks (*) and double asterisks identifies the ORFs belonging to the Glycosyl-hydrolases 16 super family. The double asterisks (**) shows the ORF exhibiting strong homology of the potential ORF to glucosidase gene. (B) The gene (rectangles) clusters from *C. lusitaniae* ATCC 42720. The black arrows show the orientation of the genes. The bold annotations represent the genes of *C. lusitaniae* ATCC 42720 that shows similarity with the corresponding ORFs on BgluFos-G10. The continuous line between ORFs represents the intergenic spacer regions.

In the ChiFos-C21 clone, a gene cluster with high homology to CLUG_0520 to CLUG_0530 of *Clavispora lusitaniae* ATCC 42720 was identified. However, in this cluster we could only identify genes related to CLUG_0520, CLUG_05021, CLUG_05022, CLUG_05024, CLUG_05027 and CLUG_0530. None of these genes showed any homology to known chitinase enzymes. Another gene cluster ranging from CLUG_00307 to CLUG_00314 from *Clavispora lusitaniae* ATCC 42720 was also predicted but we could only identify a fragment sequence that shows homology to the genes CLUG_00307, CLUG_00309, CLUG_00313 and CLUG_00314 (Supplementary Table 5.1).

5.3.3 Grape juice whole metagenomic sequencing

The Roche GS-FLX 454 pyrosequencing of the metagenomes revealed a sequence size of 92,570,157 bp accounting for 175,616 reads (Supplementary Figure 5.3). Streptophyta (mainly *Vitis vinifera*) to be the dominant taxa accounting for 57% of the functional gene pool in the whole metagenome while the ascomycota and basidiomycota, accounted for 28% and 4%, respectively. Functional analysis of the sequence data showed that genes encoding enzymes involved in carbohydrate metabolism and transport accounted for 4.6% of the functional gene pool (Figure 5.3).

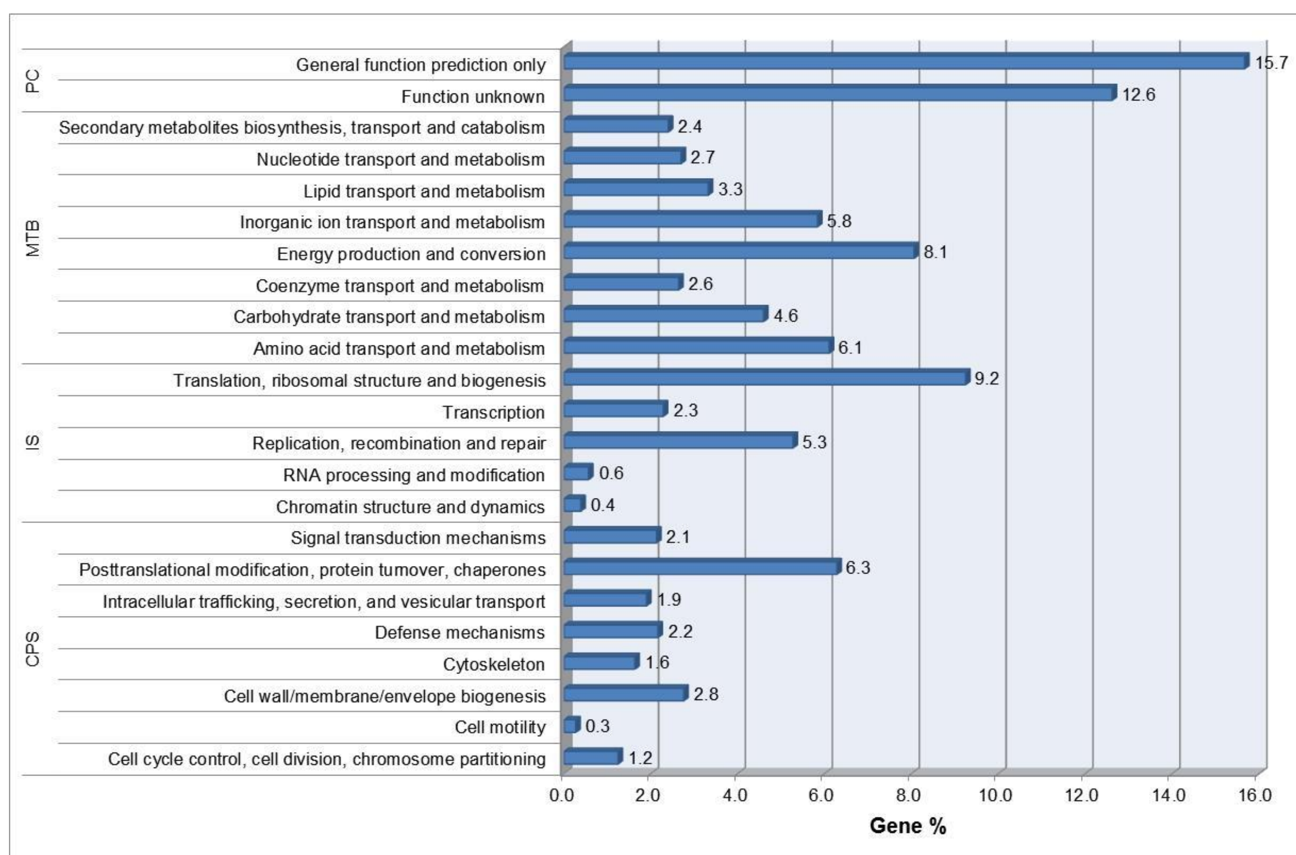

Figure 5.3. COG-based annotation of the genes from the Cabernet Sauvignon grape must metagenome, showing the distribution of genes related to cellular processes (CPS), Information storage (IS), Metabolism (MTB) and Poorly Characterized genes (PC). The values immediately next to rectangular horizontal bars represent the relative abundance of each of the genes present in the gene pool.

Genes encoding glycosidases were the most abundant within the carbohydrate transport and metabolism gene pool, followed by 6-phosphofructokinase and chitinase encoding genes (Figure 5.4). Taxonomic assignment of the fungal gene pool revealed that the genes were mainly derived from non-*Saccharomyces* yeasts and filamentous fungi (Table 5.2). The yeast derived genes identified in the current study exhibited sequence similarity to chitinases, aspartic protease

and exo-glucanases from common wine yeasts including *Metschnikowia pulcherrima*, *S. cerevisiae* and *Debaryomyces hansenii*.

