Biochemical characterization and evaluation of the oenological attributes of *Lachancea* species

by

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Declaration

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Summary

Previously thought to be the catalyst for spoilt wine, several non-*Saccharomyces* yeasts are now commercially available for winemaking. Increasing investigations into this group of microorganisms has emphasized their oenological potential in terms of their contribution to the organoleptic profile of the resulting wines. However, their sub-optimal fermentation performances, force their combined inoculation with the main wine yeast *Saccharomyces cerevisiae*. Among the non-*Saccharomyces* yeasts, *Lachancea thermotolerans* has been reported to bring about an overall improvement in wine acidity through the production of L-lactic acid and reduction of acetic acid as well as increased glycerol and 2-phenylethanol levels, which can impart a sweet taste and flowery aromas to wine, respectively. Its secretion of hydrolytic enzymes of oenological relevance has also been reported. Other species of the *Lachancea* genus (*L. fermentati* and *L. lanzarotensis*) have also been isolated from the fermentation environment, but have not received the same degree of focus as *L. thermotolerans*. The aim of this study was to investigate the oenological potential of these species, regarding their expression of oenologically relevant enzymes, their fermentation attributes and the expression and location of β-glucosidase during fermentation of synthetic and real grape must (Muscat d'Alexandrie).

All the *Lachancea* spp. illustrated β-glucosidase activity in initial plate screenings, while none exhibited protease or pectinase activities. *L. thermotolerans* and *L. fermentati* also displayed higher tolerance to ethanol and SO_2 when exposed to varying concentrations of these inhibitory compounds. Higher fermentation performance was demonstrated by *L. thermotolerans* strains and *L. fermentati*, which performed much better in Muscat grape juice, and was the only *Lachancea* spp. to complete the fermentation in monoculture. During these fermentations, all three *Lachancea* spp. illustrated β-glucosidase activity, where the highest levels were expressed by a *L. thermotolerans* strain, Y940. Most of the enzyme activity originated from the cell walls of the yeasts, while lower levels were isolated in the intracellular region and none determined to be released into the medium. The mixed culture fermentations resulted in wine products with significantly different chemical compositions compared to the *S. cerevisiae* fermentation. *L. thermotolerans* and *L. lanzarotensis* mixed culture fermentations resulted in similar wines with increased production of common compounds including isoamyl alcohol, butanol and ethyl phenylacetate. *L. fermentati* seemingly produced wines dissimilar to the other *Lachancea* spp., producing levels of acetic acid perhaps flagging this species as a potential spoilage microorganism. This study provided novel information on relatively uninvestigated *Lachancea* spp., where their oenological potential was highlighted by the ability to express β-glucosidase enzymes during fermentations and producing wines significantly different from *S. cerevisiae*.

Opsomming

‘n Aantal nie-*Saccharomyces* giste, voorheen aan gedink as die kataliste vir bederfde wyn, is nou kommersieël beskikbaar vir wynbereiding. In diepte ondersoeke na hierdie groep mikro-organisms het hul wynkundige potensiaal, in terme van hul bydra tot die organoleptiese profiel van die gevolglike wyn, beklemtoon. Alhoewel, hul sub-optimale fermentasie gedrag forseer hul gekombineerde inokulasie saam met die hoof wyn gis *Saccharomyces cerevisiae.* Onder die nie-*Saccharomyces* giste, word berig dat *Lachancea thermotolerans* ‘n algehele verbetering in wyn suurheid te weeg bring deur die produksie van L-melksuur en ‘n verlaging in asynsuur asook ‘n verhoging in gliserol en 2-fenieletanol vlakke, wat onderskeidelik ‘n soet smaak en blommerige aroma aan die wyn kan verleen. Die afskeiding van hidrolitiese ensieme van wynkundige belang is ook al berig. Ander spesies van die *Lachancea* genus (*L. fermentati* en *L. lanzarotensis*) is ook al uit fermentasie omgewings ge-isoleer, maar hul is nie so in deeglik soos *L.* thermotolerans bestudeer nie. Die doel van hierdie studie was om die wynkundige potensiaal van hierdie spesies te ondersoek, met betrekking tot die uitdrukking van wynkundige relevante ensieme, hul fermentasie eienskappe en die setel van β-glukosidase gedurende fermentasie in sintetiese en egte druiwe sap (Muscat d’Alexandrie).

Al die *Lachancea* spp. het β-glukosidase aktiwiteit getoon in die plaat siftings, maar het geen protease of pektinase aktiwiteit getoon nie. *L. thermotolerans* en *L. fermentati* het ‘n hoë toleransie vir etanol en SO_2 vertoon in ‘n inhibiese sifting met verkillende konsentrasies van hierdie inhibitoriese komponente. *L. thermotolerans* rasse en *L. fermentati* het ook hoër fermentasie prestasie getoon; hierdie spesies was die enigste *Lachancea* spp. wat as monokulture die fermentasie voltooi het en goeie prestasie in Muscat druiwe sap getoon het. Gedurende hierdie fermentasies het al drie *Lachancea* spp. β-glukosidase aktiwiteit getoon, met die hoogste vlakke wat deur *L. thermotolerans* ras Y940 uitgedruk is. Meeste van die ensiem aktiwiteit het in die selwand voorgekom, terwyl laer vlakke uit die intrasellulêre area ge-isoleer is en geen ensiem aktiwiteit in die medium as uitgeskeide ensiem voorgekom het nie. Die gemengde kultuur fermentasies het gelei tot wynprodukte met beduidend anderse chemiese samestelling in vergelyking met *S. cerevisiae* fermentasie. *L. thermotolerans* en *L. lanzarotensis* gemengde kultuur fermentasies het soortgelyke wyne met hoër produksie van algemene verbindings, insluitend isoamiel alkohol, butanol, en etiel fenielasetaat, opgelewer. *L. fermentati* het oënskynlik wyne geproduseer wat heelwat anders is as die ander *Lachancea* spp., met vlakke van asynsuur wat moontlik ‘n aanduiding is dat die spesie ‘n potensiele bederf mikroorganisme is. Hierdie studie het nuwe inligting oor die relatief onbestudeerde *Lachancea* spp. getoon, waar hul wynkundige potensiaal uitgelig is deur die vermoë om β-glukosidase ensieme uit te druk gedurende fermentasies en om wyne te produseer wat beduidend anders is as die van *S. cerevisiae*.

This thesis is dedicated to my family, for their unwavering love and encouragement, and to the memories of August Henry Herbst and Maureen Martha Herbst

Biographical sketch

Tristan Jade Porter was born in Cape Town, South Africa, on 9 October 1993. She attended Table View Primary and High School, where she matriculated in 2011. Tristan then enrolled at the University of Cape Town in 2012 and obtained a BSc degree in 2014, majoring in Genetics and Microbiology. In 2015, she then enrolled for a BSc Honours degree in Wine Biotechnology at the Institute for Wine Biotechnology, Stellenbosch University, after which she continued with an MSc.

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Preface

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately and is written according to the style of the journal *South African Journal of Enology and Viticulture*.

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CHAPTER 1

General introduction and project aims

Chapter 1 – General introduction and project aims

1.1 Introduction

Wine fermentations are very complex environments, with various microorganisms playing diverse roles in the overall microbial process of converting grape juice to wine (Padilla *et al*., 2016). Yeasts play a fundamental role in this process and the beginning stages of the fermentation are dominated by the non-*Saccharomyces* yeasts, naturally present on the surfaces of grapes and subsequently in the grape must. Majority of these yeast species are however poor fermenters and were previously considered as the source of spoilage, where their presence and proliferation was linked to stuck, sluggish or spoilt wines (Bisson, 1999; Domizio *et al.,* 2011). In contrast, *S. cerevisiae*, a highly efficient fermenter and not having been linked to spoilt wine, is utilized as the main fermentation agent; where the main objective for winemakers is to inoculate this species and allow its dominance over the non-*Saccharomyces* yeasts, for a reliable and uniform wine product (Padilla *et al*., 2016; García *et al.,* 2016). With increasing research into wine-related non-*Saccharomyces* yeasts, several species were discovered to in fact possess desirable characteristics, in terms of their ability to positively impact wine production as well as wine aroma and flavour (Ciani & Maccarelli, 1997; Beltran *et al.,* 2002; Romano *et al.,* 2003; Fleet, 2008; Jolly *et al.,* 2014). These yeasts are able to positively contribute to the organoleptic quality of wine through the production of several primary and secondary metabolites, including the highly aromatic higher alcohols and esters (Cano-López *et al.,* 2010; Cordero-Bueso *et al.,* 2013).

Mixed culture fermentations with *S. cerevisiae* and non-*Saccharomyces* yeast are therefore often utilized to introduce the diverse/enhanced production of various compounds while simultaneously ensuring the reliable completion of the fermentation due to the presence of *S. cerevisiae* (Ciani *et al.,* 2006; Jolly *et al.,* 2014). The implementation of specific non-*Saccharomyces* yeasts is often utilized for the purpose of modifying certain characteristics of the wine. For instance, *L. thermotolerans* has been inoculated to enhance the acidity of wine (Kapsopoulou *et al.,* 2007; Padilla *et al.,* 2016). This drift toward utilizing mixed culture inoculation has led to the commercialization of several yeast species often isolated from the wine fermentation environment, including *Lachancea thermotolerans*, *Pichia kluyveri*, *Metschnikowia pulcherrima* and *Torulaspora delbrueckii*, whereby their metabolic heterogeneity offers the opportunity to overcome shortcomings seen when utilizing merely *S. cerevisiae*, such as monotonous wine products (Belda *et al.,* 2016). Additional studies have highlighted beneficial characteristics by many non-*Saccharomyces* species, often not possessed by *S. cerevisiae*, such as the expression of β-glucosidase, α-L-arabinofuranosidase, β-D-xylosidase, protease and pectinase enzymes (Manzanares *et al.,* 1999; Strauss *et al.,* 2001; Cordero Otero *et al.,* 2003; Ciani *et al.,* 2010; Comitini *et al.,* 2011). These enzymes have the potential to significantly enhance the varietal flavour of wine as well as aid in increasing the efficiency of grape pressing,

clarification of the must and increasing substance extraction, contributing to wine colour and aroma (Fernández *et al.,* 2000; Garcia *et al.,* 2002). Several of these beneficial characteristics have been attributed to *L. thermotolerans*. Already shown to be a fairly good fermenter in comparison to the other non-*Saccharomyces* yeasts, increasing the oxygen availability in the fermentation has shown to increase the persistence of *L. thermotolerans* even more (Shekhawat *et al.,* 2017). Increased persistence has been determined to allow *L. thermotolerans* to have an increasingly significant impact on the quality of wine, through the reduction of volatile acidity and increase in overall acidity, glycerol and 2-phenylethanol, which can impart a flowery aroma to the wine (Comitini *et al.,* 2011; Gobbi *et al.,* 2013; Benito *et al.,* 2016). *L. thermotolerans* has also been reported to possess enzymes of oenological interest, particularly β-glucosidase (Romo-Sánchez *et al.,* 2010; Comitini *et al.,* 2011; Cordero-Bueso *et al.,* 2013; Belda *et al.,* 2016).

While several wine-related species including the highly investigated *L. thermotolerans*, have been shown to generally possess beneficial characteristics for wine production (Gobbi *et al.,* 2013; Balikci *et al.,* 2016; Beckner Whitener *et al.,* 2017), two other species in the *Lachancea* genus (also isolated from the wine fermentation environment) – namely *L. fermentati* and *L. lanzarotensis* have not received the same attention. While *L. fermentati* has been shown to possess strong fermentative behaviour like *L. thermotolerans* (Romano & Suzzi, 1993; Romano *et al.,* 1997), this species noticeably lacks extensive investigation. The same can be noted for *L. lanzarotensis*, which has yet to be investigated, most likely due to its more recent isolation and identification. Increasing the scope of research into potential yeast species, already isolated from the wine fermentation environment, has the potential to identify yeasts able to impart significant variation, negative or positive, to the wine product. Research could also provide increasing information of the *Lachancea* genus as a whole, and its potential role in wine fermentations.

1.2 Project aims

The aim of this study was to investigate the phenotypic traits and oenological potential of *Lachancea* spp. strains associated with the wine fermentation environment with focus on *L. thermotolerans*, *L. fermentati* and *L. lanzarotensis*.

The specific objectives of the study were as follows:

1. To screen *Lachancea* spp. for the production of hydrolytic enzymes of oenological relevance, in particular proteases, glycosidases and pectinases
2. To assess tolerance to SO_2 and ethanol and determine H_2S production in *Lachancea* spp./strains

3. To determine the fermentation attributes of selected strains of *L. thermotolerans, L. lanzarotensis* and *L. fermentati* in monoculture and co-culture sequential fermentations with *S. cerevisiae* in both synthetic and real grape must
4. To evaluate the β-glucosidase activity, as well as the cellular location thereof, during monoculture and co-culture sequential fermentations with *S. cerevisiae* in both synthetic and real grape must

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CHAPTER 2

Literature Review

Lachancea yeast species: origin, biochemical characteristics and oenological significance

Chapter 2 - *Lachancea* yeast species: origin, biotechnological characteristics and significance

2.1 Introduction

Yeasts form part of a large and diverse group of microorganisms belonging to the kingdom Fungi. Following the discovery of their metabolic activities and role in fermentation in the 19th century, employing yeasts during various processes became the standard (Mattanovich *et al.,* 2014; Türker, 2014). Although *Saccharomyces cerevisiae* is commonly synonymous with yeasts, a wide variety of yeast species, estimated at a total population of 150,000, occur ubiquitously in nature (Hawksworth, 2004). Of this large population, only a small percentage has been identified, and an even smaller portion (approximately a dozen) play a commercial role on the industrial level (Türker, 2014). While there has been various yeast species shown to possess potentially beneficial characteristics for different biotechnological processes, their characterization commonly remains on the laboratory scale. This indicates the extent of untapped opportunity that lies with diverse yeast species, which may have the potential to change modern day bioprocesses (Deak, 2009).

Yeasts are routinely involved in many modern biotechnological applications including the production of metabolites and recombinant proteins as well as *in vivo* biotransformations (Mattanovich *et al.,* 2014). Most commonly, yeast species have been associated with the fermentation of alcoholic beverages and food. Although *S. cerevisiae* has been acknowledged as the most efficient yeast species for the transformation of grape juice to wine, in recent years, attention has shifted toward the potential of non-conventional or non-*Saccharomyces* yeasts, specifically regarding their involvement in the winemaking process (Ciani & Comitini, 2011; Padilla *et al.,* 2016). Positive contributions from many yeast species have been noted, regarding enzymatic activity and metabolite production (Cordero Otero *et al.,* 2003; González-Royo *et al.,* 2014). This new light shone on this group of yeasts has subsequently led to the commercialization of several strains (Belda *et al.,* 2016). However, of the wide variety of non-*Saccharomyces* yeasts associated with this environment, only a small percentage has been commercially utilized for wine fermentations to bring about the so-called diverse and complex wine products. Further characterization of a wider variety of yeast species, isolated from this environment, can result in increased knowledge not only of yeast species but perhaps also entire genera for the potential benefit of the wine industry. A currently commercialized yeast is *L. thermotolerans*, which has shown increasing potential due to being a fairly good fermenter, producer of various compounds enhancing wine flavour and aroma as well as various strains expressing enzymes of oenological interest (Beckner Whitener *et al.,* 2015, 2016; Varela, 2016). This review intends to summarize the information available on the yeast genus *Lachancea*, regarding the characteristics of the comprising

species and their potential role in biotechnological processes, specifically that of wine fermentations.

2.2 Systematics and taxonomy of the genus *Lachancea*

2.2.1 Historical overview of *Lachancea* species classification

The genus *Lachancea* is a reasonably young genus, first proposed by Kurtzman (2003), following a reclassification of several yeast genera based on genetic relatedness rather than phenotypic similarities. The genus now comprises species that were previously classified in the genera *Zygosaccharomyces*, *Kluyveromyces* and *Saccharomyces*. Historically, these species were clustered into their respective genera based on morphology of vegetative cells and sexual states as well as fermentation and growth tests common for yeast identification (Kurtzman & Robnett, 2003; Fell *et al.*, 2004). The unreliability of such tests can be noticed by the reclassification of *L. fermentati* a total of six times. Indeed, *Zygosaccharomyces fermentati* (Naganishi, 1928) was changed to *S. cerevisiae* in 1952 (Lodder & Kreger-van Rij, 1952), back to *Z. fermentati* in 1954 (Kudryavtsev, 1954), to *S. montanus* in 1956 (Phaff *et al.,* 1956), to *Torulaspora manchurica* in 1975 (van der Walt & Johannsen, 1975), back to *Z. fermentati* again in 1977 (von Arx *et al.,* 1977) and finally to *L. fermentati* in 2003 (Kurtzman, 2003) as discussed below.

At the beginning of the 21st century, Kurtzman and Robnett (2003) employed a multigene sequence analysis strategy to develop a dataset for various yeasts comprising nucleotide sequences of unlinked genes; 18S rDNA, ITS1-5.8S rDNA-ITS2 and 26S rDNA, translation elongation factor 1α (EF-1α), actin-1 and RNA polymerase II nuclear genes as well as cytochrome oxidase II (COX II) mitochondrial genes. This dataset led to the reclassification of various yeast species and the introduction of the genus *Lachancea* (Kurtzman, 2003). Included into this proposed novel genus were the species *Lachancea cidri* (formerly *Zygosaccharomyces cidri*), *Lachancea fermentati* (*Zygosaccharomyces fermentati* etc.), *Lachancea thermotolerans* (*Zygosaccharomyces thermotolerans* and *Kluyveromyces thermotolerans*), *Lachancea kluyveri* (*Saccharomyces kluyveri*) and *Lachancea waltii* (*Kluyveromyces waltii*). *L. thermotolerans* was selected as the type species for this genus. Following the proposal of the *Lachancea* genus by Kurtzman (2003), several other species including *L. meyersii*, *L. dasiensis*, *L. nothofagi*, *L. mirantina*, *L. lanzarotensis* and *L. quebecensis* were subsequently isolated, identified and placed within this genus as well. An increase in the frequency of yeast species added into the *Lachancea* genus can be noticed following its proposal in 2003 (**Figure 2.1**), which could be due to the increase in ease and reliability of species classification due to genetic sequencing.