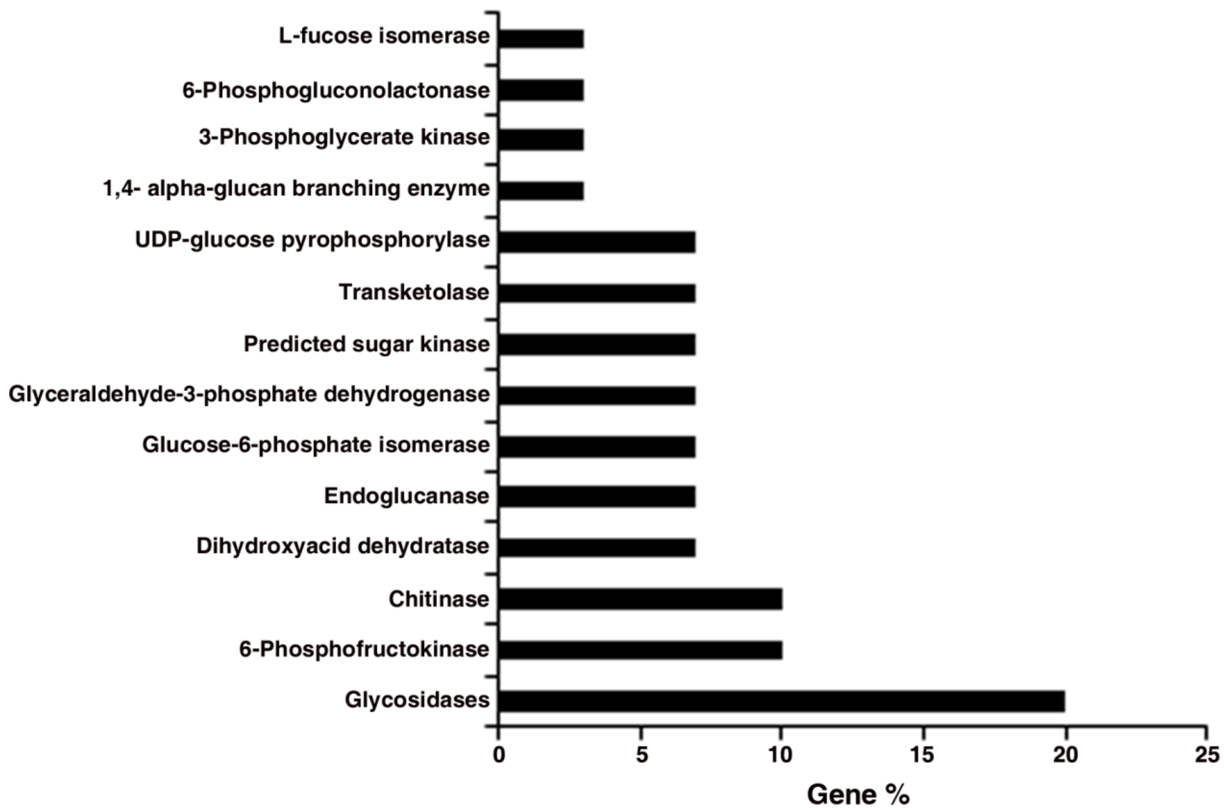

Figure 5.4. The relative abundance and distribution of genes involved in carbohydrate transport and metabolism in the Cabernet sauvignon metagenome.

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Table 5.2. Putative fungal hydrolases identified from the carbohydrate transport and metabolism gene pool of the Cabernet sauvignon whole metagenome. The specific gene fragment lengths for each of the closest relatives are also being denoted.

Sequence ID	Fragment length (bp)	Predicted enzyme	Closest relative	% identity	Length of the specific gene in the closest relative (bp)
H551VLF01A094D	510	Chitinase	*Debaryomyces hansenii*	44	1,182
H551VLF01BBMN2	449	Chitinase	*Metschnikowia pulcherrima*	79	1,080
H551VLF01A6SRB	543	Glucosidase II	*Scheffersomyces stipitis*	47	2,736
H551VLF01A4E06	395	β-glucosidase	*Scheffersomyces stipitis*	78	2,214
H551VLF01AMBHY	536	β-glucosidase	*Aspergillus clavatus*	42	1,326
H551VLF01A4E7Y	491	β-glucosidase	*Rhizomucor miehei*	57	4,063
H551VLF01AI9Q2	475	β-glucosidase	*Cryptococcus neoformans*	54	2,573
H551VLF01BWCP7	521	β-1,3-exoglucanase	*Ampelomyces quisqualis*	69	2,349
H551VLF01ADDS1	447	β-1,3-glucosidase	*Pyrenophora tritici-repentis*	88	1,992
H551VLF01BIDGN	527	β-1,3-glucosidase	*Aspergillus niger*	87	2,583
H551VLF01A59K3	505	oligo-1,6-glucosidase	*Talaromyces marneffei*	73	1,827
HQ7JFPR01BIPL7	499	β-1,3-glucosidase precursor	*Talaromyces stipitatus*	48	2,023

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Table 5.2 (cont.)

Sequence ID	**Fragment length (bp)**	**Predicted enzyme**	**Closest relative**	**% identity**	**Length of the specific gene in the closest relative (bp)**
HQ7JFPR01AIP93	547	β-glucosidase	*Talaromyces marneffei*	44	2,535
HQ7JFPR01A3AMV	494	β-glucosidase	*Aspergillus terreus*	69	2,586
HQ7JFPR01AFNUE	706	β-glucosidase	*Aspergillus clavatus*	69	1,326
HQ7JFPR01AU0VN	418	β-glucosidase	*Scheffersomyces stipitis*	58	2,214
HQ7JFPR01A7K2B	551	β-glucosidase	*Paecilomyces* spp.	60	2,968
H551VLF01AK6ZL	524	β-glucosidase	*Aspergillus niger*	50	1,988
H551VLF01BIW20	467	β-glucosidase	*Aspergillus flavus*	68	1,794
HQ7JFPR01BFNG8	542	Chitinase	*Wickerhamomyces ciferrii*	42	1,269
HQ7JFPR01BHOWG	533	Aspartic protease	*Metschnikowia pulcherrima*	52	1,137
HQ7JFPR01ASQKL	497	Endo-1,3(4)-β-glucanase	*Talaromyces marneffei*	58	2,511
HQ7JFPR01BHN4Z	583	Exo-glucanase	*Saccharomyces cerevisiae*	55	1,344
H551VLF01A5JYC	405	Endoglucanase	*Trichoderma* spp.	61	1,257
H551VLF01A1Y8U	484	Hydrolase	*Baudoinia compniacensis*	79	2,996
H551VLF01BFKJN	544	Endopeptidase	Mucor *circinelloides* f. *circinelloides*	66	1,316

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5.4 Discussion

The winemaking process is primarily governed by an array of enzymatic activities that drive the fermentation kinetics involved in the bioconversion of grape juice to wine. These enzymes are derived from grapes and the microbiota present on/in the grape/must/wine (46). Our screening through cultivation-based approaches (Chapter 4) has shown a wide range of diversities in the enzymatic activities between the non-*Saccharomyces* yeasts. Chitinase and glucanase activities were predominant followed by β-glucosidase activity exhibited only by *Metschnikowia chrysoperlae*. Our observations were consistent with earlier studies where it has also been shown that wine hosts various extracellular hydrolytic enzymes such as glucanases, amylases, pectinases, β-glucosidases and acid proteases originating from different non-*Saccharomyces* yeasts (7, 40). In the current study, two metagenomic approaches were employed to evaluate the functional potential of the grape must microbiome.

Whole metagenome shotgun sequencing was used to determine the functional potential of the wine microbiome. Using this approach we found a wide range of DNA sequences that showed homology to an array of genes encoding hydrolytic enzymes. As depicted in Figure 5.4, in the carbohydrate transport and metabolism gene pool, glycosidases were the dominant enzymes followed by chitinases and endoglucanases. The fungal partial sequences of the genes encoding these enzymes were found to originate mainly from filamentous fungi and non-*Saccharomyces* yeasts (Table 5.2) similar to the results obtained from plate assays. When a fungal community profiling based on the rRNA gene sequences and predicted metabolic genes was performed, the presence of non-*Saccharomyces* yeast genera such as *Clavispora*, *Metschnikowia*, *Kazachstania, Torulaspora*, *Lachancea*, *Candida*, *Pichia* and *Kluyveromyces* in the whole metagenome sequence was confirmed but the number of fragments per species was very low (1-2 hits) (Supplementary Figure 5.1 and 5.2). This could explain why, in spite of the presence of these yeast species in the whole metagenome, most of the hydrolytic enzymes displayed (Table 5.2) were found to originate from filamentous fungi.