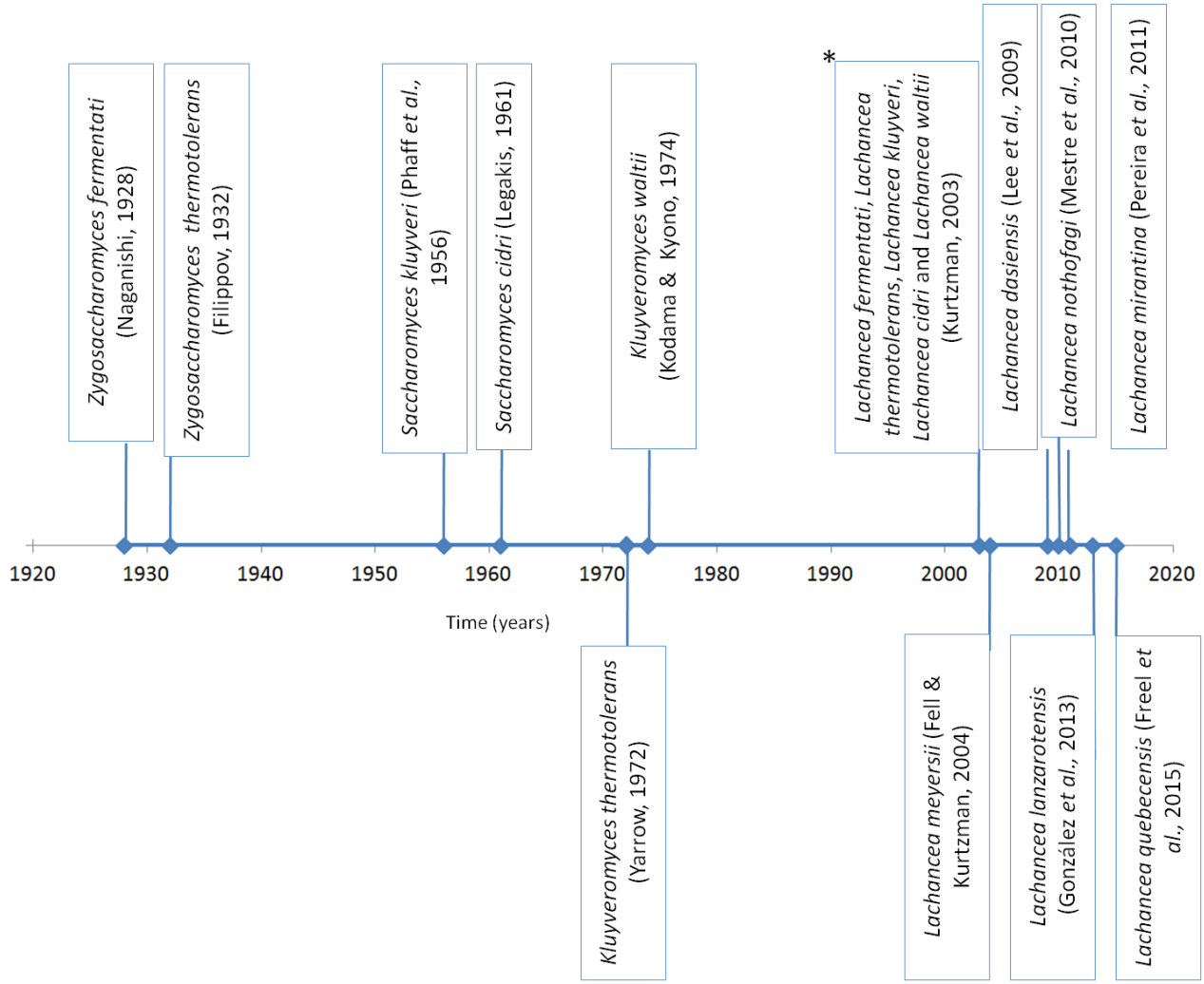

Figure 2.1 Timeline depicting the initial taxonomic characterization, the eventual re-characterization of several *Lachancea* spp. (*) and the subsequent addition of recently isolated yeast species to the *Lachancea* genus

2.2.2 The phylogeny of *Lachancea* species

Currently, 11 species have been placed into the *Lachancea* genus, ranging from close to more distant relatedness. Construction of a phylogenetic tree, using the 546 bp variable region of the D1/D2 of the 26S rRNA (**Figure 2.2**) illustrates the presence of four main clusters. Following the isolation of more *Lachancea* spp., the relationships between the species have been adjusted, where certain species, such as *L. thermotolerans* and *L. meyersii*, have been realized to be further apart on the phylogenetic tree in comparison to what was previously understood. In contrast, species such as *L. thermotolerans* and *L. quebecensis* as well as *L. cidri* and *L. fermentati* remained in tight clades. The continuation of species addition to the *Lachancea* genus suggests these phylogenetic relationships are likely to be further adjusted in the future. This type of adjustment is evident following the proposal of the *Lachancea* genus by Kurtzman (2003), where *L. kluyveri* was proposed as

perhaps eventually serving as the type species of a sister genus due to its distant relation to the other *Lachancea* species. However, with the identification of several other *Lachancea* species since then, it appears such a statement could be true for *L. mirantina* instead (**Figure 2.2**). The evolutionary range in diversity in the *Lachancea* spp. highlights *Lachancea* as a potential ideal model system for investigating the genomic evolution of closely and distantly related lineages within the same genus.

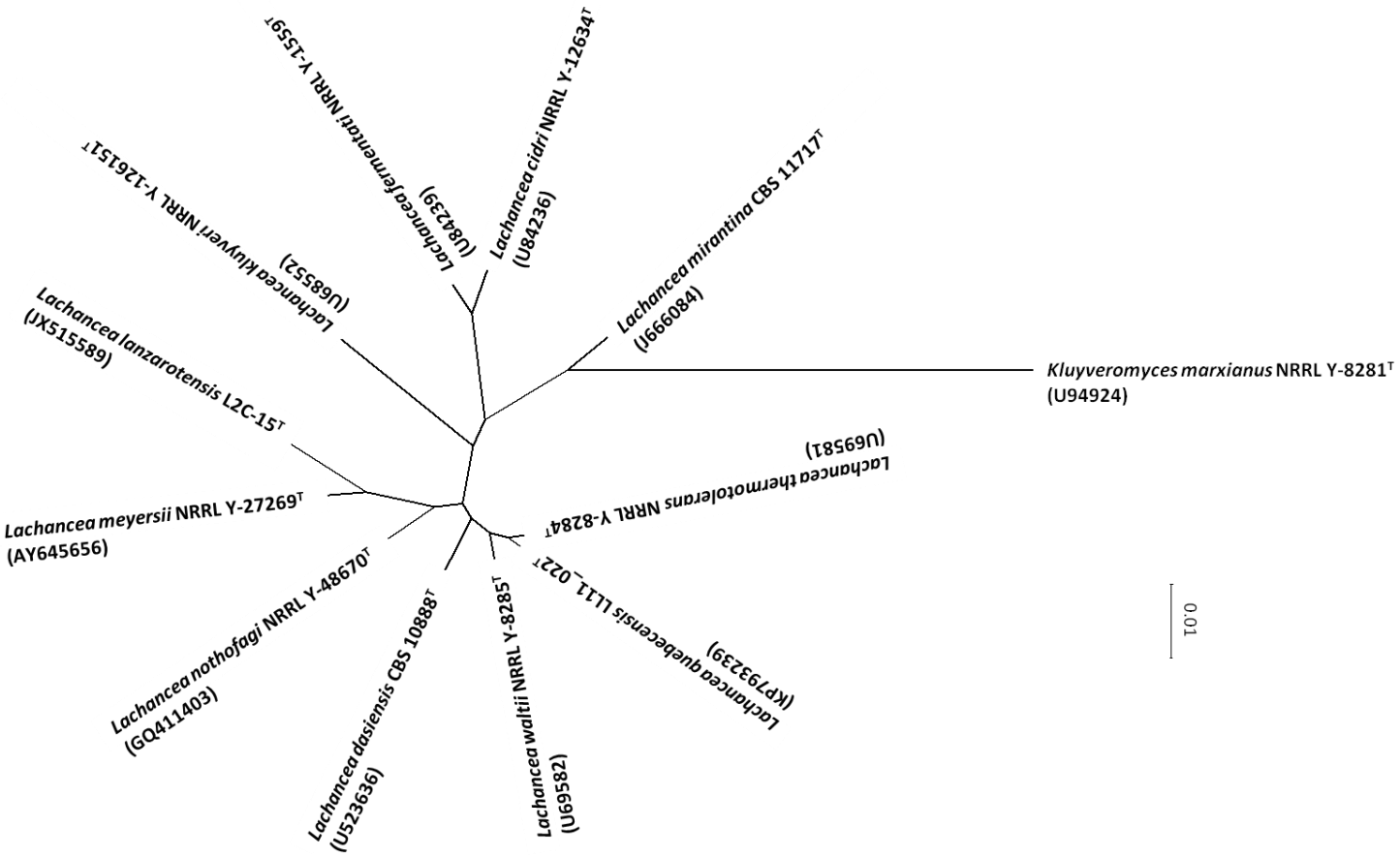

Figure 2.2 The phylogenetic relationships of the *Lachancea* spp. was inferred using the Neighbour-Joining method and based on the 546 bp of the D1/D2 domain of the 26S rRNA. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and the evolutionary analyses were conducted in MEGA7. The outgroup species used was *Kluyveromyces marxianus*

While multigene sequencing has allowed the reclassification of several yeast species and rediscovering of their phylogeny, sequencing of the mitochondria and nuclear genomes has provided deeper insight into their respective similarity/discrepancy and evolution. The increasing availability of mitochondrial (mt) and nuclear genomes of various yeast species provides the opportunity to investigate intra- and interspecies evolution. Specifically, the

sequencing of mitochondrial (mt) DNA, due to its higher mutation rate in comparison to the nuclear genome, has allowed the observation of large sets of yeast species and subsequently provided insight into the evolution of an entire phylum, for example *Saccharomycotina* (Freel *et al.,* 2014). Initially investigated were *L. kluyveri* and *L. thermotolerans*, both of which have been reported to share the occurrence of higher variation in their mitochondrial (Jung *et al.,* 2012; Friedrich *et al.,* 2015) and nuclear genomes (Friedrich *et al.,* 2012; Jung *et al.,* 2012; Freel *et al.,* 2014, 2015).

Jung *et al.* (2012) investigated the mt genome diversity of 18 *L. kluyveri* strains and observed variation in intron content, resulting in mt genome size differentiation (50.1 – 53.7 kb), while the protein-coding regions were syntenic. The whole genome analysis also showed a higher rate of differentiation (in the form of SNPs and indels) in the intergenic regions as opposed to the protein coding region. This was suggested to be due to purifying selection and the subsequent removal of most indels and non-synonomous differences from the mt encoding regions. Freel and colleagues (2014) then investigated intraspecific species diversity by sequencing the genomes of 50 *L. thermotolerans* isolates from diverse geographical and substrate locations. The mt genome sizes of *L. kluyveri* and *L. thermotolerans* varied greatly in size (23,584 bp versus 51kb) but a consistent number of genes (35) were found, of which 8 encode proteins. Proving the size differences in the mt genomes is due to differences in intron number and sizes. While both species showed similar levels of intraspecific divergence, as determined by their nuclear genomes, *L. thermotolerans* illustrated higher mt genome conservation (coding regions having lower rate of substitutions). This suggests that even between yeast species of the same genus, varying factors can drive mt genome evolution (Freel *et al.,* 2014).

Intraspecific analysis by both Freel *et al.* (2014) and Jung *et al.* (2012) for *L. thermotolerans* and *L. kluyveri* respectively further supported the hypothesis that species evolution is associated with geographical location. While Jung *et al.* (2012) more clearly supports this hypothesis due to clearer separations of isolates based on their originating locations, this report did only investigate 18 isolates in comparison to the 50 isolates investigated by Freel *et al.* (2014). This smaller gene/sample pool could explain the increased association found. In contrast, both reports found no significant association between the substrates from which the yeasts were isolated (e.g. tree exudates or fruit) and the phylogeny. Friedrich *et al.* (2012) investigated interspecific variation by analyzing the mt genomes of several *Lachancea* spp., namely *L. dasiensis*, *L. nothofagi*, *L. mirantina*, *L. meyersii* and *L. fantastica*. *L. fantastica* nomen nudum (CBS 6924) is not yet a valid species within the *Lachancea* genus, with no available information on its origin. Similar results were however obtained when analyzing the mt genomes of these *Lachancea* spp., as seen with the intraspecies analysis, with overall *Lachancea* purifying selective pressures noted.

Therefore, overall, while showing high levels of synteny in their protein coding regions, differentiation lies in the intergenic regions and intron content of the *Lachancea* spp. and mt genome sequence similarity can be linked to their geographical locations.

2.3 *Lachancea* yeast species isolation and biochemical characteristics

2.3.1 Natural habitats from which *Lachancea* spp. were isolated

Lachancea species have been isolated from a wide variety of ecological niches over time, including plants, insects, soil as well as the processing of food and beverages (**Figure 2.3**). Although diverse, it can be noticed that the *Lachancea* spp. have most frequently been isolated from fermentation environments, including grape must, Champús, Moutai-Daqu, Cachaça, cocoa and apple fermentations among others. Of the *Lachancea* spp., *L. thermotolerans* and *L. fermentati* have noticeably been isolated most frequently and from the widest diversity of ecological niches (**Figure 2.3**). *L. fermentati* has been isolated from grains, tree bark, cactus, olive mill wastewater among other habitats, while *L. thermotolerans*, while isolated from less diverse habitats, has been found in lake water and fruit juice. Both species have additionally been isolated in cocoa fermentations, olive paste and pomace, grape berries and most frequently, grape must. In contrast to this extensive diversity, several *Lachancea* spp. have only been isolated once (in single reports), these include *L. dasiensis*, *L. nothofagi*, *L. quebecensis, L. mirantina* and *L. lanzarotensis*. This could suggest to a lack of data on the habitats from which these yeasts are able to proliferate or alternatively their inability to grow in varied habitats.

Three strains of *L. dasiensis* were isolated from leaves of the fern *Angiopteris lygodiifolia* Rosenst, the palm *Arenga engleri* Beccari (both in Dasi, Taoyuan, Thailand 2006) and forest soil in Sinyi, Nantou in 2007, respectively (Lee *et al.,* 2009). Also associated with plants, six novel yeast strains were isolated from tree bark, exudate and the ectomycorrhizosphere (zone of influence of mycorrhizae) of *Nothofagus* species in Patagonia, Argentina in 1999, 2007 and 2008. These strains were placed in the novel species *L. nothofagi*. The various *L. nothofagi* strains were isolated from three different *Nothofagus* species (*N. nervosa*, *N. antartica* and *N. pumilio*), over a wide latitudinal range (40 – 50° S) and over time; this indicates its likely association with *Nothofagus* species in Andean Patagonia (Mestre *et al.,* 2010). Five of the six strains were isolated from fluxes and bark of the different *Nothofagus* species while the sixth was isolated from adjacent soil samples. Plant exudates are able to influence these substrates and attract insects (feeding and breeding grounds created), and acquire a complex microbial community, which in turn is able to modify the surrounding environment's microbiota (Mestre *et al.,* 2010). This led to the proposal that these yeasts could be associated with insects, perhaps drosophilids that

frequently visit sap fluxes and tree bark (Mestre *et al.,* 2010). This could therefore be the case for the wide variety of *Lachancea* strains that have been isolated from tree exudates. In fact, *L. kluyveri* and *L. fermentati* were previously isolated from the intestinal canal of *Drosophila* wild species (Phaff *et al.,* 1956). The latest species to join the *Lachancea* genus is *L. quebecensis*. Over the course of two years, samples from tree exudates, bark, soil, insects, slugs, flowers and fruits were collected, majority taken in Québec (Canada), and the yeast species identified. Of the many yeasts isolated, four were determined as *Lachancea* spp. and isolated from maple and oak bark (Freel *et al.,* 2015), these strains were placed in the novel species; *L. quebecensis*. Interestingly, *L. quebecensis* strains grow better at lower temperatures than other *Lachancea* spp., including *L. thermotolerans*. This characteristic could suggest to its adaptation to the low temperate Québec and surrounding areas (Freel *et al.,* 2015). Also isolated only once, *L. mirantina* was isolated from the Cachaça fermentation process, a Brazilian alcoholic beverage produced from the fermentation of sugarcane juice (Pereira *et al.,* 2011) and *L. lanzarotensis* from grape must and berries in Lanzarote, Spain (González *et al.,* 2013).

Observing the phylogenetic relationships of the *Lachancea* spp. and the habitats from which they have been isolated, reveal similarities and differences. For example, the closely related *L. thermotolerans* and *L. quebecensis* have not yet been isolated from a common ecological niche, correlating to the report from Freel *et al.* (2014) and Jung *et al.* (2012). In contrast, the closely related *L. waltii*, *L. dasiensis*, *L. nothofagi* and *L. quebecensis* have all been isolated from tree bark and exudates. Similarly, *L. fermentati* and *L. cidri*, species forming a tight clade, have often been isolated from beverage fermentation environments. With the possible continuous isolation of the *Lachancea* spp. in a wider variety of habitats, a correlation between their genetic relatedness and the habitats in which they proliferate may appear. Because yeast species are continuously being isolated and characterized, conclusions made about their ability to proliferate in certain environments based on where they have been found are unreliable. The particular characteristics of the *Lachancea* spp. allowing their survival and proliferation in certain environments have not yet been investigated. The assimilation, expression and fermentation capabilities, as well as their genetic backgrounds could however provide some insight into why certain yeast species are isolated from particular habitats while others are not.