Furthermore, pair-wise comparison of the partial chitinase-encoding gene sequence from the whole metagenome (ID: H551VLF01BBMN2) and those obtained from the yeast isolates in Chapter 4 revealed 69.30%, 44.98% and 41.71% identity to the partial chitinase sequences of *M. chrysoperlae*, *H. pseudoburtonii* and *P. burtonii,* respectively, whereas the sequence ID: H551VLF01A094D exhibited 48.26%, 71.76% and 48.26% to *M. chrysoperlae*, *H. pseudoburtonii* and *P. burtonii,* respectively. In addition, the sequence ID: HQ7JFPR01BFNG8, closely related to the chitinase of *Wickerhamomyces cifferi* (42% identity), showed 43.80%, 49.44% and 75% identity to *M. chrysoperlae*, *H. pseudoburtonii* and *P. burtonii* partial chitinase sequence, respectively. This diversity of enzymes revealed through direct metagenome sequencing was also observed in other

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hydrolases. For instance, the data display a diversity of glucanases derived from filamentous fungi and non-*Saccharomyces* yeasts, among them β-1,3-exoglucanases that have been shown to play a role in mycoparasitic fungal interactions. For instance, a sequence ID: H551VLF01BWCP7 exhibited homology to β-1,3-exoglucanase from *Ampelomyces quisqualis*. This fungus has previously been reported to produce β-1,3-exoglucanase enzyme during mycoparisitism (37) and is used as a biocontrol agent against grapevine powdery mildew disease (12). Another fragment, sequence ID: H551VLF01A5JYC, showed homology to exoglucanase from *Trichoderma* species. Various species of *Trichoderma* have been reported to exhibit mycoparisitism and have been extensively used as biocontrol agents. The mycoparisitism of *Trichoderma* spp. has been reported to be mediated through lytic enzymes like endoglucanases, chitinases and proteases (6, 34, 38). Our metagenomic sequences also revealed a few sequences that showed close homology to the β-glucosidase gene from *Aspergillus* spp. and *Talaromyces* spp. *Aspergillus* spp. has been previously reported as potential agent biocontrol agent (36, 44) and also shown to secrete β-glucosidase (45, 47). *Talaromyces* sp. is not a frequent fungus isolated from grape. It has however been reported that this fungus exhibits glucosidase activity (30). Only a few sequences of genes encoding enzymes involved in protein degradation were retrieved. Interestingly, one sequence ID: HQ7JFPR01BHOWG exhibited 52% identity to the aspartic protease from *M. pulcherrima* (35). All these observations show that the genes retrieved from the metagenome exhibit low percentage identity with the available sequences from our yeast isolates (Chapter 4), suggesting that the whole metagenome sequences originate from different microorganisms. This implies that whole metagenomic sequencing could reveal novel biocatalysts that have not yet been retrieved from cultured microorganisms. However, the sequence data obtained from shotgun sequences still only provides a snapshot of the functional potential of the wine mycobiota since only partial gene sequences were obtained and the sequence coverage/depth was not enough to give a near representation of the entire metagenome. Indeed, only 92.5 Mb of sequence data were retrieved, representing approximately 7 yeast genome sizes. Nevertheless, the data show that glucosidases are the most predominant glycosyl hydrolases in the grape must ecosystem. Indeed, most screening attempts have similar trends even though the focus tends to be on non-*Saccharomyces* yeasts (7, 26, 40, 46). Moreover, the glycosyl hydrolase-encoding genes showed a percent similarity ranging from 42 to 88% to known fungal species (Table 5.2). This finding was not surprising because the lack of fully annotated non- *Saccharomyces* genomes pose a challenge in gene prediction and annotation of the metagenomes. Many of the non-*Saccharomyces* yeast genomes are not sequenced and those which are (Supplementary Table 5.4), are not fully annotated yet.

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Apart from shotgun whole metagenome sequencing, a fosmid library was constructed in the current study and screened for chitinases, β-glucosidases, β-1,4-, β-1,3-glucanases and β-1,6-glucanases, acid proteases and pectinases. Similar to the shotgun sequencing data, the fosmid library also revealed a high frequency of glucosidases. In addition, chitinases were found to be more prevalent among the fosmid clones. End sequencing of the positive fosmids revealed that 68.75% of the ascomycota showed close homology to *S. cerevisiae* while the rest 31.25% shows homology to non-*Saccharomyces* species. Although, this finding clearly shows that there is a dominance of species population that is related to *S. cerevisiae*, the end-sequencing data were not very clean to confirm this statement. The sequence analysis of one of the β-glucosidase expressing clones, BgluFos-G10, revealed that the clone harboured two ORFs with high sequence similarity to family 16 glucosyl hydrolases. This family encompasses various glucanases including fungal Kre6-glucanase (23). The latter protein (cd02180) is closely related to laminarinase and has been previously identified as an important component of the cell wall β-1,6-glucan synthesis pathway in *Saccharomyces cerevisiae* (20). Orthologues of this protein display glucosidase activity and have been also shown to play a role in β-D-glucan biosynthetic processes. This could explain why the clone displayed a strong β-glucosidase activity. However, studies have reported that this category of enzyme is more involved in yeast cell wall assembly and cell septation as primary function (1, 29). Consequently it is possible that the gene retrieved from the fosmid does not have a direct influence on wine quality. In order to verify our hypothesis, it would be necessary to retrieve the full sequence of ORF4, clone it and express it in a heterologous host.

Although the ChiFos-C21 fosmid clone showed extracellular chitinase activities *in vitro*, the assembled sequence fragments did not show any homology to known chitinase genes. We indentified a group of sequence fragments that exhibited high homology to a gene cluster from CLUG_05020 to CLUG_05030 of *Clavispora lusitaniae* ATCC 42720. However, we could not detect sequence fragments of ChiFos-C21 that show homology to the genes CLUG_05023, CLUG_05025, CLUG_05026, CLUG_05028 and CLUG_05029. The sequence depth was not sufficient to allow retrieval of all the genes that make up the insert in this fosmid. We could also retrieve another sequence cluster that shows homology to the genes ranging from CLUG_00307 to CLUG_00314 but we could only identify sequence contigs with homology to CLUG_00307, CLUG_00309, CLUG_00313 and CLUG_00314, none of which encode chitinases. However, in the close vicinity of these genes, another gene cluster ranging from CLUG_00319 to CLUG_0022 encode various hydrolases. Among them, CLUG_00319 which is a GH18 superfamily protein, exhibits chitinase type II activity (cd06548). Therefore, we can speculate that a similar gene encoding a chitinase in our ChiFos-C21 was missed out in our sequencing due to the lack of sequencing depth. Furthermore, a pair-wise alignment of the fosmid-derived chitinase fragment (CLUG_00319) with that derived from the whole metagenome revealed 71.33% identity between

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the 2 fragments, which suggests that they could originate from different organisms. Since the metagenome-derived fragment has high similarity with *M. pulcherrima*, the DNA fragment present in clone ChiFos-C21 could originate from *Metschnikowia* sp. This would not be surprising since *Clavispora* has been shown to be the sister genus of *Metschnikowia* and fall in the family of *Metschnikowiaceae* (21).