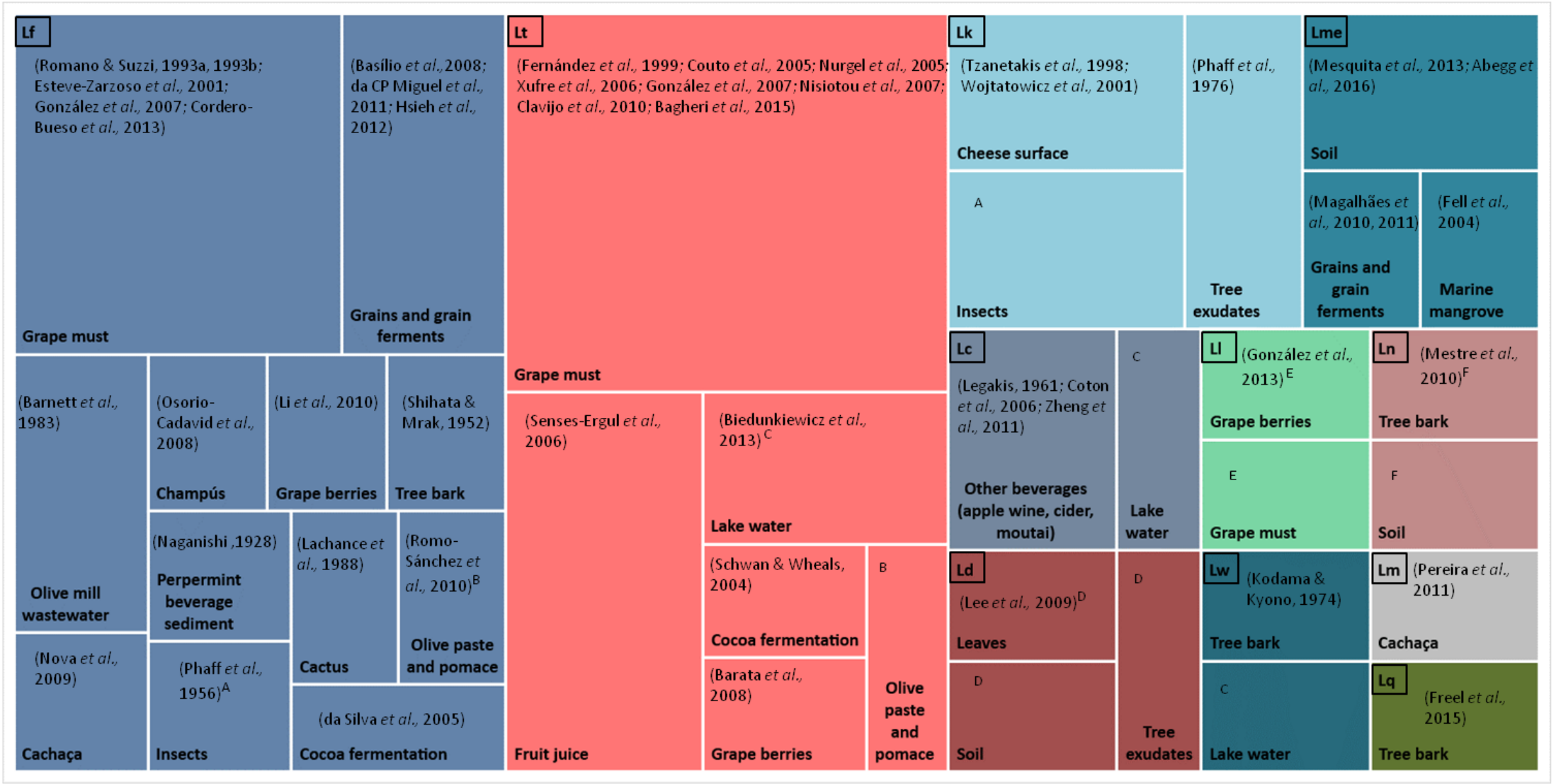

Figure 2.3 Various habitats and ecological niches from which classified *Lachancea* spp. have previously been isolated; Lf: *L. fermentati*, Lt: *L. thermotolerans*; Lk: *L. kluyveri*, Lc: *L. cidri*; Ld: *L. dasiensis*, Lme: *L. meyersii*, Ll: *L. lanzarotensis*, Lw: *L. waltii*, Ln: *L. nothofagi*, Lm: *L. mirantina* and Lq: *L. quebecensis*. The segment colours separate the *Lachancea* spp., and the size of each segment represents the frequency each species has been isolated from a particular habitat. The references for the corresponding reports are noted and common references illustrated by corresponding letters.

2.3.2 Biochemical traits of *Lachancea* spp.

Following the reclassification of several yeast species into the newly proposed genus *Lachancea*, Fell *et al.* (2004) highlighted that members of this genus possess certain common substrate assimilation and fermentation characteristics. However, following the characterization of several new species into this genus, these common characteristics no longer hold true. Typically, *Lachancea* species can ferment glucose and at least one other sugar as well as assimilate raffinose, ethanol (with the exception of *L. dasiensis*, *L. nothofagi* and *L. mirantina*) and mannitol. They are also typically unable to assimilate nitrate, lactose, soluble starch, L-, D-arabinose, D-ribose (except *L. quebecensis*), L-rhamnose, D-glucosamine, N-*acetyl*-D-glucosamine (except *L. mirantina*), methanol, erythritol, galactitol, citrate, inositol and hexadecane. Information regarding hexadecane assimilation for *L. dasiensis* and *L. lanzarotensis*, citrate assimilation for *L. nothofagi* and D-glucosamine assimilation for *L. mirantina* remain unavailable. Another noted characteristic was the production of 1-4 ascospores following conjugation (Fell *et al.,* 2004; Lee *et al.,* 2009; Mestre *et al.,* 2010; Pereira *et al.,* 2011; González *et al.,* 2013; Freel *et al.,* 2015).

Table 2.1 Reported assimilation profiles for the *Lachancea* yeast species

Assimilation profiles	LF	LT	LK	LC	LW	LM	LD	LN	LMi	LL	LQ
L-Sorbose	V	+	V	+	+	W/-	-	-	-	-	V
Inulin	V	V	-	V	-	V	-	-	S	-	+
2-Keto-D-gluconate	V	V	V	-	-	V	-	V	-	nd	V
Ethanol	+/S	V	+	+	+	S	-	-	-	+/W	S
Glycerol	V	+	V	+	-	S	-	+	+	V	+
D-Galactose	+/V	+/V	+	+	-	-/W	+	D	S	+/W	+
Maltose	+/W	+	+	+/V	-	+	+	+	S	+	+
Trehalose	+/W	+	+/V	+	-	+	W	+	S	+	V
Melezitose	+	+	+/V	-/V	-	+	+	+	-	+	+
Succinate	+/V	V	+/V	+	-	-	-	-	-	-	nd
DL-Lactate	+/W	-	+/V	+/V	-	-	-	-	S	-	+
Melibiose	-/V	-/V	+	+	-	-	+	-	-	-	+
∂-Methyl-D-glucoside	+	+	+	+	-	+	nd	nd	nd	nd	nd
Growth on:											
50% D-Glucose	+	+	+/V	+/V	+	-/V	+	V	-	+	+
0.01% Cyclohexamide	+	-	-	+	+	-	+	-	-	-	-
10% NaCl	v/+/s	-/V	-	+	-	+	-	V	-	+	S

nd: no data available; D: Delayed growth; S: slow growth; V: variable growth; W: weak growth; -: negative; +: positive; LF: *L. fermentati*; LT: *L. thermotolerans*; LK: *L. kluyveri*; LC: *L. cidri*; LW: *L. waltii*; LM: *L. meyersii*; LD: *L. dasiensis*; LN: *L. nothofagi*; LMi: *L. mirantina*; LL: *L. lanzarotensis*; LQ: *L. quebecensis*

Carbon assimilation, depending on organic sources of carbon for energy and growth, is an important form of identification utilized for taxonomic grouping of yeast species and has been utilized over decades, with the first study perhaps performed by Beijerinck in 1889

(Wickerham & Burton, 1948; Obasi *et al.,* 2014). **Table 2.1** summarizes the assimilation profiles for the *Lachancea* spp. and the contradictory results from various reports have been highlighted (Fell *et al.,* 2004; Lee *et al.,* 2009; Mestre *et al.,* 2010; Pereira *et al.,* 2011; González *et al.,* 2013; Freel *et al.,* 2015). This contradiction suggests intra- and interspecies differentiation. Although these carbon sources were chosen for taxonomic reasons, the ability for the yeasts to assimilate these compounds provides information of their genetic background and their ability to proliferate under certain environmental conditions. However, it must be noted that there is a lack in literature regarding the synthesis of compounds by *Lachancea* spp., and therefore conclusions on which genes are present and involved in compound assimilation and synthesis cannot accurately be made from these simple assimilation profiles. Furthermore, making a connection between the habitats from which these yeasts have been isolated and their ability to assimilate certain compounds becomes difficult. Even though a species may be unable to assimilate a compound does not negate its potential ability to synthesize it, thereby enabling its proliferation in a specific habitat. A noticeable profile is that of *L. walti*, where the strains analyzed are unable to assimilate inulin, 2-keto-D-gluconate, glycerol, D-galactose, maltose, trehalose, melezitose, succinate, lactate, melibiose or ∂-Methyl-D-glucoside as well as grow on media supplemented with 10% NaCl. This suggests to its stricter nutritional requirements, and therefore perhaps to its limited proliferation in varied habitats. However, this cannot be assumed without evaluating its ability to synthesize these compounds.

2.4 Oenological potential of *Lachancea* spp.

As discussed above, amongst the members of the genus *Lachancea*, *L. thermotolerans* and *L. fermentati* have been associated with grape must and wine fermentation processes in several wine producing countries and over many years, while *L. lanzarotensis* has only recently been isolated. Consequently, *L. thermotolerans* and to a lesser extent, *L. fermentati*, have been studied and characterized and their oenological traits described.

2.4.1 Biochemical traits of oenological interest

Observing the reported assimilation profiles (at least for the strains analyzed) for the wine-related *Lachancea* spp. (**Table 2.1**) the data suggest potential biochemical characteristics allowing adaptation to the harsh winemaking conditions. All three of the yeast species have been shown to assimilate trehalose and unlike many of the carbon sources, utilized mainly for proliferation, trehalose metabolism has been linked to stress resistance in yeast cells. Under stress, trehalose concentration can reach as high as 35% of the dry weight of the cells (Wiemken, 1990). This was however reported for *S. cerevisiae* cells and not *Lachancea*

spp., where trehalose has been seen to protect *S. cerevisiae* cells when exposed to lethal ethanol concentrations of over 10% (v/v) - a common environment of wine fermentations (Bandara *et al.,* 2009). The intracellular uptake of ethanol during carbohydrate fermentation places the yeast cells in a stressful environment. A mechanism of survival involves metabolic responses including the accumulation of trehalose, likely through its membrane-stabilizing action (Sebollela *et al.,* 2004). Investigation into whether this is the metabolic process followed by *Lachancea* spp., is therefore required to understand the link between trehalose accumulation and stress resistance as well as whether *Lachancea* spp. are able to synthesize trehalose, enabling their survival in stressful conditions, such as a wine fermentation.

The strains of the three wine-related yeast species have also been seen to grow on the 50% D-glucose supplemented medium (**Table 2.1**), suggesting to their increased osmotic tolerance, which is an important characteristic for yeast in wine fermentations. Grape must contains high concentrations of sugars, or osmotically active substances, which can lead to the cells entering hyperosmotic shock. Yeast cells adapt by cell wall and cytoskeleton modification or the synthesis/uptake of an osmolyte such as glycerol (Bauer & Pretorius, 2000). *L. fermentati* and *L. lanzarotensis* strains displayed variability in glycerol assimilation while various *L. thermotolerans* strains showed the ability to assimilate this compound. Investigation of the yeast species behaviour in wine fermentations, specifically for *L. thermotolerans* and *L. fermentati* has however provided more information on their adaptation capabilities and is discussed below.

2.4.2 *L. thermotolerans* - behaviour during fermentation of grape juice and contribution to resulting wines

2.4.2.1 *L. thermotolerans* - fermentation kinetics

While a range of investigations have been performed to evaluate the oenological impact of *L. thermotolerans*, these reports have been carried out in different grape matrices, fermentation conditions and utilizing various yeast strains. Therefore, drawing conclusions from these studies regarding the behaviour of the *L. thermotolerans* species as a whole would be inaccurate, however certain trends, irrespective of the changing conditions, can be noticed. The single inoculation of *L. thermotolerans* into white and red grape must has been shown to result in the presence of residual sugars and lower ethanol concentrations, due to its lower fermentation rate (Mora *et al.,* 1990; Ciani *et al.,* 2006; Cordero-Bueso *et al.,* 2013; Gobbi *et al.,* 2013; Mostert & Divol, 2014; Balkci *et al.,* 2016). For the completion of the fermentation, co-culture or sequential inoculation with *S. cerevisiae* is therefore necessary.

L. thermotolerans has been seen to persist till the middle to end stages of the fermentation during mixed culture fermentations with *S. cerevisiae*. The consequence of inoculating *L. thermotolerans* is the resulting lowered fermentation rate, which has been seen for both simultaneous and sequential inoculation strategies. Although, when adding *L. thermotolerans* and *S. cerevisiae* simultaneously the cumulative CO_2 loss is increasingly similar by the end of the fermentation. All simultaneous fermentations, irrespective of varying dosage, have consequently resulted in similar ethanol and residual sugar levels in the wine compared to their respective *S. cerevisiae* controls (Ciani *et al.,* 2006; Kapsopoulou *et al.,* 2007; Comitini *et al.,* 2011; Gobbi *et al.,* 2013; Balikci *et al.,* 2016; Benito *et al.,* 2016). Sequential fermentations have in general been shown to result in the increased persistence of *L. thermotolerans* in the mixed fermentations, where the later addition of *S. cerevisiae* has allowed the *L. thermotolerans* yeast cells to reach higher concentrations and subsequently illustrate increased competitiveness (Ciani *et al.,* 2006; Kapsopoulou *et al.,* 2007; Gobbi *et al.,* 2013; Balikci *et al.,* 2016; Benito *et al.,* 2016; Beckner Whitener 2017). Specifically how long the *L. thermotolerans* is able to persist and dominate in the fermentations has however varied, which was explained by Kapsopoulou *et al.* (2007) as being dependent on the ability for *L. thermotolerans* to reach a critical cell population, namely 7 log cfu/mL, prior to the inoculation of *S. cerevisiae*. Upon critical analysis, while this hypothesis was true in several reports (Nurgel *et al.,* 2005; Ciani *et al.,* 2006; Gobbi *et al.,* 2013; Balikci *et al.,* 2016), it has also been invalidated in others (Benito *et al.,* 2016; Beckner Whitener *et al.,* 2016, 2017). This therefore suggests the dominance and competitive nature of *L. thermotolerans*, to be strain- or condition-dependent. The death of this yeast species can also be attributed to various biotic factors.

The decline in *L. thermotolerans* during fermentations has also been attributed to the impact of parameters such as higher temperatures (Ciani *et al.,* 2006; Gobbi *et al.,* 2013; Balikci *et al.,* 2016), lack of oxygenation (Holm Hansen *et al.,* 2001; Shekhawat *et al.,* 2017), cell-to-cell contact with *S. cerevisiae* (Nissen & Arneborg, 2003; Nissen *et al.,* 2003) and the production of toxic compounds by *S. cerevisiae* (Albergaria *et al.,* 2010). Therefore, further investigation into the ability of various *L. thermotolerans* strains to survive till the end of fermentations, in spite of these inhibitory factors, could unveil interesting characteristics and provide insight into traits which could further uncover additional commercial-potential yeasts.

2.4.2.2 *L. thermotolerans* – impact on wine quality

Although *L. thermotolerans* does not consistently persist to the end of the fermentation, it has still been determined to positively influence the analytical and sensorial profile of wine. Various investigations have been performed analyzing *L. thermotolerans* in co-culture

fermentations with *S. cerevisiae*. These investigations have been carried out in diverse grape matrices, utilizing different strains of both species and different inoculation dosages and strategies (**Table 2.2**). The determined metabolite concentrations in the resulting wines are thus different for each report; however, certain trends are noticeable when comparing the co-culture fermentation to those completed with *S. cerevisiae* alone. For instance, co-culture fermentations with *L. thermotolerans* typically result in lower volatile acidity and higher titratable and total acidity. This is generally attributed to the reduction in acetic acid and increase in D,L-lactic acid levels, respectively (Mora *et al.,* 1990; Ciani *et al.,* 2006; Kapsopoulou *et al.,* 2007; Comitini *et al.,* 2011; Gobbi *et al.,* 2013; Balikci *et al.,* 2016; Beckner Whitener *et al.,* 2016; Benito *et al.,* 2015, 2016). Glycerol, able to impart a sweet taste and impact mouth fullness (Nieuwoudt *et al.,* 2002), was also seen to increase with *L. thermotolerans* inoculation (Kapsopoulou *et al.*, 2007; Gobbi *et al.,* 2013; Benito *et al.,* 2015, 2016). These features appear to be independent of the strain and fermentation conditions (**Table 2.2**), however are influenced by the biomass and persistence of *L. thermotolerans*. Prolonging the persistence of *L. thermotolerans*, generally through the implementation of sequential inoculation strategies, has resulted in the respective increase/decrease of these compounds (Kapsopoulou *et al.,* 2007; Comitini *et al.,* 2011; Gobbi *et al.,* 2013; Balikci *et al.,* 2016; Benito *et al.,* 2016; Beckner Whitener *et al.,* 2017). In contrast to these observed trends, the production of ethanol and acetaldehyde varied within the different reports (Ciani *et al.,* 2006; Kapsopoulou *et al.*, 2007; Gobbi *et al.,* 2013; Benito *et al.,* 2015, 2016; Balikci *et al.,* 2016).

The production of various volatile compounds can significantly impact the overall perception of wine. Critical analysis of various reports (**Table 2.2**) found 2-phenylethanol to increase irrespective of the varying fermentation conditions, with a fold increase between 1.22 – 1.91 relative to the *S. cerevisiae* monoculture fermentations (Comitini *et al.,* 2011; Gobbi *et al.,* 2013; Beckner Whitener, 2015; Benito *et al.,* 2016). The concentrations of this compound were reported above its sensory detection thresholds and would therefore be able to impart flowery/pollen aromas to the wine (Li *et al.,* 2008). Regarding the ester production, the only trend common in the varying reports was the increase in ethyl lactate (buttery, cream aroma) and ethyl hexanoate (green apple and anise aroma) as well as the reduction in phenylethyl acetate (fruity aroma) (Gobbi *et al.,* 2013; Benito *et al.,* 2015, 2016). Sensory analysis performed on the co-culture fermented wines reported on the increased overall impression and total acidity (Gobbi *et al.,* 2013; Benito *et al.,* 2016). Beckner Whitener *et al.* (2016, 2017), also analyzing sensory perception, highlighted the role differing grape must could play in determining the wine quality. *L. thermotolerans* inoculated Shiraz wines were determined as more distinct than Sauvignon blanc wines when compared to those produced by *S. cerevisiae* alone. *L. thermotolerans* inoculation was seen to enhance

the production of 1-ethyl-1h-pyrorole-2-carboxaldehyde, which is beneficial to Shiraz wines due to imparting a smokey/roasted aroma.