Overall, our findings suggest that the sequencing depth was not sufficient to allow retrieval of all the genes that constitute the fosmid ChiFos-C21 insert. In order to confirm this hypothesis, ChiFos-C21 should be re-sequenced. Moreover, both the fosmid clones, BgluFos-G10 and ChiFos-C21, have shown close homology to *C. lusitaniae*. In support of these findings our shotgun sequence data also seem to detect a higher abundance of genes closely related to *Clavispora* (Supplementary Figure 5.2).

In conclusion, our metagenomic study confirms that grape juice is a rich reservoir of valuable biocatalysts. Enzymes from oenological relevance were successfully retrieved from both data sets (functional screening and direct sequencing).Further investigations should include retrieving full gene sequences and thereafter test their expression during the wine fermentation. The activity of the corresponding enzymes under wine-making condition should also be assessed in order to evaluate their impact of their activities on the sensory properties of wine.

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5.5 References

1. **Adams, D. J.** 2004. Fungal cell wall chitinases and glucanases. Microbiology **150:**2029-35.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-402.
3. **Barata, A., M. Malfeito-Ferreira, and V. Loureiro.** 2012. Changes in sour rotten grape berry microbiota during ripening and wine fermentation. Int J Food Microbiol **154:**152-61.
4. **Barata, A., M. Malfeito-Ferreira, and V. Loureiro.** 2012. The microbial ecology of wine grape berries. Int J Food Microbiol **153:**243-59.
5. **Bueso, C. G., B. E. Zarzoso, J. M. Cabellos, G. M. Díaz, and T. Arroyo.** 2012. Biotechnological potential of non-*Saccharomyces* yeasts during isolated during spontaneous fermentations of Malvar (Vitis vinifera cv.L.). Eur Food Res Technol**:**1-15.
6. **Cabaroglu, T., S. Selli, A. Canbas, J. P. Lepoutre, and Z. Günata.** 2003. Wine flavor enhancement through the use of exogenous fungal glycosidases. Enzyme Microbial Technol **33:**581-587.
7. **Charoenchai, C., G. H. Fleet, P. A. Henschke, and B. E. N. Todd.** 1997. Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. Aust J Grape Wine Res **3:**2-8.
8. **Chung, E. J., H. K. Lim, J. C. Kim, G. J. Choi, E. J. Park, M. H. Lee, Y. R. Chung, and S. W. Lee.** 2008. Forest soil metagenome gene cluster involved in antifungal activity expression in *Escherichia coli*. Appl Environ Microbiol **74:**723-30.
9. **Da, R. R., V. Palacios, M. Combina, M. E. Fraga, O. R. De, C. E. Magnoli, and A. M. Dalcero.** 2002. Potential ochratoxin A producers from wine grapes in Argentina and Brazil. Food Addit Contam **19:**408-14.
10. **Diguta, C. F., B. Vincent, M. Guilloux-Benatier, H. Alexandre, and S. Rousseaux.** 2011. PCR ITS-RFLP: A useful method for identifying filamentous fungi isolates on grapes. Food Microbiol **28:**1145-54.
11. **Falk, S. P., D. M. Gadoury, R. C. Pearson, and R. C. Seem.** 1995. Partial Central of Grape Powdery Mildew by the Mycoparasite *Ampelomyces-Quisqualis*. Plant Disease **79:**483-490.
12. **Fleet, G. H.** 2003. Yeast interactions and wine flavour. Int J Food Microbiol **86:**11-22.
13. **Fleet, G. H., and G. M. Heard.** 1993. Yeasts - growth during ferementation. Wine Microbiology and Biotechnology**:**27-54.
14. **Fleet, G. H., Prakittchaiwattana, C., Beh, A.L., Heard, G.M.** 2002. The yeast ecology of wine grapes. Biodiversity and Biotechnology of wine yeasts**:**1-17.

Stellenbosch University https://scholar.sun.ac.za

15. **Jolly, N., O. Augustyn, and I. S. Pretorius.** 2006. The role and use of non-*Saccharomyces* yeasts in wine production. S Afr J Enol Vitic **27:**15-39.
16. **Jolly, N. P., C. Varela, and I. S. Pretorius.** 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res **14:**215-37.
17. **Kent, W. J.** 2002. BLAT-the BLAST-like alignment tool. Gen Res **12:**656-664.
18. **Kurita, T., Y. Noda, T. Takagi, M. Osumi, and K. Yoda.** 2010. Kre6 protein essential for yeast cell wall beta-1,6-glucan synthesis accumulates at sites of polarized growth. J Biol Chem **286:**7429-38.
19. **Lachance, M. A.** 2011. Clavispora Rodrigues de Miranda (1979). The yeasts A Taxonomic study **21:**349-353.
20. **Lagace, L. S., and L. F. Bisson.** 1990. Survey of yeast acid proteases for effectiveness of wine haze reduction. Am J Enol Vitic **41:**147-155.
21. **Lesage, G., and H. Bussey.** 2006. Cell wall assembly in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev **70:**317-43.
22. **Lorenz, P., K. Liebeton, F. Niehaus, and J. Eck.** 2002. Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space. Curr Opin Biotechnol **13:**572-7.
23. **Loureiro, V., and M. Malfeito-Ferreira.** 2003. Spoilage yeasts in the wine industry. International Journal of Food Microbiology **86:**23-50.
24. **Maturano, Y. P., L. A. Rodriguez Assaf, M. E. Toro, M. C. Nally, M. Vallejo, L. I. Castellanos de Figueroa, M. Combina, and F. Vazquez.** 2012. Multi-enzyme production by pure and mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts during wine fermentation. Int J Food Microbiol **155:**43-50.
25. **Meyer, F., D. Paarmann, M. D'Souza, R. Olson, E. M. Glass, M. Kubal, T. Paczian, A. Rodriguez, R. Stevens, A. Wilke, J. Wilkening, and R. A. Edwards.** 2008. The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics **9:**386.
26. **Michlmayr, H., C. Schumann, N. M. da Silva, K. D. Kulbe, and A. M. del Hierro.** 2011. Isolation and basic characterization of a beta-glucosidase from a strain of *Lactobacillus brevis* isolated from a malolactic starter culture. J Appl Microbiol **108:**550-9.
27. **Mouassite, M., N. Camougrand, E. Schwob, G. Demaison, M. Laclau, and M. Guerin.** 2000. The 'SUN' family: yeast SUN4/SCW3 is involved in cell septation. Yeast **16:**905-19.
28. **Nakkharat, P., and D. Haltrich.** 2006. Purification and characterisation of an intracellular enzyme with beta-glucosidase and beta-galactosidase activity from the thermophilic fungus *Talaromyces thermophilus* CBS 236.58. J Biotechnol **123:**304-13.

Stellenbosch University https://scholar.sun.ac.za

29. **Pancher, M., M. Ceol, P. E. Corneo, C. M. Longa, S. Yousaf, I. Pertot, and A. Campisano.** 2012. Fungal endophytic communities in grapevines (*Vitis vinifera* L.) respond to crop management. Appl Environ Microbiol **78:**4308-17.

30. **Parish, M. E., and D. E. Caroll.** 1985. Indigenous yeasts associated with Muscadine (*Vitis routundifolia*) grapes and must. Am J Enol Vitic **36:**165 - 169.