While certain trends can be determined from the various fermentations performed by *L. thermotolerans*, overall, there seems to be more commonality in the production of primary metabolites than that of secondary metabolites, where a higher dependence on yeast strain and fermentation conditions is noticeable. The investigations performed for *L. thermotolerans* (**Table 2.2**) have provided insight into the yeast species fermentation capabilities and potential sensorial impact, however because various fermentation conditions or parameters are not kept constant between the different studies, it is difficult to conclude what actual impact *L. thermotolerans* has on wine quality. Future investigations, where single parameters are changed and the impact on *L. thermotolerans* is evaluated could provide an opportunity where the optimal conditions for this species are determined, to ultimately produce better quality and distinct wine products using *L. thermotolerans*.

Table 2.2 Pure and co-culture fermentations with varying strains of *L. thermotolerans* and *S. cerevisiae* under varying conditions

Yeast strain		Inoculation strategy (day)	Dosage (cfu/mL)		Scale of fermentation	Grape variety or media	Reference
Lt	Sc		Lt	Sc			
*From former work	/	Pure	10^6	/	Large scale (100 L)	Unsterilized Manto Negro grape must	Mora *et al.*, 1990
M8	DBVPG 101	Simultaneous Sequential (4)	10^6	10^6	Small scale (200 mL)	Paterurized Pinot grigio grape must	Ciani *et al.*, 2006
TH941	SCM952	Simultaneous Sequential (1) Sequential (2) Sequential (3)	5 x 10^5	5 x 10^6	Small scale (200 mL)	Sterile grape must	Kapsopoulou *et al.*, 2007
101**	EC1118	Simultaneous	10^7	10^7, 10^5, 10^3	Small scale (500 mL)	Pasteurized grape must	Comitini *et al.*, 2011
CLI 1219		Pure	10^6	/	Small scale (400 mL)	Filter sterilized Malvar grape must	Cordero-Bueso *et al.*, 2013
101**	/ EC1118	Pure Simultaneous Sequential (1) Sequential (2) Simultaneous Sequential (2)	10^7 10^7	/ 10^6	Medium scale (1L) Industrial scale (10 hL)	Pasteurized Sangiovese grape must	Gobbi *et al.*, 2013
IWBT 1326***	/ VIN13	Pure Sequential (1)	2 x 10^7	/ 2 x 10^5	Small (600 mL)	Synthetic grape juice	Mostert & Divol 2014
Concerto TM	/ /	Pure	10^6	/ /	Small (55 mL)	Syrah grape must Sauvignon grape must	Beckner Whitner *et al.*, 2015

Concerto TM	EC1118	Sequential (2)	10^6	10^7	Medium scale (4 L)	Sterilized Riesling grape must	Benito *et al.,* 2015
CBS 2860	/ *From former work	Pure Simultaneous Sequential (1) Sequential (2) Sequential (3)	5 x 10^6 5 x 10^6	/ 5x 10^6	Small scale (800 mL)	Sterilized white grape must cv. Emir	Balikci *et al.,* 2016
Concerto TM	Enoferm, M2, Lallemand	Sequential (Sc added when 2% ethanol v/v/ reached)	10^6	10^6	Large scale (10 L)	Sauvignon blanc grape must	Beckner Whitener *et al.,* 2016
CECT 12672	CECT 12512	Simultaneous Sequential (4)	2.95 x 10^7	1.18 x 10^7	Medium scale (3.9 L)	Sterilized Airén grape must	Benito *et al.,* 2016
Concerto TM	Enoferm, M2, Lallemand	Sequential (Sc added when 2% ethanol v/v/ reached)	10^6	10^6	Large scale (10 L)	Shiraz grape must (SO_2 addition for sterilization)	Beckner Whitener *et al.,* 2017

Inoculation of Lt: *L. thermotolerans* and Sc: *S. cerevisiae* strains in varying dosage and grape must
* Strains not specified in report, noted as strain isolated in previous work
** Strain from culture collection of the Dipartimento di Scienze della Vita e dell'Ambiente DiSVA of the Polytechnic University of Marche
*** Strain from culture collection of Institute of Wine Biotechnology (IWBT), Stellenbosch University

2.4.3 *L. fermentati* - behaviour during fermentation of grape juice and contribution to resulting wines

Extensive investigation into *L. fermentati* as a potential wine yeast is notably limited in literature, where a few studies were performed in the 1990s and more recently in 2013 (Cordero-Bueso *et al.,* 2013). *L. fermentati* was previously thought to form part of the *Zygosaccharomyces* genus, and possess a close relation to *Z. bailii*, a known wine spoilage yeast species (Romano & Suzzi, 1993b). This affiliation with a spoilage microorganism could explain the limited investigation regarding its potential positive contribution to wine quality.

2.4.3.1 *L. fermentati* - fermentation kinetics

Currently, various strains of *L. fermentati* have only been investigated in monoculture fermentations and were shown to possess high fermentation vigour (Romano & Suzzi, 1993b; Romano *et al.,* 1997). Cordero-Bueso *et al.* (2013) reported a 2.2 ± 0.7% of daily sugar consumption for *L. fermentati*, superior to that of a *L. thermotolerans* strain. The competitive nature of this species was illustrated by Romano *et al.* (1997), where *L. fermentati* strains replaced yeast species including *Kloeckera apiculata*, *Candida stelata* and *Candida valida* during the middle stages of spontaneous fermentations and were present at the end of the fermentation with *S. cerevisiae*. While this data suggests *L. fermentati* as a potential wine yeast species, able to ferment grape must and persist till the later stages of the fermentation, the resulting residual sugars from these fermentations were not specified.

Only Cordero-Bueso *et al.* (2013) reported on the residual sugar levels of *L. fermentati* monoculture fermentations and while it was lower than many other strains analyzed, co-culture with *S. cerevisiae* would be necessary to complete the fermentation.

2.4.3.2 *L. fermentati* – impact on wine quality and potential biotechnological advantage

Monoculture fermentations with *L. fermentati* have been reported to result in low acetaldehyde, H_2S, SO_2 and acetic acid production as well as, in comparison to *S. cerevisiae*, increased volatile acidity and lowered titratable acidity (Romano & Suzzi, 1993b; Romano *et al.,* 1997; Cordero-Bueso *et al.,* 2013). The production of various volatile compounds can also impact the quality of wine. *L. fermentati* has very limited research into the production of volatile compounds and critical analysis of the common compounds suggests strain differentiation regarding the production of higher alcohols, Isoamyl alcohol and propanol (Romano & Suzzi 1993a; Romano *et al.,* 1997; Romano *et al.,* 2003; Cordero-Bueso *et al.,* 2013). The production of isobutanol could potentially be medium dependent, where low levels were reported when fermented in synthetic medium and levels comparable to *S. cerevisiae* when fermenting grape must (Romano & Suzzi, 1993a; Romano *et al.,* 1997; Romano *et al.,* 2003; Cordero-Bueso *et al.,* 2013). Increased investigations into the volatiles produced by *L. fermentati*, during monoculture and co-culture fermentations, will provide an opportunity to gage insight into what one may expect from *L. fermentati* as a species.

Various *L. fermentati* strains have also been shown to flocculate (Suzzi *et al.,* 1992; Romano & Suzzi, 1993b). Flocculation of yeast cells often involve the binding of flocculins (lectin-like proteins) on the cell walls of flocculent cells that selectively bind to the mannose residues in adjacent yeast cell walls (Verstrepen *et al.,* 2003). This behaviour can aid in the efficiency of grape must clarification and processing (Rossouw *et al.,* 2015). The various *L. fermentati* strains analyzed by Romano and Suzzi (1993b), for their ability to flocculate and their degree of flocculation, illustrated strain dependence. Of the 14 analysed, in real grape juice four strains illustrated moderate and extreme flocculation. The same four strains were determined to flocculate in synthetic must as well, however to a lesser degree (poor to very flocculent). Suzzi *et al.* (1992) investigated the flocculation behaviour of three flocculating *L. fermentati* strains and specifically the effect of proteinase and sugar addition. *L. fermentati* flocculation was determined to be highly proteinase- and trypsin-resistant, however, an irreversible inhibitory effect on flocculation was seen when mannose was present. This suggests that the free mannose in the media was able to bind to and occupy the flocculin receptors, thus preventing direct interaction with the mannose of adjacent yeast cells and subsequently flocculation (Verstrepen *et al.,* 2003). The flocculating behaviour of several *L. fermentati* strains could potentially benefit wine production; however, like the fermentation

behaviour and organoleptic contributions of this yeast species, increased research is required.

2.4.4 *L. thermotolerans* and *L. fermentati* - extracellular enzyme production

In addition to the production of various primary and secondary metabolites, yeast species can also influence wine aroma as well as the technological process of wine production through the expression of various extracellular enzymes. Several of these enzymes are produced and transported to the periplasmic space and secreted to the extracellular medium, where they are able to interact with grape derived aroma precursor compounds (Strauss *et al.,* 2001). Enzymes of oenological relevance commonly investigated for wine yeasts include protease, pectinase and glycosidases. Of the *Lachancea* spp. such enzymatic activities have been analyzed for *L. thermotolerans* and *L. fermentati*, while *L. lanzarotensis* noticeably lacks this information.

2.4.4.1 Protease and pectinase enzyme activity

Unstable protein of grape origin in wine, forming a haze, is an issue often faced during the ageing of white and rosé wines. While not noxious, the appearance of protein haze in wine can result in consumer rejection. It is therefore common for winemakers to utilize protein removal techniques, such as bentonite fining. It has however been reported that not all the proteins are adsorbed and utilization of bentonite can lead to reduced wine volume (5 – 20%) and alteration of the wine sensory characteristics, due to loss of aroma and flavour compounds (Lagace & Bisson, 1990). An alternative method involves exploiting the yeasts ability to express proteases. Proteases are able to hydrolyse proteins into smaller and more soluble compounds, which can aid in wine clarification and stabilization (Fernández *et al.,* 2000). Another advantage of protein hydrolysis is the resulting increase in amino acids and peptides, aiding in the prevention of stuck or sluggish fermentations due to the resulting increase in assimilable nitrogen (Lagace & Bisson, 1990; Fernández *et al.,* 2000). Another enzyme with oenological relevance is pectinase. Pectinolytic enzymes, through the degradation of pectin, can aid in enhancing the efficiency of grape pressing and clarification of the must as well as increasing substance extraction, contributing to wine colour and aroma (Fernández *et al.,* 2000).

As summarized in **Table 2.3**, of the various *L. thermotolerans* strains analyzed, negative results were reported for 100 and 97.98% of these strains for protease and polygalacturonase activities, respectively (Sakai *et al.,* 1984; Schwan *et al.,* 1997; Romo-Sánchez *et al.,* 2010; Alimardani-Theuil *et al.,* 2011; Comitini *et al.,* 2011; Cordero-Bueso *et al.,* 2013; Mostert, 2013; Belda *et al.,* 2016). This therefore suggests most *L. thermotolerans* strains to lack this enzyme activity, however it must be recognised that varying methodology

was utilized to measure this activity, regarding the substrates and conditions (for example pH). Regarding the activities for *L. fermentati*, although also illustrating strain dependence, drawing any form of conclusions from the available data is difficult due to the overall limited number of screenings performed. Cordero-Bueso *et al.* (2013) reported a *L. fermentati* strain to lack polygalacturonase activity, while da Silva *et al.* (2005) reported positive polygalacturonase activity for another strain. Romo-Sánchez *et al.* (2010) investigated the enzyme activity of nine *L. fermentati* strains and found none to illustrate polygalacturonase activity under the conditions tested. In addition, from what has been found in literature, there is an overall lack of information regarding the protease activity for *L. fermentati* strains. As illustrated, the reports investigating enzyme activity depict strain dependent expression and it cannot be ignored that many of these investigations utilize varying substrates and techniques, which can ultimately effect the induction and/or intensity of the respective enzymes (Fernández-González *et al.,* 2003; Hernández *et al.,* 2003).

Table 2.3 Summarized results for the various *L. thermotolerans* and *L. fermentati* strains protease, polygalacturonase and β-glucosidase activity screenings

Enzyme activity	*L. thermotolerans*		*L. fermentati* *	
	Positive activity	Negative activity	Positive activity	Negative activity
Protease	-	100%	-	
No. of strains	98			
Polygalacturonase	2.02%	97.98%	9.09%	90.91%
No. of strains	97		11	
β-Glucosidase	13.27%	86.73%	80%	20%
No. of strains	111		10	

***As far as could be determined, no *L. fermentati* strains have been screened for protease and very few for polygalacturonase and β-glucosidase activities

2.4.4.2 Glycoside hydrolase enzyme activity

The contribution by yeast species to wine quality has previously been noted specifically due to the production of sensorially significant, yeast metabolism derived, volatile compounds including esters, alcohols and acetates (Jolly *et al.,* 2014). However, a large proportion of varietal flavours of wines are due to the presence of grape derived volatile aromatic compounds (Cabaroglu *et al.,* 2003). Monoterpenes, due to their pleasant floral notes, play an essential role in the aroma profiles of wines, especially that of white varieties, for example Muscat, Gewürztraminer and Riesling. A large proportion of the varietal aroma compounds do however appear glycosidically conjugated in young wines, resulting in their inability to contribute to the aroma of wine, and therefore form a large group of untapped aroma and flavour potential (Manzanares *et al.,* 1999; 2000). These compounds exist largely in wines as aroma precursors; either monosaccharide glucosides (β-D-glucopyranosides) or disaccharide glycosides (6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides or 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides) and possess the ability to contribute to the wine

aroma upon liberation (Günata *et al.,* 1988). These volatile compounds can be liberated from their precursors by acid or enzymatic hydrolysis.

High temperature acid hydrolysis does however introduce the possibility of monoterpenol rearrangement and potentially the formation of alternative compounds in place of the fruity and floral desired monoterpenes, while enzymatic hydrolysis has been reported to induce minimal changes (Günata *et al.,* 1988; Skouroumounis & Sefton, 2000). Enzymatic hydrolysis is therefore the beneficial option for monoglucoside or disaccharide glycoside hydrolysis. Monoglucoside hydrolysis is performed by β-glucosidases, resulting in the release of the monoterpenol and glucose (**Figure 2.4 A**). This enzyme does not however possess endoglucanase activity and therefore disaccharide glycosides, possessing more than the one bound sugar moiety cannot be hydrolyzed by only β-glucosidases and requires additional action by other glycoside hydrolases. Glycoside hydrolases are involved in the sequential cleaving of monoterpenol disaccharide precursors and, as illustrated in **Figure 2.4 B**, involves the sequential mode of action of several enzymes, depending on the bound sugar molecule: α-L-arabinofuranosidase, α-L-rhamnopyranosidase or β-D-apiofuranosidase and β-D-glucosidase (Günata *et al.,* 1988). Firstly, the (1 → 6) linkage of the diglycoside molecules is cleaved by α-L-arabinofuranosidase, α-L-rhamnopyranosidase or β-D-apiofuranosidase, resulting in the release of α-L-arabinose, α-L-rhamnose or β-D-apiose, respectively, as well as the monoterpenyl β-D-glucoside. The β-D-glucosidase enzyme is thereafter able to access the glucoside and release the monoterpenol along with β-D-glucose. The ability of the glycoside hydrolases to execute the release of monoterpenols depends on their affinity for grape-derived terpenoid aglycones and their activity under winemaking conditions, for example low pH, fermentation temperatures, high initial sugar and increasing ethanol levels. Commercially prepared enzymatic mixtures, such as Lallzyme® Cuvée blanc (produced by Lallemand with advertised main pectinase and concentrated β-glucosidase activity), are often used for the purpose of enhancing aroma complexity and the release of aroma compounds through the introduction of these enzymes. Composition of commercial mixtures are however often unknown and irregular, making it difficult to control the subsequent impact on wine quality (Roche *et al.,* 2005). For this reason, exploiting yeasts that are able to produce functional glycosidases; which are capable of releasing the aromatic monoterpenes from their non-aromatic glycosidically bound precursors could provide a more reliable and efficient method than the addition of enzyme preparations.

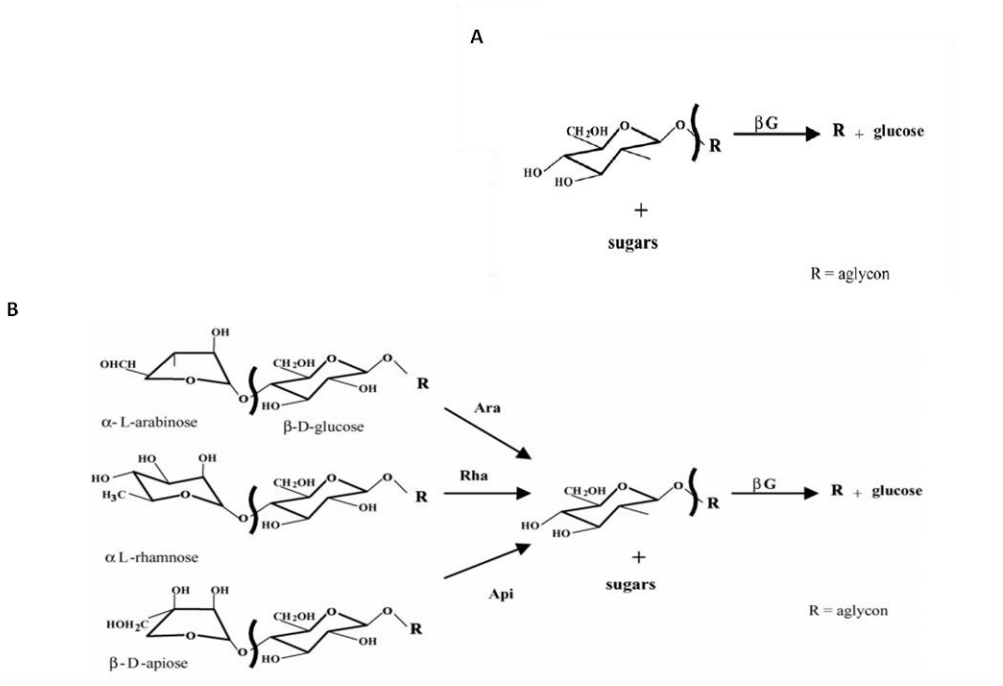

Figure 2.4 Hydrolysis of monosaccharide glucosides (A) and the sequential hydrolysis of disaccharide glycosides (B), Ara: α-L-arabinofuranosidase, Rha: α-L-rhamnopyranosidase; Api: β-D-apiofuranosidase; βG: β-D-glucopyranosidase; R: monoterpenes, sesquiterpenes, norisoprenoids, benzene derivatives, aliphatic alcohols (adapted from Palmeri & Spagna 2007)

As summarized in **Table 2.3**, screenings of various *L. thermotolerans* strains found only 13.27% of the strains to possess β-glucosidase activity, whereas 86.73% lacked the activity. Forming a large percentage of these results are the 88 *L. thermotolerans* strains analyzed by Belda *et al.,* (2016), of which a large portion illustrated negative activity. The reports that investigated β-glucosidase activity were performed on plates, where arbutin was utilized as the substrate for enzyme activity and although the pH was different for some, the methodology was similar. Therefore, analysis of several *L. thermotolerans* strains illustrated varying results; suggesting strain differentiation (Rosi *et al.,* 1994; Romo-Sánchez *et al.,* 2010; Comitini *et al.,* 2011; Cordero-Bueso *et al.,* 2013; Mostert 2013; Belda *et al.,* 2016).