31. **Pocock, K. F., P. B. Hoj, K. S. Adams, M. J. Kwiatkowski, and E. J. Waters.** 2003. Combined heat and proteolytic enzyme treatment of white wines reduces haze forming protein content without detrimental effect. Aus J Grape Wine Res **9:**56-63.

32. **Punja, Z. K., and R. S. Utkhede.** 2003. Using fungi and yeasts to manage vegetable crop diseases. Trends Biotechnol **21:**400-7.

33. **Reid, V. J., L. W. Theron, M. du Toit, and B. Divol.** 2012. Identification and partial characterization of extracellular aspartic protease genes from Metschnikowia pulcherrima IWBT Y1123 and Candida apicola IWBT Y1384. Applied and Environmental Microbiology **78:**6838-49.

34. **Rosada, L. J., J. R. Sant'anna, C. C. Franco, G. N. Esquissato, P. A. Santos, J. P. Yajima, F. D. Ferreira, M. Machinski, B. Correa, and M. A. Castro-Prado.** 2013. Identifcation of *Aspergillus flavus* isolates as potential biocontrol agents of aflatoxin contamination in crops. J Food Prot **76:**1051-1055.

35. **Rotem, Y., O. Yarden, and A. Sztejnberg.** 1999. The mycoparasite *Ampelomyces quisqualis* expresses exgA encoding an exo-beta-1,3-glucanase in culture and during mycoparasitism. Phytopathology **89:**631-638.

36. **Sanz, L., M. Montero, J. Redondo, A. Llobell, and E. Monte.** 2005. Expression of an alpha-1,3-glucanase during mycoparasitic interaction of *Trichoderma asperellum*. FEBS J **272:**493-9.

37. **Schroder, C., S. Elleuche, S. Blank, and G. Antranikian.** 2014. Characterization of a heat-active archaeal beta-glucosidase from a hydrothermal spring metagenome. Enzyme Microb Technol **57:**48-54.

38. **Strauss, M. L. A., N. P. Jolly, M. G. Lambrechts, and P. van Rensburg.** 2001. Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. J Appl Microbiol **91:**182-190.

39. **Tatusov, R. L., M. Y. Galperin, D. A. Natale, and E. V. Koonin.** 2000. The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res **28:**33-6.

40. **Tatusov, R. L., D. A. Natale, I. V. Garkavtsev, T. A. Tatusova, U. T. Shankavaram, B. S. Rao, B. Kiryutin, M. Y. Galperin, N. D. Fedorova, and E. V. Koonin.** 2001. The COG database: new developments in phylogenetic classification of proteins from complete genomes. Nucleic Acids Res **29:**22-8.

Stellenbosch University https://scholar.sun.ac.za

41. **Tatusova, T., S. Ciufo, B. Fedorov, K. O'Neill, and I. Tolstoy.** 2014. RefSeq microbial genomes database: new representation and annotation strategy. Nucleic Acids Res **42:**D553-9.

42. **Tiwari, C. K., J. Parihar, and R. K. Verma.** 2011. Potential of *Aspergillus niger* and *Trichoderma viridae* as biocontrol agents of wood decay. J Indian Acad Wood Sci **8:**169-172.

43. **Vaithanomsat, P., M. Songpim, T. Malapant, A. Kosugi, W. Thanapase, and Y. Mori.** 2011. Production of beta-Glucosidase from a Newly Isolated *Aspergillus* Species Using Response Surface Methodology. Int J Microbiol **2011:**949252.

44. **van Rensburg, P., and I. S. Pretorius.** 2000. Enzymes in Wine making: Harnessing natural catalysts for efficient biotransformations - A Review. S Afr J Enol Vitic **21:**52-73.

45. **Watanabe, T., T. Sato, S. Yoshioka, T. Koshijima, and M. Kuwahara.** 1992. Purification and properties of *Aspergillus* niger beta-glucosidase. Eur J Biochem **209:**651-9.

46. **Yeh, Y. F., S. C. Chang, H. W. Kuo, C. G. Tong, S. M. Yu, and T. H. Ho.** 2013. A metagenomic approach for the identification and cloning of an endoglucanase from rice straw compost. Gene **519:**360-6.

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Appendix

Sup. Table 5.1: Representation of the DNA sequences after assembling of the contigs obtained from C21 fosmid clone after 454-pyrosequencing. The closest protein super families, closest protein and the closest relatives are also being depicted (- denotes 'not applicable')

DNA sequence serial numbers	Number of contigs assembled	Length of the contigs after assembly (bp)	Closest similar protein super families	Closest protein	Closest relative	% identity	Protein accession number
1	11161	3898	ATPase-I	Hypothetical protein CLUG_00309	*Clavispora lusitaniae* ATCC 42720	74	XP002619150.1
2	426	1205	Arrest in_C	Hypothetical protein CLUG_00314	*Clavispora lusitaniae* ATCC 42720	76	XP002619155.1
3	271	831	DnaJ	Hypothetical protein CLUG_05030	*Clavispora lusitaniae* ATCC 42720	84	EMG49827
4	83	557	No putative conserved domain has been detected	Hypothetical protein CLUG_05025	*Clavispora lusitaniae* ATCC 42720	73	XP002615010.1
5	222	547	Hydrolase_like2	Hypothetical protein CLUG_00309	*Clavispora lusitaniae* ATCC 42720	75	XP002619150.1
6	88	516	Cyto_heme_lyase	Hypothetical protein CLUG_05021	*Clavispora lusitaniae* ATCC 42720	67	XP002615006.1
8	72	453	No putative conserved domain has been detected	Hypothetical protein CLUG_00314	*Clavispora lusitaniae* ATCC 42720	75	XP002619155.1
9	111	451	No putative conserved domain has been detected	Hypothetical protein CLUG_05022	*Clavispora lusitaniae* ATCC 42720	52	XP002615007.1
13	55	406	AMN1	Hypothetical protein CLUG_04841	*Clavispora lusitaniae* ATCC 42720	42	XP002614826.1
14	148	419	Cation_ATPase_N	Hypothetical protein CLUG_00309	*Clavispora lusitaniae* ATCC 42720	68	XP002619150.1
15	189	403	No putative conserved domain has been detected	Hypothetical protein G210_5613, partial	*Candida maltose* Xu316	59	EMG50961.1
16	81	376	DoxX	Hypothetical protein CLUG_05020	*Clavispora lusitaniae* ATCC 42720	73	XP002615005.1
17	51	338	No putative conserved domain has been detected	Hypothetical protein CLUG_05022	*Clavispora lusitaniae* ATCC 42720	55	XP002615007.1
18	68	336	ABC_ATPase	Hypothetical protein CLUG_05027	*Clavispora lusitaniae* ATCC 42720	98	XP002615012.1
22	3	230	ATPase-IID_K-Na	Hypothetical protein CLUG_00309	*Clavispora lusitaniae* ATCC 42720	78	XP002619150.1
23	12	178	Pmev_kin_ERG8	Hypothetical protein 24CLUG_05024	*Clavispora lusitaniae* ATCC 42720	62	XP002615009
25	8	308	No putative conserved domain has been detected	Hypothetical protein CLUG_00307	*Clavispora lusitaniae* ATCC 42720	53	XP002619148.1
26	2	267	Pepsin_Retropepsin_like	Aspartic protease	*Metschnikowia pulcherrima*	99	AFK08696
27	4	226	No putative conserved domain has been detected	Hypothetical protein CLUG_00314	*Clavispora lusitaniae* ATCC 42720	73	CCE43603

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Sup. Table 5.2 Representation of the end sequencing of the putative fosmid clones. The Fw and Rv represents the forward and the reverse primers respectively used for end sequencing. The clones marked with asterisk (*) subjected for Next Generation sequencing. (-) denotes no sequencing results.

Enzymatic activities	Clones	Closest relatives	Identity	Closest relatives	Identity
Glucosidases	G2	*S. cerevisiae* (Fw)	93%	*S. cerevisiae* (Rv)	90%
	G5	*S. cerevisiae* (Fw)	93%	*S. cerevisiae* (Rv)	97%
	G7	-	-	-	-
	G10*	*Schefferomyces stipitis*, CBS 6054 chromosome 1 (Fw)	74%	Helleborus x hybridus FRUITFULL-like protein (FL1) mRNA, partial cds, (Rv)	96%
	G12	*Candida tenuis* ATCC 10573 hypothetical protein partial mRNA (Fw)	77%	-	-
	G13	-	-	*Mes musculus* (Rv)	81%
	G15	*Listonella anguillarum* (Fw)	96%	*Candida tropicalis* (Rv)	70%
	G16	Mucor *circinelloides* (Fw)	73%	-	-
	G19	-	-	*Aplysia californica* (sea hare) (Rv)	78%
	G20	*Flavobacterium psychrophilum* (Fw)	96%	*Pseudomonas putida* NBRC 141 (Rv)	74%
	G23	*Vitis vinifera* (Fw)	88%	*Asclepias syriaca* (Rv)	80%
Chitinases	C1	*Panthelops hodgsanii* (Fw)	100%	-	-
	C2	*Streptococcus parasanguinis* ATCC (Fw)	93%	-	-
	C3	*Candida pseudolambica* strain (Fw)	76%	-	-
	C4	*Asclepias syriaca* (Fw)	79%	-	-
	C5	*Asclepias syriaca* (Fw)	92%	*Vitis Vinifera* (Rv)	88%
	C6	*Botryotinia fuckeliana* (Fw)	89%	*Belliella baltica* (Rv)	88%
	C7	*S. cerevisiae* YJM993 (Fw)	95%	*S. cerevisiae* (Rv)	87%
	C8	*S. cerevisiae* (Fw)	84%	*S. cerevisiae* (Rv)	75%
	C9	*S. cerevisiae* (Fw)	92%	*S. cerevisiae* (Rv)	70%
	C10	-	-	-	-
	C11	*S. cerevisiae* S288c chromosome ii, complete genome (Fw)	90%	*S. cerevisiae* (Rv)	86%
	C12	*S. cerevisiae*/ Uncultured fungus clone (Fw)	86%/86%	*Listonella anguillarum* (Rv)	96%
	C13	*S. cerevisiae* JM993 chromosome XI genomic sequence (Fw)	94%	*S. cerevisiae* (Rv)	94%
	C14	*S. cerevisiae* YJM993 Chromosome XVI genomic sequence (Fw)	91%	*S. cerevisiae* (Rv)	86%
	C15	*S. cerevisiae* YJM993 chromosome XV genomic sequence (Fw)	92%	*Candida tropicalis*(Rv) / *Trichoderma reesei* (Rv)	70%/(68%)
	C16	*Kluyveromyces lodderae* (Fw)	73%	-	-
	C17	-	-	*Homo Sapiens* (Rv)	86%
	C18	-	-	*Mus Musculus* (Rv)	84%
	C19	*S. cerevisiae*/ Uncultured fungus clone (Fw)	74%	*Neospora canizum* (Rv)	93%
	C20	-	-	*Homo Sapiens* nuclear sequence of mitochondrial origin (Rv)	92%
	C21*	*Kazachstania naganishi* CBS 8797 (Fw)	88%	*Lodderomyces elongisporus* NRRL YB-4239 (Rv)	76%
	C22	Predicted *Pseudopodoces humilis* N-acetyl transfarese 9 (Fw)	-	*Orniothorhynchus anatinus* (Rv)	88%

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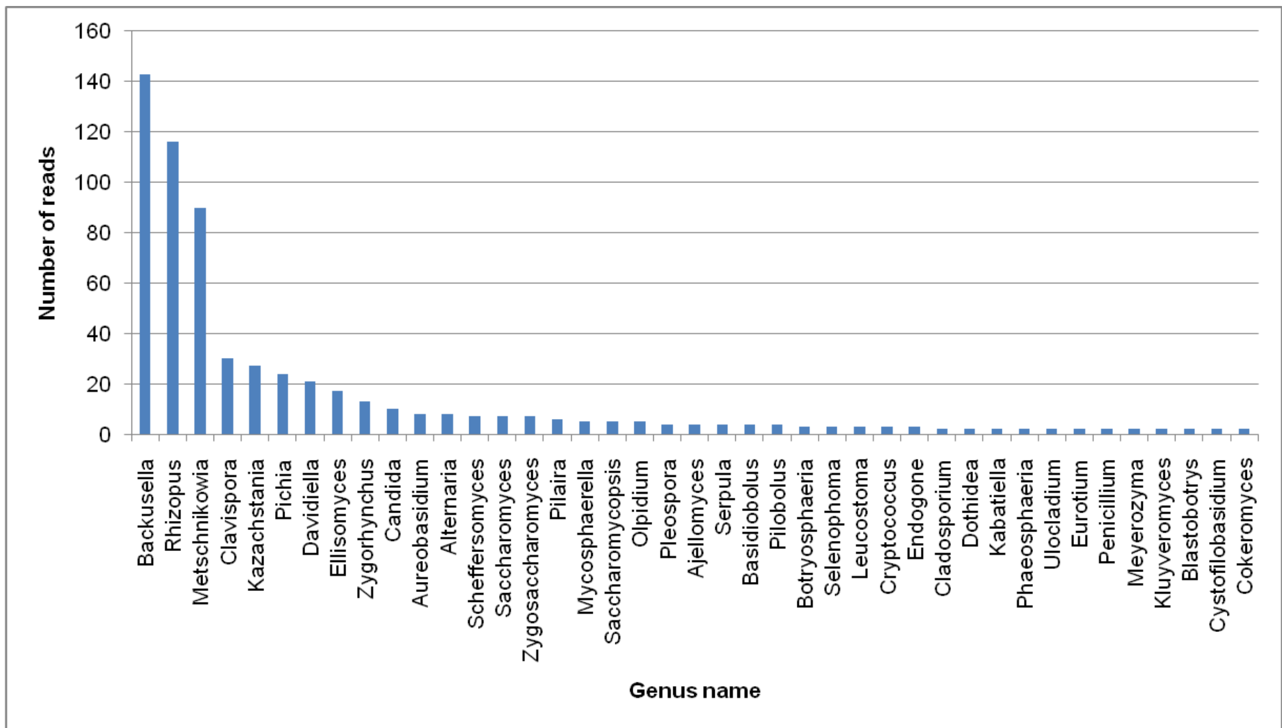
Sup. Table 5.3 Raw data set for the whole metagenome sequencing