Cordero-Bueso *et al.* (2013) investigated the β-glucosidase activity of one *L. fermentati* strain, which was also reported positive. Romo-Sánchez *et al.* (2010) analysed 9 strains of *L. fermentati* and found seven and two strains to possess and lack this activity, respectively. As discussed above, these screenings cannot accurately represent what may occur in a wine fermentation, due to differing conditions in which activity is tested and the consistent use of artificial substrates that do not represent the natural substrates in a wine fermentation. While β-glucosidase has been analyzed during wine fermentations for other yeast species (Fia *et al.,* 2005), it has not yet been investigated for *Lachancea* spp. This information could provide more insight into whether *Lachancea* spp. are able to produce this

enzyme under the harsh conditions of a wine fermentation and subsequently impact wine quality.

2.5 Conclusion

Evaluating the *Lachancea* species has provided interesting information on intra- and interspecies genetic diversity and the subsequent effects of selective pressures guiding evolution. These yeast species have also been isolated from extensively diverse habitats, suggesting to their ability to adapt to these environmental conditions even when genetically similar. However, the majority of the *Lachancea* spp. have received very little attention, only having been isolated and characterized, thus potential impactful information remains unexplored.

Increasing focus has shifted onto non-*Saccharomyces* yeasts and their potential usefulness to various biotechnological processes, specifically that of wine fermentations. As of now, only three *Lachancea* species have been associated with grape berries and grape must – namely *L. thermotolerans, L. fermentati* and most recently *L. lanzarotensis*. Even though these species have all been isolated from the wine environment, research has specifically focussed on the fermentation behaviour/potential of *L. thermotolerans* as well as its expression of oenologically relevant enzymes. In contrast, information is lacking for *L. fermentati*, even though it has been found in this environment and shown the potential to possess robust fermentative capabilities. *L. fermentati* additionally has very limited data available regarding its expression of enzymes of oenological relevance. The investigations into enzyme activity for *L. thermotolerans* and *L. fermentati* have also been performed under conditions dissimilar to that of a wine fermentation. This therefore cannot be used to determine the actual potential of these yeast species. Lastly, *L. lanzarotensis* lacks any information of oenological relevance and therefore potentially vital information on its contribution, positive or negative, to the fermentation and resulting wine quality remains unclear.

Future investigation into the fermentative capabilities of the *Lachancea* spp., under real fermentation conditions, their survival when in the presence of various other indigenous yeast species as well as their impact on the flavour and aroma of wine could result in not only expanding the knowledge of the genus but also in the beneficial use of these species in various biotechnological processes, from which they have already been isolated.

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CHAPTER 3

Research Results

Evaluation of the biochemical characteristics and fermentation attributes of yeast species of the genus *Lachancea*

Chapter 3 – Evaluation of the biochemical characteristics and fermentation attributes of yeast species of the genus *Lachancea*

3.1 Introduction

The recent increase in research of wine-related non-*Saccharomyces* yeasts aims to find a way of partially returning to the traditional method of wine production, where the wide variety of indigenous yeasts contributed to the overall complexity and varietal flavours of the wine product. Inoculating non-*Saccharomyces* yeasts into the fermentation aims to increase the ability to bring aspects of that history back, however in a controlled manner (Ciani & Comitini, 2011; Padilla *et al.,* 2016; Varela, 2016). Non-*Saccharomyces* yeasts can potentially influence the primary/varietal and secondary/fermentation aroma of wine through the production of hydrolytic enzymes and metabolites, respectively (Ciani *et al.,* 2009; García *et al.,* 2016). Varietal aroma mainly consists of volatile sulphur compounds, methoxypyrazines, C_{13}-norisoprenoids and terpenes (Ebeler & Thorngate, 2009). Monoterpenes play a significant role in the aroma profiles of certain wines, especially those of Muscat, the most prominent of which include geraniol, linalool, nerol, citronellol and α-terpineol in this grape variety (Mateo & Jiménez, 2000). A large proportion of these primary aroma compounds do however occur in grape juice in bound, non-aromatic forms (monoglucoside and diglycoside complexes) and require hydrolysis to effectively contribute to the wine aroma (Dimitriadis & Williams, 1984; Günata *et al.,* 1985). In Muscat d'Alexandrie grape must, the ratio between bound to free (aromatic) monoterpenes have been reported as high as 5:1 (Williams *et al.,* 1982), leaving a large portion of potential aroma unavailable. Glycolytic enzymes act to hydrolyze these complexes, where α-L-arabinofuranosidase, α-L-rhamnopyranosidase or β-D-apiofuranosidase (depending on the conjugated sugar moiety) and β-D-glucosidase work in succession to hydrolyze diglycoside complexes, while β-glucosidases act alone to release volatile monoterpenes from monoglucoside complexes (Günata *et al.,* 1988).

While *Vitis vinifera* possesses endogenous glycosidases, the functionality of these enzymes is reduced by glycerol and low pH, parameters common in wine fermentations. Another factor hindering the applicability of these enzymes is their narrow aglycone specificity, showing the inability to hydrolyze sugar conjugates of flavourful monoterpenes such as linalool (Aryan *et al.,* 1987). As a result, research focus subsequently shifted to exploiting wine-related yeasts shown to express this activity, particularly β-glucosidase, which found non-*Saccharomyces* yeasts to generally display stronger activity than the common wine yeast *Saccharomyces cerevisiae* (Rosi *et al.,* 1994; Mateo & Di Stefano,

1997; Strauss *et al.,* 2001; Cordero Otero *et al.,* 2003). Indeed, Rosi *et al.* (1994) screened 153 *S. cerevisiae* strains of which only one illustrated β-glucosidase activity.

While monoterpenes are a focus of research due to their floral notes and low odour thresholds, majority of the wine aroma compounds arise during the fermentation (Padilla *et al.,* 2016). Non-*Saccharomyces* yeasts have also been shown to impact the secondary/fermentation aroma and quality of wine through the production of primary metabolites such as ethanol and glycerol, but more prominently through the production of secondary metabolites such as higher alcohols, esters and volatile fatty acids. Increasing investigations have proposed mixed culture fermentations with particular non-*Saccharomyces* yeasts and *S. cerevisiae* to bring about unique aromatic characteristics. Inoculation of specific non-*Saccharomyces* yeasts have been performed with the aim of modifying certain aspects of the wine, such as enhancing wine acidity with *Lachancea thermotolerans* (Kapsopoulou *et al.,* 2007; Comitini *et al.,* 2011; Gobbi *et al.,* 2013).

L. thermotolerans is commonly associated with the wine fermentation environment and has been identified to lead to reduced production of acetic acid and enhanced production of L-lactic acid, glycerol and 2-phenylethanol in mixed culture fermentations with *S. cerevisiae* (Kapsopoulou *et al.,* 2005; Comitini *et al.,* 2011; Balikci *et al.,* 2016). These positive attributes have subsequently led to the commercialization of a strain from this yeast species (*L. thermotolerans* Concerto). Regarding its contribution to the varietal flavour of wine, various studies have highlighted the ability for *L. thermotolerans* to express β-glucosidase activity, while a lack of α-L-arabinofuranosidase activity has been noted, suggesting its aptitude for releasing monoterpenols from monoglucoside complexes in grape must (Rosi *et al.,* 1994; Romo-Sánchez *et al.,* 2010; Comitini *et al.,* 2011; Cordero-Bueso *et al.,* 2013; Mostert 2013; Belda *et al.,* 2016). However, there is a lack in literature regarding the expression of these enzymes by *L. thermotolerans* during actual wine fermentations, where factors such as pH, glucose and temperature could impact enzyme expression and activity. In contrast to *L. thermotolerans*, other species of the *Lachancea* genus such as *L. fermentati* and *L. lanzarotensis*, also isolated from the wine fermentation environment, have received little to no research into their oenological potential.

The current study aimed to investigate the potential of *Lachancea* species as co-inoculants in wine fermentations and their overall impact on wine composition. Furthermore, the study focused on the *Lachancea* spp.'s ability to express active β-glucosidase during fermentations and the subsequent impact on the levels of free monoterpenes.

3.2 Materials and Methods

3.2.1 Yeast strains and growth conditions

3.2.1.1 Yeast strains

Various strains of the *Lachancea thermotolerans* and *Lachancea fermentati* obtained from CBS, Chr Hansen and the culture collection of the Institute for Wine Biotechnology (Stellenbosch University) as specified in **Table 3.1**, were screened for enzymatic activities of oenological interest. Also screened were various *Lachancea lanzarotensis* isolates, not having undergone any typing (molecular or otherwise). These isolates originated from Sauvignon blanc grape samples – Morning star vineyard, Elgin, South Africa; 2012-2015. Positive controls used for the enzyme screenings included, *Schwanniomorphus polymorphus* var. *africanus* CBS 8047 (Cordero Otero *et al.,* 2003), *Saccharomyces paradoxus* RO88 (Mocke, 2005), *Metschnikowia pulcherrima* IWBT Y1123 (Theron *et al.,* 2017), *Saccharomyces cerevisiae* V517-5A (Unité de Recherche Œnologie, Institut des Sciences de la Vigne et du Vin, Villenave d'Ornon, France), *S. cerevisiae* VIN13car1::XYN4 (unpublished, courtesy of Dr A Zietsman, IWBT) and *Saccharomyces bayanus* (Vivace, Renaissance Yeast, Vancouver, Canada). For the generation of comparable data, the commercial yeast *Saccharomyces cerevisiae* VIN13 (Anchor Yeast, Cape Town, South Africa) was also screened for enzymatic activity and utilized during fermentations. This yeast strain was additionally used as the positive control for the sulphur dioxide (SO_2) and ethanol tolerance assays.

3.2.1.2 Growth conditions

All yeast strains were cultivated on Wallerstein Laboratory (WL) nutrient agar or Yeast Extract Peptone Dextrose (YPD) media at 25°C. Prior to enzyme screening and fermentation assays, the yeasts were grown in YPD broth at 25°C (50 rpm) until the cells reached late exponential/early stationary phase of growth. Cells were harvested for 10 min at 2800 *x g* (20°C) and the pellet resuspended in saline (0.9% w/v NaCl). For the *L. thermotolerans* and *L. fermentati* strains, flocculation was observed in YPD media and therefore following harvesting of the cells, the pellets was treated with 50 mM Ethylenediaminetetra-acetic acid (EDTA) (pH 8.0) and saline in order to divide flocculated yeast cells, after which the pellets were washed in saline and resuspended once again in saline.

Table 3.1 Non-*Saccharomyces* yeast strains/isolates investigated in this study, where certain strains were selected, based on their enzyme screening results, for further analysis regarding their fermentation capabilities

Yeast species	Origin of isolation	Strain/Isolate
Lachancea thermotolerans	Sauvignon blanc, Stone wall, Somerset West	IWBT Y1202
	Sauvignon blanc, Stone wall, Somerset West	IWBT Y1109
	Chardonnay, Nelson's creek, Paarl	IWBT Y1017
	Muscat d'Alexandre. Jason's hill, Rawsonvill	IWBT Y1240
	Chardonnay, Stonewall, Somerset West	IWBT Y1326
	Sauvignon blanc, Stone wall, Somerset West	IWBT Y1206
	Sauvignon blanc, Stone wall, Somerset West	IWBT Y1197
	Chardonnay, Sir Lowry's Pass	IWBT Y1038
	Sauvignon blanc – Chardonnay blend, Stonewall, Somerset West	IWBT Y1295
	Sauvignon blanc (2014), Elgin Valley, Morningstar vineyard	IWBT Y513
	Chenin blanc (2011), Riebeeck Casteel	IWBT Y905
	Shiraz (2010), Nietvoorbij vineyard, Stellenbosch	IWBT Y940
	Chardonnay, Sir Lowry's Pass	IWBT Y1220
	Chr. Hansen	Concerto™
L. lanzarotensis	Grape, microvinification and winery samples – Lanzarote, Canary Islands	CBS 12615
	Sauvignon blanc grape samples – Morning star vineyard (Elgin, South Africa) 2012-2015	IWBT Y992-1 IWBT Y992-6 IWBT Y992-4 IWBT Y992-5 IWBT Y992-2 IWBT Y992-3
Lachancea fermentati	Sauvignon blanc juice (2016), Welgevallen	IWBT Y515
Saccharomyces cerevisiae	Anchor Yeast	VIN13

IWBT: Institute for Wine Biotechnology (Stellenbosch University); CBS: *Central Bureau of* Fungal *Cultures*

3.2.2 Screening for enzymatic activities and H_2S production

The yeast cells were prepared as described above and the absorbance (OD_{600nm}) was determined. At an OD of 0.1, 5 µL of the yeast strains and respective positive controls were spotted, in triplicate, onto the selected agar plates prepared for enzyme activity screenings. Alternatively, the yeast strains were streaked onto selective media for evaluation of H_2S production. The plates were incubated at 30°C for 4-5 days, after which positive/negative activity or H_2S production was visually inspected as summarized in **Table 3.2** and **Table 3.3** respectively.

3.2.2.1 β-Glucosidase and β-xylosidase activity

β-Glucosidase activity was determined on arbutin selective medium, containing 1% (w/v) yeast extract, 2% (w/v) peptone and 0.5% (w/v) arbutin, with the pH adjusted to 3.5. To this selective medium, 20 mL 1% filter sterilized ammonium ferric citrate and 250 mL 4X autoclaved bacteriological agar was added, as described by Albertin *et al.,* (2016), modified from Strauss *et al.,* (2001). *S. polymorphus* var. a*fricanus* DSM 8047 was used as the positive control. Positive β-glucosidase activity was visualized as a brown colour in and/or a brown halo surrounding the colony.

Also evaluating β-glucosidase activity, an additional substrate was utilized; 4-methylumbelliferyl-β-D-glucoside (4-MUG). The selective media contained; 0.17% Yeast Nitrogen Base (YNB) -without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 0.5% xylose, 2% bacteriological agar and with the pH adjusted to 5.5. This media was prepared according to Manzanares *et al.,* (1999), with the modification of the substrate addition; 10% filter sterilized MUG was added to the media. Hydrolysis of MUG resulted in the liberation of the 4-methylumbelliferone fluorescent compound which could be visualized as a halo, under UV radiation (260 nm), which was monitored daily. Utilizing the same method described above, β-xylosidase activity was analyzed, however utilizing 4-methylumbelliferyl-β-D-xyloside (4-MUX) as substrate. *S. cerevisiae* VIN13car1::XYN4 was utilized as the positive control.

3.2.2.2 Polygalacturonase Activity

Polygalacturonase activity was evaluated on selective media containing 1.25% (w/v) polygalacturonic acid dissolved in 0.68% (w/v) potassium phosphate with 0.67% (w/v) YNB and 1% (w/v) Glucose, with the pH adjusted to 3.5 and 2% (w/v) agar as described by Albertin *et al.,* (2016), modified from McKay (1988). *S. paradoxus* RO88 was used as the positive control and the colonies washed off with distilled water (dH_2O) and the plates flooded with 6 M HCl (Mostert, 2013). The formation of a clear halo surrounding the colonies was observed as positive polygalacturonase activity.

3.2.2.3 Protease Activity

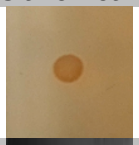
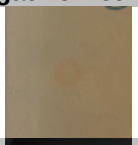
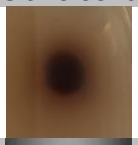
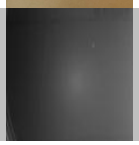
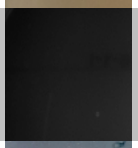
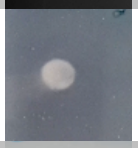
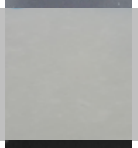
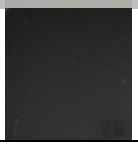
Protease activity was evaluated using a selective medium according to Balinksi *et al.* (1988). Seventy millilitres Citrate phosphate buffer (0.05 M and pH 3.5), containing 44.2 mL 0.2 M Na_2PO_4 and 25.8 mL 0.1 M citric acid was prepared, to which 10% (w/v) skim milk was added. Sixty millilitres phosphate buffer (24 g/L KH_2PO_4 and 35 g/L $Na_2HPO_4.7H_2O$) was added to the skim milk solution and microwaved until beginning to simmer. A 480 mL medium containing 4.8 g glucose, 3.36 g YNB (without amino acids and ammonium

sulphate) and 9.6 g bacteriological agar was prepared separately and added to the skim milk solution. *M. pulcherrima* Y1123 was used as the positive control and positive protease activity was visualised as a clear halo surrounding the colonies.

3.2.2.4 H_2S production

The production of Hydrogen sulphide (H_2S) was evaluated using the selective media – 4.5% (w/v) Bismuth Sulfite Glucose Glycine Yeast Agar, heated for sterilization (180°C to dissolve). Individual colonies of the yeast strains were streaked, in triplicate, onto the selective media. High and low H_2S producing yeast strains, *Saccharomyces cerevisiae* V517-5A and *Saccharomyces bayanus* respectively, were also streaked onto the media as controls. The formation of a brown colour in the colonies and/or surrounding the colonies was indicative of H_2S production. The range of H_2S production was subsequently based on the respective colour intensities of the positive and negative controls (**Table 3.3**).

Table 3.2 Morphologies depicting positive and negative activity and corresponding positive controls

Enzyme activity	Positive Activity	Negative Activity	Positive control
Glucosidase - arbutin			
Glucosidase – 4-MUG			
Polgalacturonase	-		
Protease	-		
β-xylosidase			

No morphology of positive activity are shown for enzyme assays which resulted in no positive readings for the strains analyzed

Table 3.3 Rating system utilized for determining relative production of H_2S

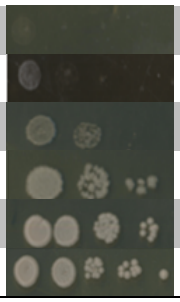
Rating	H_2S production
-	
+	
++	
+++	
++++	

3.2.3 Screening ethanol and SO_2 tolerance

3.2.3.1 Ethanol tolerance

The OD_{600nm} was determined for the yeast strains and an OD_{600nm} of 1 in saline prepared for each. A serial dilution was thereafter prepared from 10^0 to 10^{-4}, with the OD_{600nm} = 1 prepared solution representing no dilution (10^0). Five µL of each dilution was then spot plated onto the prepared media (in triplicate). The media was composed of 50 g/L YPD agar media, with the pH adjusted to 3.5. Following the autoclaving of the media it was allowed to cool after which absolute ethanol was added to create ethanol percentages of 0 (control), 5, 7 and 10% (v/v). *S. cerevisiae* VIN13 was employed as the positive control. The plates were then incubated at 30°C and the colony growth for each serial dilution monitored for 6 days. Tolerance to ethanol was evaluated as indicated in **Table 3.4**.

Table 3.4 Rating system utilized for determining relative tolerance to ethanol

Rating	Explanation	Example
-	No growth observed	-
Slow/delayed	Limited growth at 10^0 dilution	
+	Growth at 10^0 dilution	
++	Growth at 10^{-1} dilution	
+++	Growth at 10^{-2} dilution	
++++	Growth at 10^{-3} dilution	
+++++	Growth at 10^{-4} dilution	

3.2.3.2 SO_2 tolerance

The SO_2 tolerance was evaluated for the yeast strains, with *S. cerevisiae* VIN13 again employed as the positive control. A solution containing 0.67% (w/v) YNB and 2% (w/v) glucose solution (pH 3.5) was prepared and autoclaved. Following autoclaving, SO_2 was added to the media at final concentrations of 4.22, 8.32, 10, 15, 20 and 25 mg/L total SO_2 by adding the required volume of potassium metabisulphate. The corresponding molecular SO_2 levels in the YNB media were determined; where y= 0.0122x – 0.0015; y: molecular SO_2

mg/L and x: total SO_2 mg/L (Usseglio-Tomasset, 1984). The prepared media was then distributed to test tubes (7 mL). In duplicate, the yeast strains were inoculated at an OD_{600nm} of 0.1 and incubated at 22°C at 50 rpm. Growth was monitored daily for 6 days and OD_{600nm} taken, utilizing both the spectrophotometer and the microtiter plate spectrophotometer (BioTek) where appropriate.

3.2.4 Microfermentations

3.2.4.1 Synthetic grape juice fermentations

Based on the initial enzyme activity screening as well as the respective tolerance levels, five *Lachancea* species strains viz. *L. thermotolerans* Concerto™, *L. thermotolerans* IWBT Y940, *L. lanzarotensis* CBS 12615, *L. lanzarotensis* IWBT Y992-5, *L. fermentati* IWBT Y515 as well as *S. cerevisiae* VIN13 were further characterized with regard to fermentation properties.

Fermentations were performed in 250 mL of synthetic grape juice, the composition of which is described in **Table 3.5** (Henschke & Jiranek, 1993). The fermentations were performed in triplicate (biological repeats), at 25°C, under anaerobic conditions and at 60 rpm. The monoculture fermentations were inoculated with a final cell density of 1 x 10^6 CFU/mL and the co-culture (sequential) fermentations were inoculated with the *Lachancea* strains at 1 x 10^7 CFU/mL 48 h prior to the addition of *S. cerevisiae* VIN13 at 1 x 10^6 CFU/mL. Fermentation progress was monitored by weighing the flasks daily and the yeast growth by measuring optical density or determining viable counts on WL agar. The end of fermentation was assumed when the weight loss was constant over two days.

Table 3.5 Synthetic grape juice chemical composition

Carbon Sources (g/L)		**Nitrogen Sources (mg/L)**		**Trace Elements (µg/L)**	
Glucose	100	NH_4Cl	120	$MnCl_2.4H_2O$	200
Fructose	100	Alanine	100	$ZnCl_2$	135
Acids ($g.l^{-1}$)		Arginine	750	$FeCl_2$	30
KH Tartrate	2.5	Asparagine	150	$CuCl_2$	15
L-Malic acid	3	Aspartic acid	350	H_3BO_3	5
Citric acid	0.2	Glutamine	200	$Co(NO_3)_2.6H_2O$	30
Salts ($g.l^{-1}$)		Glutamic acid	500	$NaMoO_4.2H_2O$	25
K_2HPO_4	1.14	Glycine	50	KIO_3	10
$MgSO_4.7H_2O$	1.23	Histidine	150	**Vitamins ($mg.l^{-1}$)**	
$CaCl_2.2H_2O$	0.44	Isoleucine	200	Myo-inositol	100
Lipids/oxygen		Leucine	300	Pyridoxine.HCl	2
Ergosterol	0.1 g	Methionine	150	Nicotinic acid	2
Tween 80	5 ml	Phenylalanine	150	Ca Pantothenate	1
		Proline	500	Thiamin.HCl	0.5
		Serine	400	PABA.K	0.2
		Threonine	350	Riboflavin	0.2
		Tryptophan	100	Biotin	0.125
		Tyrosine	20	Folic acid	0.2
		Valine	200		
		Lysine	250	pH adjusted to 3.5 with 2 M KOH	

3.2.4.2 Muscat d'Alexandrie grape juice fermentations

Frozen Muscat d'Alexandrie grape juice was thawed and thermovinfied by heating at 70°C for 15 min. A sample of the sterilized juice was plated onto YPD agar to assess viable yeast load. The juice had the following characteristics; pH 3.40, 186.83 g/L reducible sugars, 26.2 mg/L ammonia and 160.63 mg N/L primary amino nitrogen (as determined utilizing Megazyme (Bray, Ireland) kits). Sixty millilitres of the juice was dispensed into 100 mL spice jars and inoculated with monocultures or with co-cultures. The fermentations were performed and monitored as described for the synthetic grape juice.

3.2.5 β-glucosidase activity throughout fermentations

3.2.5.1 Protein extraction from synthetic grape juice fermentations

Samples were withdrawn at the beginning, middle and end of fermentation in order to determine β-glucosidase activity. Intracellular and cell wall-associated proteins were extracted according the protocol by Dunn & Wobbe (1992) with modifications. The extracellular matrix (i.e. cell-free supernatant) was also investigated for enzymatic activity.

The yeast cells were harvested via centrifugation from the respective samples and 1 g of yeast cell wet weight was seen as equivalent to 1 vol. The yeast cells were suspended in 2-4 vol ice-cold distilled water and centrifuged for 5 min at 1500 *g* (4°C). The pellet was then resuspended in 3 vol zymolyase buffer, prepared by combining 50 mM Tris.Cl (pH 7.5), 10 mM $MgCl_2$, 1 M sorbitol and 1X protease inhibitor. Zymolyase enzyme was thereafter added to the solution (200 U/mL of original packed cell volume) and the reaction incubated at 30°C for 40 min. Conversion of the yeast cells into spheroplasts was determined by performing the lysis in water technique. If incomplete, the reaction was further incubated at 30°C. The spheroplasts were centrifuged for 5 min at 1500 *g* and the supernatant provided the sample containing cell-wall associated proteins. The spheroplasts were then washed gently by resuspending the pellet in 2 vol ice-cold zymolyase buffer and centrifuged for 5 min at 1500 *g.* The wash step was repeated three times.

The spheroplasts were gently dislodged in 2 vol lysis buffer and swirled in lysis buffer 10 to 20 times. Lysis buffer contained 50 mM Tris.HCl (pH 7.5), 10 mM $MgSO_4$, 1 mM EDTA, 10 mM potassium acetate, 1 X protease inhibitor mix and 1 mM phenylmethylsulfonyl fluoride (PMSF). The method for lysing the spheroplasts was modified from the protocol described by Dunn & Wobbe (1992). Sterile microbeads (100 µL) were added to the solution containing the spheroplasts and very slowly vortexed for approximately 30 s. The solution was thereafter cooled down in ice for another 30 s and this vortex-cool cycle was repeated 3 times. The lysed spheroplasts were thereafter centrifuged for 10 min at 1500 *g*. The supernatant provided the intracellular protein extract.

3.2.5.2 Protein extraction from Muscat grape juice fermentations

Protein from the cell wall and cytoplasm (whole cell) were extracted using Y-PER™ yeast protein extraction reagent (Thermoscientific). Yeast cells from the samples taken during the fermentations were collected by centrifugation (3000 *g*, 5 min at 4°C) and the cells resuspended in Y-PER reagent (volume dependent on wet cell pellet weight as determined by Thermosienctific instructions) with the addition of 1 x protease inhibitor to prevent protein degradation. The solution was slowly agitated at room temperature for 20 min, and the cell debris collected by centrifugation at 14,000 *g* for 10 min. The lysate was then utilized for protein quantification and β-glucosidase activity analysis, as described below.

3.2.5.3 Protein quantification

The protein concentration was determined using the Bradford reagent (Sigma-aldrich) with bovine serum albumin (BSA) as the standard. Bradford reagent (1 mL) was added to 20 µL of the extracted protein, and the solution was left at room temperature for 5 min after which the absorbance at 595 nm was measured against the reagent blank (water added instead of protein sample). The BSA standard solution (1 mg/mL) was serially diluted (0.2 - 0.9 mg/mL) in order to create a standard line to which the sample could be compared; this standard was created each time protein concentrations were measured due to its reliance on room temperature.

3.2.5.4 β-glucosidase quantification

Stock solution (10 mM) of p-nitrophenol was dissolved in 0.05 M (pH 5.3) citrate phosphate buffer (0.139 g/100 mL buffer) and the stock solution serially diluted (1:20 - 1:200) to create a standard curve for rate of p-nitrophenol production and corresponding absorbance readings. A substrate solution was created by preparing 1 mM 4-nitrophenyl-β-glucopyranoside in citrate phosphate buffer. For each analysis, 900 µL of the substrate solution was incubated at 50°C (10 min) after which 500 µL of 1 M Na_2CO_3 was added followed by 100 µL of each standard solution (respective dilutions created). The absorbance was measured at 400 nm for each solution, in triplicate, against a reagent blank which is prepared as described above, however in place of p-nitrophenol (standard solution), 100 µL of buffer is added to the preparation.

In order to determine β-glucosidase activity in the extracted protein samples, the protocol above was followed, however following the substrate solution incubation, 100 µL of the sample was added and further incubated at 50°C (10 min), and the reaction stopped by Na_2CO_3. The absorbance was thereafter measured (400 nm) against the reagent blank. This was performed in triplicate. Making use of the equation from the prepared standard curve

and the absorbance values, the β-glucosidase enzyme activity (nkat/mL) was determined (adapted from Mateo & Di Stefano, 1997).

3.2.6 Chemical analysis of resulting wines

3.2.6.1 Gas chromatography-flame ionization detector (GC-FID) analysis

GC-FID was performed on the resulting wines (with 2x technical and 3x biological repeats) in order to analyze major esters, higher alcohols, and volatile acids within the resulting wine. The wine with internal standard, 4-Methyl-2-Pentanol, (100 µl of 0.5 mg/ml solution in soaking solution) was extracted with diethyl ether. The injection volume was 3 µl and a DB-FFAP, 60 m x 0.32 mm x 0.5 µm f.t. column was utilized (Louw *et al.,* 2009).

3.2.6.2 High performance liquid chromatography (HPLC) analysis

HPLC was performed in order to determine the concentrations of the major sugars (glucose and fructose), organic acids (citric, tartaric, malic, succinic and acetic acid) in addition to glycerol and ethanol in the resulting wines (2x technical and 3x biological repeats). An argilent 1100 series HPLC system, Chemstation Rev. A10.02 software, an Aminex HPX-87 column (300 mm x 8.8 mm) and a Bio-Rad guard column (30mm x 4.6mm) was used (Eyéghé-Bickong *et al.,* 2012).

3.2.6.3 Head-space Gas Chromatography mass spectrometry (GC/MS) analysis

For the evaluation of monoterpenes in the Muscat juice fermented wines (2x technical and 3x biological repeats), 10 mL of the wine samples, 2.5 mL 20% NaCl and 100 µL internal standard (3 octanol and Anisol-D8 1ppm in MeOH) was added to 20 mL screw cap GC vials, in triplicate, and vortexed. Solid phase micro-extraction (SPME) of the head-space was performed using a 50/30 µm grey divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, PA) which had previously undergone conditioning for 60 min at 270°C in the GC injection port. The vials were incubated at 50°C for 5 min in the autosampler heating chamber; which was rotated at 250 rpm to allow compounds in the sample and headspace equilibrium. While these conditions were maintained, the fibre was inserted through the septa and exposed to the headspace analytes for 20 min. In the injection port, the analytes were desorbed and the fibre maintained for 1 min to prevent carryover.

Analysis was performed utilizing a Trace 1300 gas chromatograph (Thermo Scientific, Germany) system coupled to a Triplus RSH auto-sampler and a TSQ 8000 MSD mass spectrometer detector through a transfer line. A Zebron 7HG-G009-11 ZB-FFAP capillary column (30 m x 250 ID µm, 0.25 µm film thickness) (Phenomenex, USA) was also

utilized. Analyte desorption took place at 250°C for 5 min with a 50:1 split and helium as the carrier gas (initial flow rate 1 mL/min). The oven temperature was initially 50°C (3 min), which was then ramped at a rate of 10°C/min to 240°C and held for 2 min, with the total run of 24 min and the transfer line temperature of 250°C.

3.2.7 Statistical analysis

The standard deviations of the biological and technical repeats for chemical and enzymatic analysis were calculated to analyze the variability of the experiments and technical processes respectively. The data obtained was tested for normality and the statistical significance calculated through one-way analysis of variance (ANOVA). Tukey's test was utilized to calculate the significant differences between group means (for GC-FID, GCMS, HPLC and enzymatic quantitative data), with a significance level of 5%. Principal component analysis was utilized to discriminate between the means of various major volatiles present in wine and represented in correlation biplot graphs (coefficient = n/p).

3.3 Results

3.3.1 Screening for extracellular hydrolytic enzyme activity and H_2S production

Strains of *L. thermotolerans*, *L. lanzarotensis*, *L. fermentati* as well as a commercial strain of *S. cerevisiae* (used as the control for fermentations) were screened for various enzyme activities including β-glucosidase, β-xylosidase, pectinase and protease activity. Overall, none of the strains exhibited pectinase or protease activity, while within the *Lachancea* genus, β-xylosidase activity was variable between species and strains. For instance, only *L. fermentati* and *L. thermotolerans* exhibited β-xylosidase activity, however, two strains of *L. thermotolerans* did not have activity (**Table 3.6**). In contrast, β-glucosidase on arbutin was detected in all species and strains, but *L. lanzarotensis* did not display activity on 4-MUG. H_2S production was detected for all yeast strains, with *L. lanzarotensis* isolates producing notably higher levels than the other species tested (**Table 3.6**).

Table 3.6 Extracellular enzyme and H_2S production screening on substrate specific agarose plates for various yeast strains. The strains chosen for further analysis are indicated with an asterix

Yeast species	Strain/Isolate	β-glucosidase		β-D-xylosidase	H_2S production
		Arbutin	4-MUG	4-MUX	Bismuth
L. thermotolerans	Y1202	+	+	+	++
	Y1109	+	+	+	++
	Y1017	+	+	+	++
	Y1240	+	+	+	+
	Y1326	+	+	+	++
	Y1206	+	+	+	++
	Y1197	+	+	+	+
	Y1038	+	+	+	++
	Y1295	+	+	+	++
	Y513	+	+	-	++
	Y905	+	+	+	++
	Y940*	+	+	+	+
	Y1220	+	+	+	+
	Concerto*	+	+	-	+
L. lanzarotensis	CBS 12615*	+	-	-	+++
	IWBT Y992-1	+	-	-	++++
	IWBT Y992-6	+	-	-	++++
	IWBT Y992-4	+	-	-	++++
	IWBT Y992-5*	+	-	-	++++
	IWBT Y992-2	+	-	-	++++
	IWBT Y992-3	+	-	-	++++
L. fermentati	Y515*	+	+	+	+++
S. cerevisiae	VIN13*	+	+	+	+

+: Enzyme activity observed; -: No enzyme activity observed; H_2S production: Low production (+) to high production (++++) 4-MUG: 4-methylumbelliferyl-β-D-glucoside; 4-MUX: 4-methylumbelliferyl-β-D-xyloside. Note: No polygalacturonase and pectinase activity observed (not shown)

3.3.2 Ethanol and SO_2 tolerance

The relative ethanol tolerance levels of the yeast strains were analysed and the results are displayed in **Table 3.7**. All but one of the *L. thermotolerans* strains, Y1240, could survive at 10% (v/v) ethanol. *L. fermentati* Y515 and the *L. lanzarotensis* isolates were able to tolerate 7% ethanol levels, with *L. lanzarotensis* illustrating increased sensitivity at this concentration. The yeast strains ability to grow (tolerance) when exposed to varying levels of SO_2 was analyzed. The majority of the *L. thermotolerans* strains could only tolerate 4.22 mg/L total SO_2 (i.e. 0.05 mg/L molecular SO_2), with the exception of Y940, which tolerated exposure to 8.32 mg/L total SO_2 (i.e. 0.1 mg/L molecular SO_2). The same level of tolerance was observed for the various *L. lanzarotensis* isolates. In contrast, *L. fermentati* Y515 was able to proliferate when exposed to 10, 15, and 20 mg/L total SO_2; corresponding to 0.12, 0.18 and 0.24 mg/L molecular SO_2, respectively. Exposure to increasing SO_2 levels resulted in a

longer lag phase in *L. fermentati* (**Figure 3.1A**). In contrast, *S. cerevisiae*, could survive exposure to the varying levels of SO_2 and less of an impact was observed in the lag phase of its growth, with the exception of exposure to 25 mg/L, where the yeast only managed to grow after 48 hours of incubation (**Figure 3.1B**).