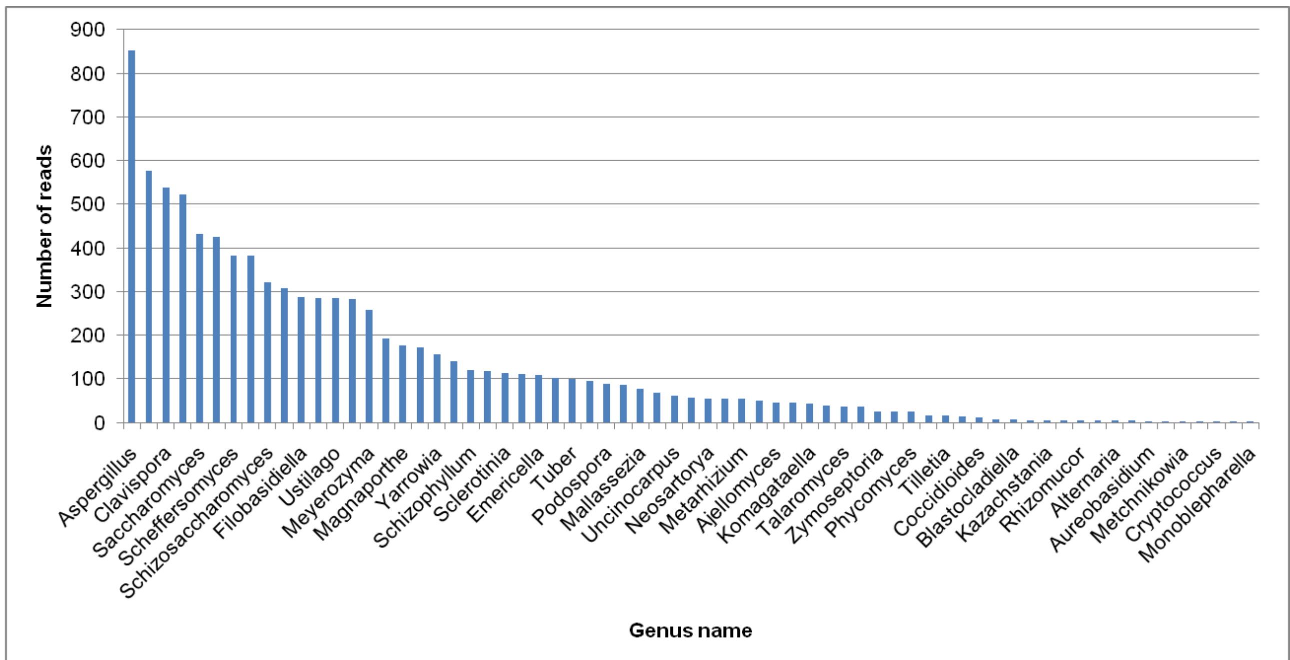
Statistical parameter	**Dataset**
Number of reads	175,616
Total number of bases (bp)	92,570,157
Mean read length (bp)	534 ± 85
Number of reads post QC	148,845
Total number of bases post QC (bp)	30,434,877
Mean read length post QC (bp)	185 ± 95
% G + C	41 ± 7%

Sup. Table 5.4 Wine related non- *Saccharomyces* yeast species whose genome has been sequenced and percent annotations (Data retrieved from (http://www.ncbi.nlm.nih.gov/genome/)

Yeast species	**Genome size (Mb)**	**Approximate percentage of assembled and annotated genes within the genomes**
Hanseniaspora vineae	11.38	25
Hanseniaspora uvarum	8	25
Wickerhamomyces anomalus	26.6	50
Meyerozyma guilliermondii	10.6	50
Kazachstania africana	11.3	75
Debaryomyces hansenii	12.1	75
L. thermotolerans	10	75
T. delbrueckii	9	75
Pichia kudriavzevii	10 – 13	25
Aureobasidium pullulans	26.6	25
Clavispora lusitaniae	12.1	50
Candida glabrata	12.3	75
Lodderomyces elongisporus	15.5	50
Brettanomyces bruxellensis	12.6	50



Sup. Figure 5.1 Fungal community profile based on rRNA gene sequences (Only genera with more than two hits were considered here)



Sup. Figure 5.2 Fungal community profile inferred from predicted metabolic genes (only genera with more than 3 hits were considered)

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Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

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CHAPTER 6

6.1 General discussion

The alcoholic fermentation of wine primarily contributes to the quality of the final product (8). While alcoholic fermentation is mainly carried out by *Saccharomyces cerevisiae*, other yeast species of non-*Saccharomyces* origin occur at variable cell concentrations and constitute the broader wine microbial consortium (7). However, the actual contribution of these non-*Saccharomyces* yeasts is not very clear in spontaneous fermentations. Some studies have shown that these non-*Saccharomyces* yeasts secrete extracellular hydrolytic enzymes (2, 12, 13) and antimicrobial peptides like killer toxins (4, 5). Whether these compounds play a major role in driving the microbial population dynamics is one of the fundamental questions that arose in the recent years. These hydrolytic enzymes have indeed been found to play a pivotal role in shaping the wine quality by contributing somehow to the final wine composition.

The current study generated insights into the wine microbial diversity and also provided an overview of the genetic make-up of the wine microbial ecosystem. The first part of the study was a pre-metagenomic exploration of the wine microbial diversity through the use of cultivation-based and cultivation-independent (ARISA) approaches in parallel. The cultivation independent approach revealed a higher microbial diversity than that revealed by plating. Moreover, when the wine microbial population dynamics was monitored during alcoholic fermentation, a decrease in the microbial diversity was noticed through ARISA. This observation was in agreement with earlier studies conducted on Slovakian wines where the authors also employed ARISA to identify the yeast isolates as well as monitor the yeast diversity and population dynamics during alcoholic fermentation (1, 3, 9, 14). However, unlike these previous studies, we also used ARISA to investigate the bacterial diversity and population dynamics in wine. In order to identify the ARISA-peaks both from the fungal and bacterial ARISA we tried to correlate the OTUs with the corresponding known fungal ITS1-5.8S rRNA-ITS2 and bacterial ITS amplicons from the online databases, respectively. Fifteen fungal and 5 bacterial OTUs could be tentatively identified. Although our results undoubtedly identified ARISA as a powerful tool for studying microbial ecology, we could not proceed with final taxonomic assignments of the ARISA-peaks generated because of the scarcity of gene sequences (especially for bacterial genes) in the online databases. Therefore there is a need for a robust database that could be used to identify the peaks.

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Certain non-*Saccharomyces* yeast isolates recovered from the same environment throughout the course of the study were screened for hydrolytic and killer activities by using plate assays. These non-*Saccharomyces* yeasts were selected based on the fact that they have not been studied previously and therefore represented an unexplored biological material. Our results showed that chitinase and glucanase activities were predominant in most of the yeast isolates. β-Glucosidase activity was only exhibited by *Metschnikowia chrysoperlae*. Additionally, *Pseudozyma fusiformata* exhibited a strong antagonistic activity against the wine spoilage yeasts *Brettanomyces bruxellensis* AWRI 1499 and *Brettanomyces anomalus* IWBT Y105. This activity was clearly not due to dsRNA viruses as has been demonstrated in another ustilaginomycete, *Ustilago maydis*. Several strains of *P. fusiformata* have been shown to display antimycotic activity against many ascomycetous and basidiomycetous yeasts and filamentous fungi. This antimycotic activity has been attributed to glycolipids and fatty acids (10, 11). However, it remains to be tested whether these compounds are also responsible for the antagonistic interactions noticed against *Brettanomyces* spp. Some of the glycolipids produced by *P. fusiformata* strains have been shown to cause plasma membrane damage and ATP leakage in desirable wine yeasts such as *S. cerevisiae*. Therefore, it would also be crucial to further investigate the antifungal compounds from the strains identified in the current study and ensure that they do not have detrimental effects against other yeasts which are of positive oenological interest. Our data also revealed that *M. chrysoperlae* also exhibited weak antagonistic activity against *Schizosaccharomyces pombe* ATCC 24844, *Pichia Kluyveri* and *Saccharomycodes ludwigii* ARC Y0154. This is the first report to demonstrate this interaction. However, earlier studies have shown that strains of the closely related *Metschnikowia pulcherrima* antagonise other fungi by producing and secreting a secondary metabolite, known as pulcherrimin. This toxic compound, binds and removed iron from the growth medium thereby causing a deficiency of iron ions for other microorganisms growing in the same medium.Our findings suggest that *P. fusiformata* and to a lesser extent *M. chrysoperlae* could be used as biocontrol agents. However, the toxic compounds produced by these yeasts would first need to be identified and tested against several grapevine pathogens and wine spoilage yeasts.