Table 3.7 Respective ethanol tolerance levels for various yeast strains. The strains chosen for further analysis are indicated with an asterix

		Ethanol tolerance		
Yeast species	**Strain/Isolate**	**5%**	**7%**	**10%**
L. thermotolerans	Y1202	++++	+++++	++
	Y1109	++++	+++	++
	Y1017	+++	++++	++
	Y1240	+++++	+++	-
	Y1326	++++	++++	+
	Y1206	++++	+++++	+
	Y1197	+++++	++++	+
	Y1038	++++	+++++	+
	Y1295	++++	++++	++
	Y513	++++	++++	+++
	Y905	++++	++++	+++
	Y940*	+++++	++++	+++
	Y1220	++++	+++	+++
	Concerto*	++++	+++	+
L. lanzarotensis	CBS 12615*	+++++	Slow/delayed	-
	IWBT Y992-1	+++++	Slow/delayed	-
	IWBT Y992-6	+++++	Slow/delayed	-
	IWBT Y992-4	++++	Slow/delayed	-
	IWBT Y992-5*	+++	Slow/delayed	-
	IWBT Y992-2	+++	Slow/delayed	-
	IWBT Y992-3	+++	Slow/delayed	-
L. fermentati	Y515*	+++++	++++	-
S. cerevisiae	VIN13*	+++++	+++++	+++

Ethanol tolerance: Growth at 10^{0} to 10^{-5} dilution; + to +++++. Slow and delayed growth on ethanol plates; small growth observed at low dilutions

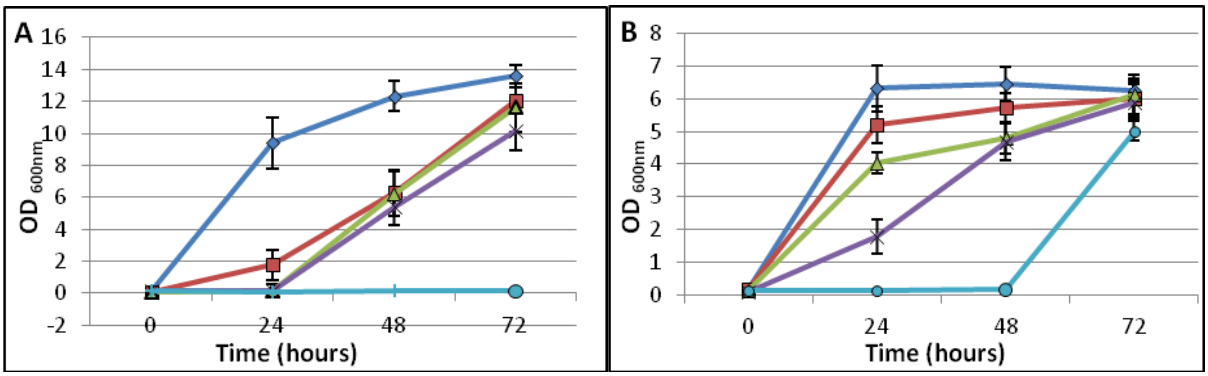


Figure 3.1 Growth kinetics of *L. fermentati* Y515 (A) and *S. cerevisiae* VIN13 (B) in YNB and glucose media at 25 °C, supplemented with 0 mg/L (-◊-); 10 mg/L (-□-); 15 mg/L (-△-); 20 mg/L (-X-) and 25 mg/L (-○-) total SO_2

3.3.3 Synthetic grape juice fermentations

Selected strains (those highlighted with an asterisk in **Tables 3.6** and **3.7**) were further characterized regarding their fermentation abilities. Amongst the *L. thermotolerans* strains, Y940 was selected based on enzymatic activity, as well as tolerance to ethanol and SO_2, while Concerto was selected as a commercial strain already frequently used in winemaking. For *L. lanzarotensis*, CBS 12615 (type strain of this species), together with Y992-5 (picked among the isolates that displayed similar characteristics and were therefore hypothesized to represent a single strain), were selected to represent this species. Finally, *L. fermentati*, Y515 was the only strain available for this species.

In monoculture fermentations, *Lachancea* spp. strains displayed considerably lower fermentation rates than *S. cerevisiae* and the fermentations became sluggish toward the middle stages of the fermentations (**Figure 3.2 A**). *L. thermotolerans* Concerto was the best fermenter among the strains of the *Lachancea* genus although it released 26.91% less CO_2 than *S. cerevisiae*, while *L. lanzarotensis* showed the lowest fermentation capacity with CBS 12615 and Y992 releasing 45.53% and 55% less CO_2 than *S. cerevisiae*, respectively. In contrast, the fermentation capacity of *L. thermotolerans* Y940 and *L. fermentati* Y515 was intermediary and very similar, resulting in 36.49% and 38.69% less CO_2 released than *S. cerevisiae*, respectively. The slower fermentation rates were reflected in the residual sugar levels, where *L. thermotolerans* Concerto and the *L. lanzarotensis* strains possessed the lowest and highest residual sugar respectively and the corresponding highest and lowest ethanol yield (**Table 3.8**). Growth analysis revealed that *S. cerevisiae* grew faster and achieved the highest cell concentrations while *L. thermotolerans* Concerto displayed rapid growth in the first 2 days but ultimately maintained similar cell concentrations as the other strains that only grew up to OD of 6 (**Figure 3.3 A**). Unfortunately, due to heavy flocculation by *L. fermentati* Y515, the growth of this yeast could not be monitored.

The co-culture fermentations (**Figure 3.2 B**) exhibited similar kinetics, where generally slower fermentation rates were observed in comparison to the *S. cerevisiae* monoculture fermentation. With minimal residual sugar, the co-culture fermentations produced ethanol levels comparable to the *S. cerevisiae* monoculture (**Table 3.8**). Growth in the co-culture fermentations showed that *S. cerevisiae* behaves very similar whether it was inoculated alone or with any of the *Lachancea* yeast species, for this reason the growth kinetics of *S. cerevisiae* during co-culture fermentations have been averaged (see **Figure 3.3 B**). *L. thermotolerans* Concerto was able to survive with *S. cerevisiae* until the later stages of the fermentation, while the rest of the *Lachancea* spp. strains declined following the inoculation of *S. cerevisiae* and were no longer viable by mid-fermentation.

3.3.4 Muscat grape juice fermentations

During the monoculture fermentations in Muscat grape must, there was increased similarity between the fermentation rates of some of the yeast strains (**Figure 3.2 C**), in comparison to fermentations in the synthetic juice. *L. fermentati* displayed better fermentation tempo in Muscat, resulting in cumulative CO_2 release comparable to *S. cerevisiae*. *L. thermotolerans* Concerto performed the second best of the *Lachancea* spp., followed by the *L. lanzarotensis* strains as well as *L. thermotolerans* Y940, which showed similar CO_2 release trends to synthetic grape juice (around 40% less than *S. cerevisiae*). The *Lachancea* spp. growth began to decline mid-fermentation, like in the synthetic fermentations, however *L. thermotolerans* Concerto was seen to be able to survive till the end of the fermentation (**Figure 3.3 C**). All the *Lachancea* spp. utilized a higher proportion of the available sugar, however only *L. fermentati* was able to complete the fermentation, possessing minimal residual sugar at the end of the fermentation (**Table 3.8**). During co-culture fermentations, comparable trends occurred regarding the fermentation tempos (**Figure 3.2 D**), with the exception of *L. thermotolerans* Concerto which increased and *L. lanzarotensis* CBS 12615, which decreased (becoming more similar to *L. lanzarotensis* Y992).

During the co-culture fermentations, an increase in the rate of decline can be noticed for the *L. lanzarotensis* strains and *L. thermotolerans* Y940 (**Figure 3.3 D**), in comparison to the growth kinetics observed in synthetic fermentations. In contrast, *L. thermotolerans* Concerto performed better in this grape matrix, surviving till the end of the fermentation. Unfortunately, as stated above, due to flocculation the *L. fermentati* growth could not be monitored.

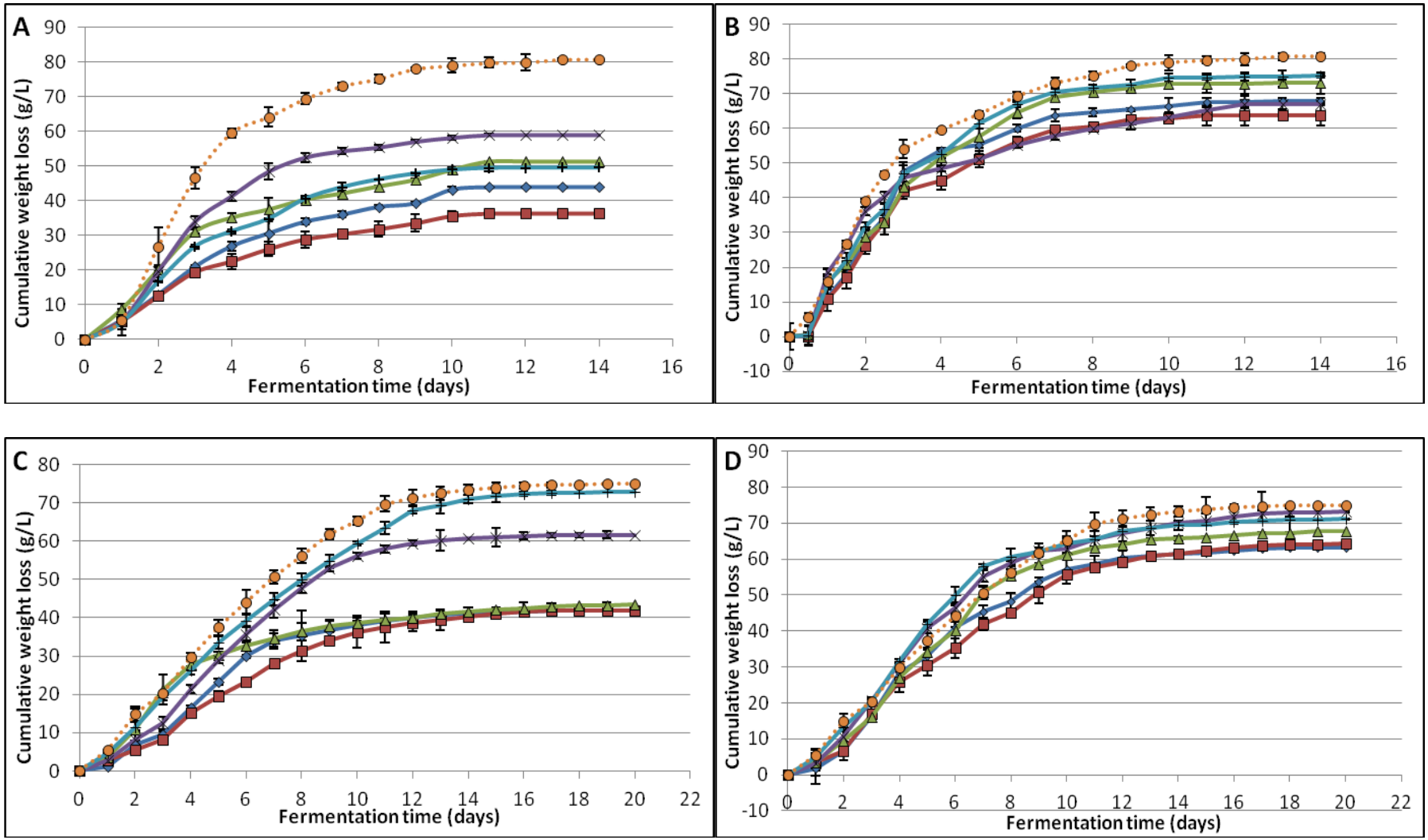


Figure 3.2 Cumulative weight loss of synthetic grape juice monoculture (A) and 48 h sequential (B) fermentations as well as Muscat grape juice monoculture (C) and 48 h sequential co-culture (D) fermentations. The fermentations were incubated at 25 °C at 60 rpm and inoculated with *L. lanzarotensis* CBS 12615 (-◊-); *L. lanzarotensis* Y992 (-□-); *L. thermotolerans* Y940 (-△-); *L. thermotolerans* Concerto™ (-X-); *L. fermentati* Y515 (-+-) and *S. cerevisiae* VIN13 (-○-) respectively

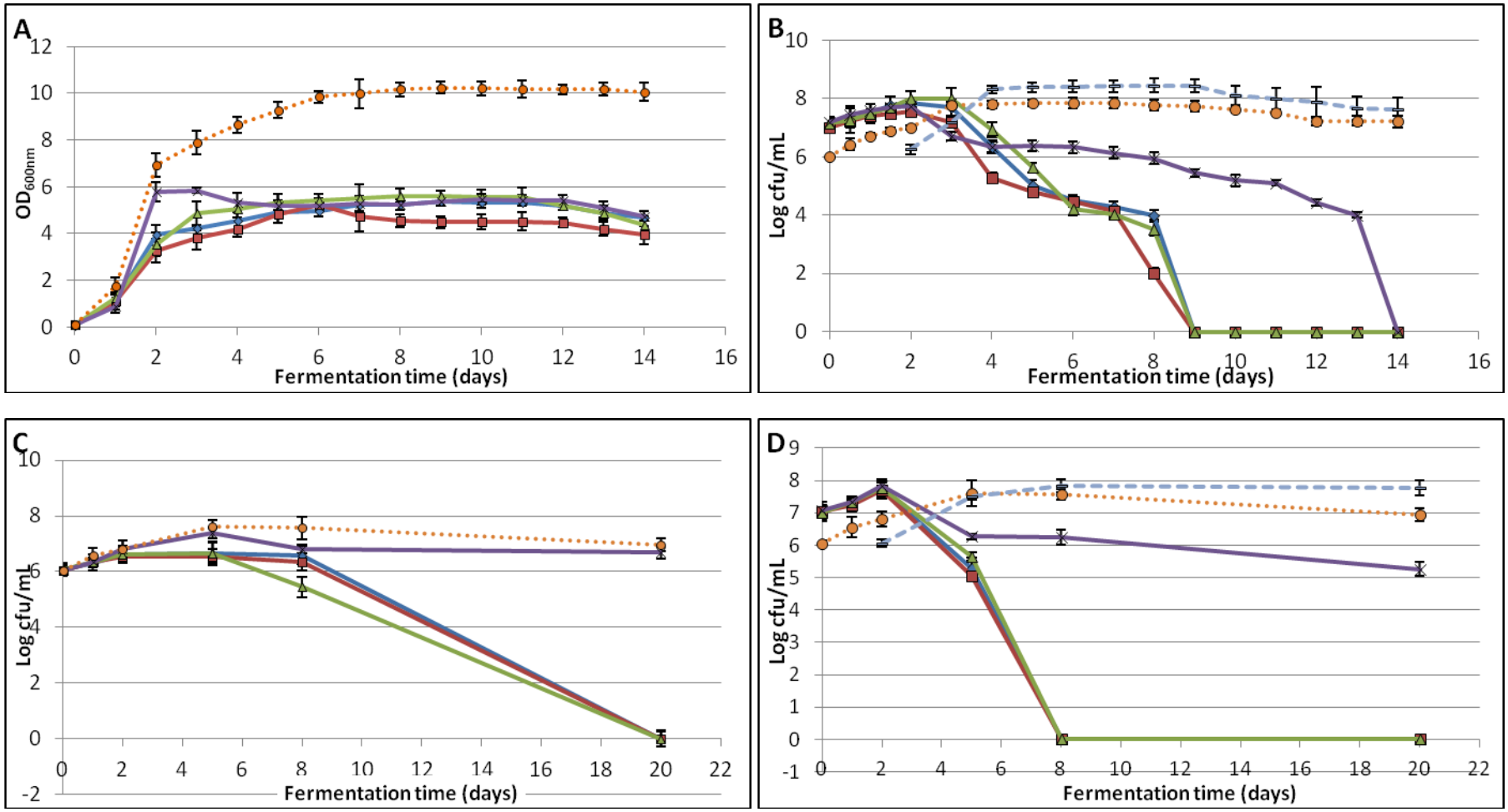


Figure 3.3 Yeast growth kinetics throughout synthetic grape juice monoculture (A) and 48h sequential (B) fermentations as well as Muscat grape must monoculture (C) and 48 h sequential (D) co-culture fermentations. The fermentations were incubated at 25 °C at 60 rpm and inoculated with *L. lanzarotensis* CBS 12615 (-◊-); *L. lanzarotensis* Y992 (-□-); *L. thermotolerans* Y940 (-△-); *L. thermotolerans* Concerto™ (-X-); and *S. cerevisiae* VIN13 (-○-) respectively. For the co-culture fermentations, *S. cerevisiae* VIN13 growth was averaged (---) and *S. cerevisiae* monoculture fermentation (-○-) acted as control. The growth kinetics were determined as absorbance (OD_{600nm}) for the synthetic monoculture fermentations and as log cfu/mL for the monoculture Muscat (due to the reduced volume) and co-culture fermentations (to monitor yeast growth by morphological difference)

Table 3.8 Residual sugar and ethanol concentrations following monoculture and co-culture Synthetic and Muscat grape juice fermentations by *Lachancea* spp. and *S. cerevisiae*

Compound	Sc VIN13	Lt Y940		Lt Concerto		Ll CBS		Ll Y992		Lf Y515	
	Synthetic grape juice fermentations										
	Mono	Mono	Co	Mono	Co	Mono	Co	Mono	Co	Mono	Co
Glucose (g/L)	1.09 ± 0.08	44.86 ± 0.74	-	9.78 ± 1.59	-	44.39 ± 1	-	42.24 ± 0.79	-	22.74 ± 1	-
Fructose (g/L)	2.40 ± 0.38	56.72 ± 0.82	-	37.18 ± 2.47	-	66.781 ± 0.652	-	63.34 ± 0.8	-	49.76 ± 1.47	0.25 ± 0.06
Ethanol (% v/v)	12.28 ± 0.31a[a]	8.93 ± 0.76bc	11.34 ± 0.77[a]	10.59 ± 1.41b	11.88 ± 0.90[a]	7.56 ± 1.83c	11.61 ± 0.34[a]	8.06 ± 0.74c	11.46 ± 1.11[a]	9.77 ± 1.97b	11.46 ± 0.69[a]
	Muscat grape must fermentations										
Glucose (g/L)	-	11.57 ± 1.55	-	6.65 ± 0.58	-	22.04 ± 1.20	-	21.98 ± 1.85	0.58 ± 0.67	0.25 ± 0.39	-
Fructose (g/L)	-	27.46 ± 4.08	-	21.47 ± 1.03	1.91 ± 1.18	49.86 ± 3.51	0.62 ± 0.11	48.09 ± 2.16	1.01 ± 1.02	3.63 ± 0.96	-
Ethanol (% v/v)	11.52 ± 0.77a[a]	10.13 ± 0.59b	11.33 ± 0.38[a]	10.05 ± 1.65ab	11.47 ± 0.09[a]	8.24 ± 1.57c	11.05 ± 0.60[a]	8.39 ± 1.70c	11.85 ± 0.64[a]	10.46 ± 1.36a	11.69 ± 0.59[a]

Data represented are that of monoculture (Mono) and 48h sequential co-culture (Co) fermentations by Sc VIN13: *S. cerevisiae* VIN13, Lt Y940: *L. thermotolerans* Y940, Lt Concerto: *L. thermotolerans* Concerto, Ll CBS: *L. lanzarotensis* CBS 12615, Ll Y992: *L. lanzarotensis* Y992 and Lf Y515: *L. fermentati* Y515. Statistically significant differences in the ethanol concentrations, according to Tukey's test ($p<0.05$), are presented. Differences between the monoculture fermentations illustrated by letters and the co-culture and *S. cerevisiae* monoculture control by superscript letters.