Finally, we attempted to further explore the entire genetic make-up of the wine microbiome through a metagenomic approach. Both whole metagenome sequencing and metagenomic library construction were employed and specifically screened for hydrolases. The data revealed a high diversity of hydrolytic enzymes, especially β-glucosidases, chitinases, glucanases both from fungal and non-*Saccharomyces* origin. As mentioned above, there is a possibility that these enzymes might play a role in the microbial interactions. The microorganisms secreting these enzymes might indeed exhibit antagonism against other yeasts. This could potentially contribute towards maintaining grapevine health by inhibiting grapevine fungal pathogens and preventing diseases. On the other hand, these enzymes can also break down various substrates derived from grapes

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during fermentation. This action might contribute significantly to the wine quality since it would influence the organoleptic properties of wine. Our dual metagenomic approaches of exploring functional potential of the wine microbiome has generated a large amount of genetic information regarding different enzymes occurring in wine. We therefore proved that each of these techniques definitely complement each other. However, we could not retrieve the complete ORFs of the hydrolases identified as the techniques employed are highly dependent on sequencing depth. In our study, this clearly prevented the full exploitation of the sequence data identified.

In summary, our study provided a broad overview of the wine microbial diversity both by culture-independent and -dependent approaches. Moreover, within the framework of the study, the functional potential of the wine microbiome has been extensively explored and wine was confirmed as a reservoir of various extracellular enzymes and antifungal peptides/proteins that can have biotechnological relevance both for the wine industry as well for other biotechnological industries.

6.2 Future perspectives

In order to use ARISA more effectively, the construction of a large sequence database should be implemented. This would aid furthering our knowledge of the wine microbial diversity and allow monitoring of population dynamics accurately.

Further investigation should also focus on retrieving the full sequences of the hydrolytic enzymes identified both from yeast isolates and metagenomic library screening of our study, clone them and verify their potential activity in wine. Moreover, as mentioned above, our screening was limited to certain specific artificial substrates; the screening spectrum could therefore be broadened by using other substrates, especially those directly derived from the wine environment and test them under different conditions such as a range of temperature/pH relevant to winemaking. Transforming our metagenomic library into eukaryotic hosts could be attempted as described in literature (6). Furthermore, it would be interesting to evaluate whether these enzymes play any role in microbial interaction and therefore somehow drive or at least contribute to the microbial population dynamics during wine fermentation. In this context, it would also be of interest to further explore the killer activity of certain yeast isolates such as *P. fusiformata* and *M. chrysoperlae* against *Brettanomyces* spp. and evaluate their potential use as biocontrol agent against wine spoilage yeasts. Finally, in order to retrieve the genes that are actually expressed under real winemaking conditions, a metatranscriptomic analysis of the wine microbiome could be envisaged. This could either be achieved through cDNA sequencing or by constructing a metatranscriptomic library followed by a function–based screening similar to that performed in this study.

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6.3 References

1. **Brezna, B., K. Zenisova, K. Chovanova, V. Chebenova, L. Krakova, T. Kuchta, and D. Pangallo.** 2010. Evaluation of fungal and yeast diversity in Slovakian wine-related microbial communities. Antonie Van Leeuwenhoek **98:**519-29.
2. **Charoenchai, C., G. H. Fleet, P. A. Henschke, and B. E. N. Todd.** 1997. Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. Aust J Grape Wine Res **3:**2-8.
3. **Chovanova, K., L. Krakova, K. Zenisova, V. Turcovska, B. Brezna, T. Kuchta, and D. Pangallo.** 2011. Selection and identification of autochthonous yeasts in Slovakian wine samples using a rapid and reliable three-step approach. Lett Appl Microbiol **53:**231-7.
4. **Ciani, M., and F. Fatichenti.** 2001. Killer toxin of *Kluyveromyces phaffii* DBVPG 6076 as a biopreservative agent to control apiculate wine Yeasts. Appl Environ Microbiol **67:**3058-63.
5. **Comitini, F., and M. Ciani.** 2011. *Kluyveromyces wickerhamii* killer toxin: purification and activity towards *Brettanomyces*/*Dekkera* yeasts in grape must. FEMS Microbiology Letters **316:**77-82.
6. **Craig, J. W., F. Y. Chang, J. H. Kim, S. C. Obiajulu, and S. F. Brady.** 2010. Expanding small-molecule functional metagenomics through parallel screening of broad-host-range cosmid environmental DNA libraries in diverse proteobacteria. Appl Environ Microbiol **76:**1633-41.
7. **Fleet, G. H., and G. M. Heard.** 1993. Yeasts - growth during ferementation. Wine Microbiology and Biotechnology**:**27-54.
8. **Jolly, N. P., C. Varela, and I. S. Pretorius.** 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res **14:**215-37.
9. **Kraková, L., K. Chovanová, K. Zenisová, V. Turkovská, B. Brezná, T. Kuchta, and D. Pangallo.** 2012. Yeast diversity investigation of wine-related samples from two different Slovakian wine-producing areas through a multistep procedure. Food Sci Technol **46:**406-411.
10. **Kulakovskaya, T. V., E. V. Kulakovskaya, and W. I. Golubev.** 2003. ATP leakage from yeast cells treated by extracellular glycolipids of *Pseudozyma fusiformata*. FEMS Yeast Res **3:**401-4.
11. **Kulakovskaya, T. V., A. S. Shashkov, E. V. Kulakovskaya, and W. I. Golubev.** 2005. Ustilagic acid secretion by *Pseudozyma fusiformata* strains. FEMS Yeast Res **5:**919-23.
12. **Maturano, Y. P., L. A. Rodriguez Assaf, M. E. Toro, M. C. Nally, M. Vallejo, L. I. Castellanos de Figueroa, M. Combina, and F. Vazquez.** 2012. Multi-enzyme production by pure and mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts during wine fermentation. Int J Food Microbiol **155:**43-50.

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13. **Strauss, M. L. A., N. P. Jolly, M. G. Lambrechts, and P. van Rensburg.** 2001. Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. J Appl Microbiol **91:**182-190.

14. **Zenisová, K., K. Chovanová, C. V. Turcovská, Z. Godálová, L. Kraková, T. Kuchta, D. Pangallo, and B. Brezná.** 2014. Mapping of wine yeast and fungal diversity in the small Carpathian wine-producing region (Slovakia): evaluation of phenotypic, genotypic and culture-independent approaches. Ann Microbiol.