3.3.5 β-glucosidase activity during synthetic grape juice fermentations

The β-glucosidase activity in the different strains was determined in monoculture and co-cultures at the beginning, middle and end stages of fermentation. The β-glucosidase activity was determined in the extracellular, cell wall and intracellular fractions of the yeast cells (**Figure 3.4**). No activity was detected in the extracellular region (not shown), and an overall higher level of activity was found to be cell wall associated in comparison to the intracellular region.

The β-glucosidase activity during the monoculture fermentations (**Figure 3.4 A** and **B**) showed similar trends where an increase was observed during the beginning stages of the fermentations after which a general decrease occurred. Comparing the highest levels of cell wall associated activity during these fermentations (beginning stages of the fermentation), showed *L. thermotolerans* Y940 to produce the highest levels of activity (**Table 3.9**), followed by *L. thermotolerans* Concerto, *S. cerevisiae* and *L. fermentati*, which behaved similarly. The *L. lanzarotensis* strains produced the lowest levels of activity. Intracellular enzyme activity was generally lower; *L. thermotolerans* and *L. fermentati* strains

produced similar levels, while *L. lanzarotensis* strains and *S. cerevisiae* produced the lowest activity. Sequential co-culture fermentations of synthetic medium (**Figure 3.4 C** and **D**), showed a limited initial increase in the activity prior to the addition of *S. cerevisiae*, followed by a considerable increase in activity, which corresponded with the increase in the population of *S. cerevisiae* and the decline in *Lachancea* growth. An overall increase in enzyme activity was determined in the co-culture in comparison to the monoculture fermentations (**Table 3.9**). The cell wall associated activity was the highest in the *L. lanzarotensis* CBS 12615 and *L. thermotolerans* Concerto co-culture fermentations, followed by *L. thermotolerans* Y940 and *L. fermentati* Y515. The Lowest activity was determined in the *L. lanzarotensis* Y992 fermentation. Overall, cell wall associated activity was higher in the co-culture fermentations in comparison to fermentations completed by *S. cerevisiae* alone. The same can be noted for the intracellular β-glucosidase activity.

3.3.6 β-Glucosidase activity during Muscat grape juice fermentations

The β-glucosidase activity observed in synthetic grape juice fermentations was validated in Muscat grape must. However, only the total activity derived from whole cell extracts was determined in this instance since it was clear that the activity was cell associated and not secreted into the extracellular environment. Similar trends were observed during the Muscat fermentations, where a peak in enzyme activity occurred at the beginning and middle stages of the monoculture and co-culture fermentations, respectively, and the co-culture fermentations resulted in a higher level of activity (**Figure 3.5**). Comparing the highest levels of activity (**Table 3.10**) also revealed similar trends to the synthetic medium fermentations; monoculture fermentations showed *L. thermotolerans* Y940 to express the highest levels of activity, followed by *L. thermotolerans* Concerto, *L. fermentati* Y515 and *S. cerevisiae*, which behaved similarly. The *L. lanzarotensis* strains expressed the lowest levels of activity.

During the co-culture fermentations, similar trends were again observed (**Table 3.10**), where the *L. thermotolerans* and *L. fermentati* fermentations exhibited high enzyme activity, the highest of which was *L. thermotolerans* Y940, and low activity for *L. lanzarotensis* Y992. The exception was seen for *L. lanzarotensis* CBS 12615 fermentation, which observed low enzymatic activity, similar to that of *L. lanzarotensis* Y992. Overall, enzymatic activity was again higher in the co-culture fermentations in comparison to *S. cerevisiae*, with *L. thermotolerans* Y940 illustrating the highest activity in both monoculture and co-culture fermentations (**Table 3.10**).

Table 3.9 Highest cell wall-associated and intracellular β-glucosidase activity (beginning stages of fermentation) throughout monoculture and co-culture synthetic juice fermentations

Yeast strain	Inoculation strategy	β-glucosidase activity (nkat/mg protein)	
		Cell wall-associated	Intracellular
LI CBS 12615	Monoculture	0.35 ± 0.04c	0.29 ± 0.04b
	Co-culture	3.31 ± 0.02[a]	0.58 ± 0.02[c]
LI Y992	Monoculture	0.48 ± 0.03c	0.24 ± 0.04b
	Co-culture	1.44 ± 0.02[c]	0.55 ± 0.01[c]
Lt Y940	Monoculture	1.40 ± 0.17a	0.68 ± 0.15a
	Co-culture	2.57 ± 0.07[b]	0.79 ± 0.03[b]
Lt Concerto	Monoculture	0.84 ± 0.13b	0.54 ± 0.07a
	Co-culture	3.15 ± 0.05[a]	0.57 ± 0.03[c]
Lf Y515	Monoculture	0.61 ± 0.04b	0.50 ± 0.06a
	Co-culture	2.66 ± 0.05[b]	0.90 ± 0.01[a]
Sc VIN13	Monoculture (control)	0.75 ± 0.07b[d]	0.18 ± 0.02b[d]

Strains inoculated; LI: *L. lanzarotensis* CBS 12615 and Y992; Lt: *L. thermotolerans* Y940 and Concerto™; Lf: *L. fermentati* Y515 and Sc: *S. cerevisiae* VIN13. 48h sequential co-culture fermentations inoculated with *Lachancea* strain and *S. cerevisiae* VIN13. Statistically significant differences, according to Tukey's test ($p<0.05$), between monoculture fermentations illustrated by letters and co-culture fermentations (incl. control) illustrated by superscript letters.

Table 3.10 Highest β-glucosidase activity (beginning stages of fermentation) throughout monoculture and co-culture Muscat fermentations

Strain	Inoculation strategy	β-glucosidase activity (nkat/mg protein)
LI CBS 12615	Monoculture	0.82 ± 0.10c
	Co-culture	1.59 ± 0.09[c]
LI Y992	Monoculture	0.73 ± 0.03c
	Co-culture	1.34 ± 0.03[c]
Lt Y940	Monoculture	1.90 ± 0.24a
	Co-culture	4.57 ± 0.04[a]
Lt Concerto	Monoculture	1.34 ± 0.19b
	Co-culture	2.32 ± 0.19[b]
Lf Y515	Monoculture	1.14 ± 0.05b
	Co-culture	2.17 ± 0.04[b]
Sc VIN13	Monoculture (control)	1.09 ± 0.07b[d]

Strains inoculated; LI: *L. lanzarotensis* CBS 12615 and Y992; Lt: *L. thermotolerans* Y940 and Concerto™; Lf: *L. fermentati* Y515 and Sc: *S. cerevisiae* VIN13. 48h sequential co-culture fermentations inoculated with *Lachancea* strain and *S. cerevisiae* VIN13. Statistically significant differences, according to Tukey's test ($p<0.05$), between monoculture fermentations illustrated by letters and co-culture fermentations (incl. control) illustrated by superscript letters.

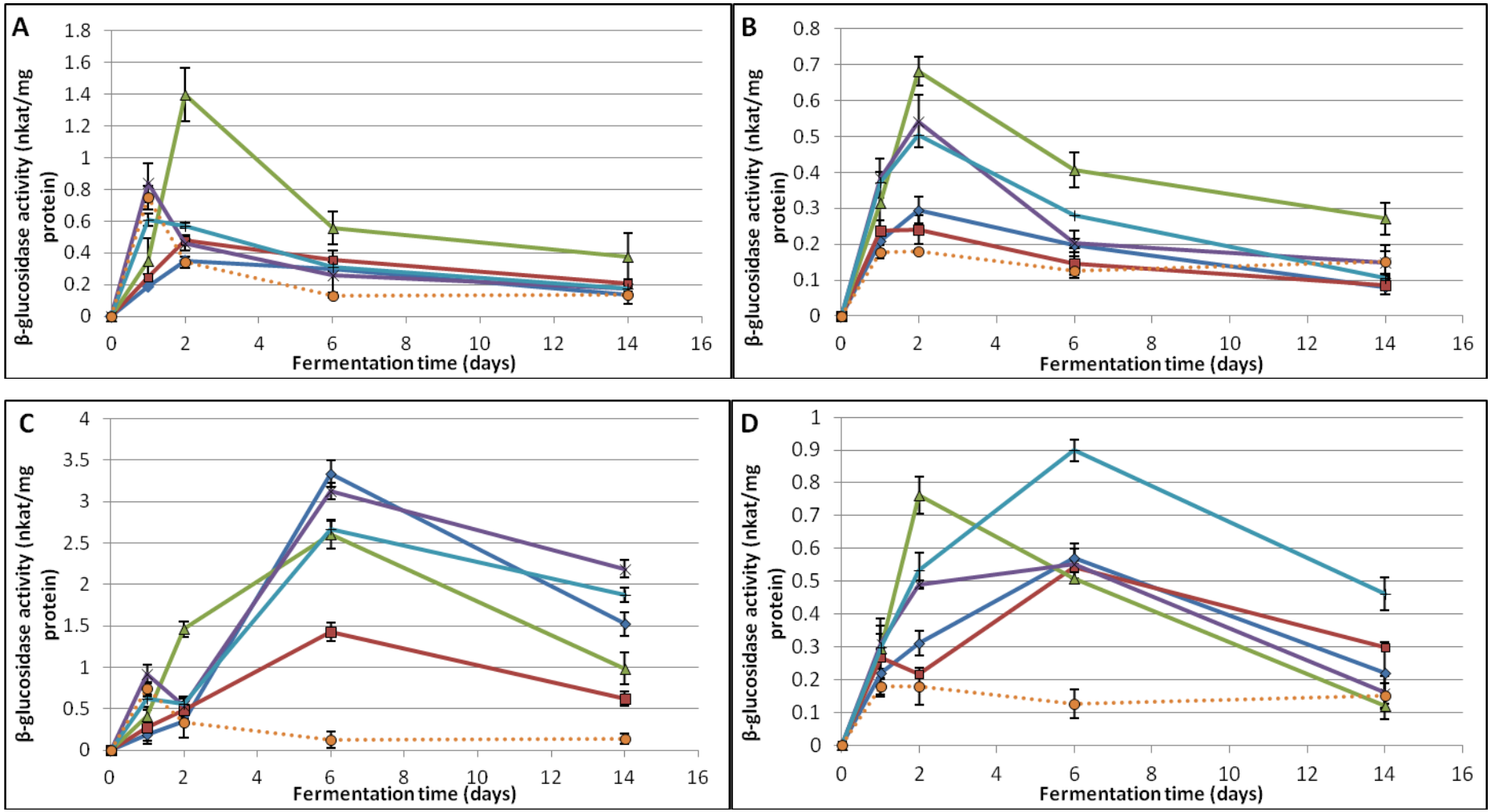


Figure 3.4 β-glucosidase activity during synthetic monoculture fermentations; in the cell wall (A) and intracellular (B) regions of the yeast cells as well as the activity in the sequential fermentations in the cell wall (C) and intracellular (D) regions. The fermentations were incubated at 25 °C at 60 rpm and inoculated with *L. lanzarotensis* CBS 12615 (-◊-); *L. lanzarotensis* Y992 (-□-); *L. thermotolerans* Y940 (-△-); *L. thermotolerans* Concerto™ (-X-); *L. fermentati* Y515 (-+-) and (control) *S. cerevisiae* VIN13 (-○-) respectively

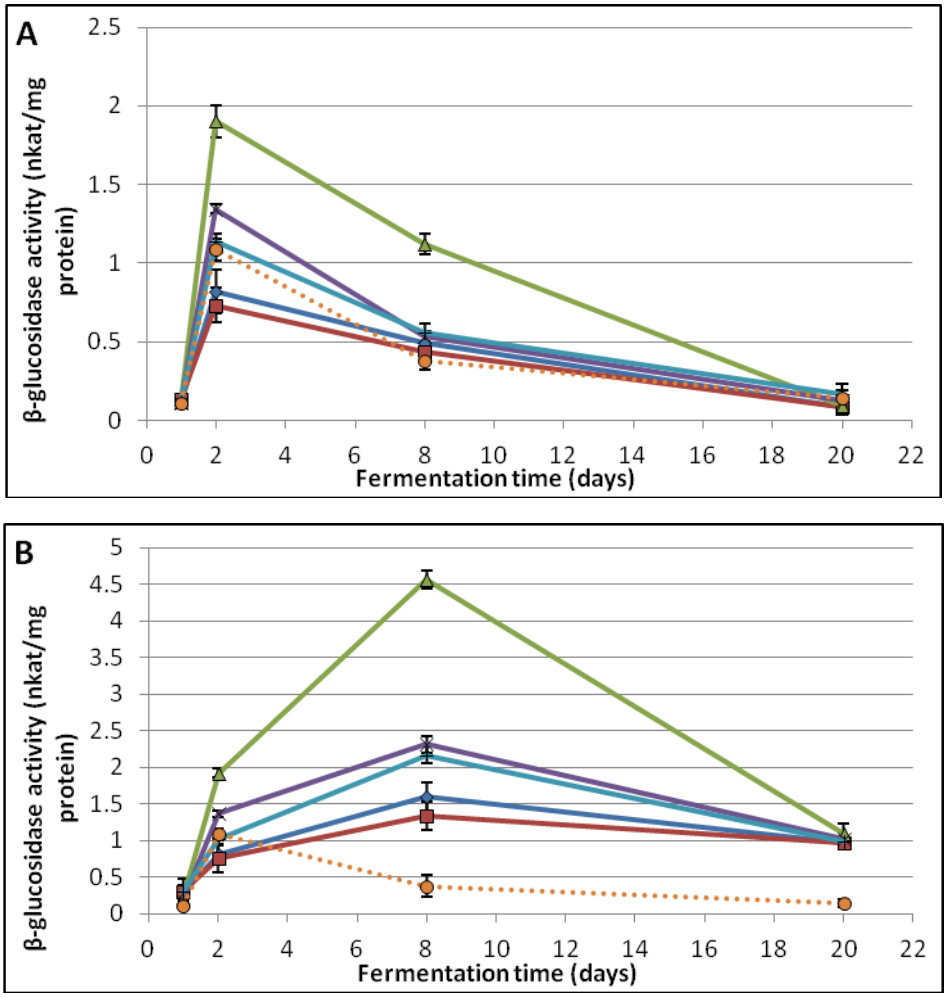


Figure 3.5 A progress curve of β-glucosidase production in monoculture (A) and 48 h sequential (B) fermentations of Muscat grape juice. The fermentations were incubated at 25 °C at 60 rpm and inoculated with *L. lanzarotensis* CBS 12615 (-◊-); *L. lanzarotensis* Y992 (-□-); *L. thermotolerans* Y940 (-△-); *L. thermotolerans* Concerto™ (-X-); *L. fermentati* Y515 (-+-) and (control) *S. cerevisiae* VIN13 (-○-) respectively

3.3.7 Chemical profiles of resulting wines

The resulting wines from the fermentations performed in both synthetic and Muscat grape juice were analyzed regarding their chemical composition utilizing GC-FID, HPLC and GC/MS. Only the results of the co-culture fermentations in synthetic and Muscat must as well as the *L. fermentati* Y515 monoculture in Muscat grape must are presented below since the other treatments did not ferment to dryness.

3.3.7.1 Organic acids and glycerol

In the wines produced from co-culture fermentation of the synthetic medium and the *S. cerevisiae* fermentation, the range of citric acid was 0.2 - 0.41 g/L, succinic acid 1.01 – 1.52 g/L and glycerol 8.08 – 9.87 g/L (**Table 3.11**). The reduction of malic acid was very similar for the majority of the co-culture fermentations and *S. cerevisiae*; reduced from 3 g/L to

between 0.82 and 0.89 g/L. In contrast, *L. thermotolerans* Concerto reduced the malic acid to 1.02 g/L. The *L. fermentati* Y515 co-culture fermentation produced the highest levels of acetic acid (1.06 ± 0.06 g/L), followed by the *S. cerevisiae* (0.63 ± 0.02 g/L), the *L. lanzarotensis* strains (0.42 – 0.51 g/L) and the lowest production by the *L. thermotolerans* strains (0.24 ± 0.02 g/L).

L. thermotolerans, L. fermentati co-culture fermentations and *S. cerevisiae* carried out in Muscat grape must resulted in similar citric acid (0.25 – 0.49 g/L) and glycerol (8.94 – 10.14 g/L) and lower succinic acid (0.56 – 0.7 g/L) levels in comparison to the synthetic fermentations. Interestingly, however, an increase in citric acid (0.78 – 0.83 g/L), succinic acid (0.83 – 0.87 g/L) and glycerol (11.13 – 11.65 g/L) was observed in the *L. lanzarotensis* fermented wines. As previously mentioned, the monoculture inoculation of *L. fermentati* Y515 was able to complete the fermentation; this resulted in lowered concentrations of citric acid (0.19 ± 0.04 g/L) and malic acid (1.66 ± 0.14 g/L) in comparison to *S. cerevisiae*. The excessive acetic acid production for *L. fermentati* Y515 fermentations were again reflected in the Muscat grape must, where monoculture and co-culture fermentations produced 0.93 ± 0.09 and 0.91 ± 0.04 g/L, respectively. This high production was followed by the *L. lanzarotensis* strains (0.75 – 0.86 g/L), *L. thermotolerans* Y940 (0.7 ± 0.02 g/L), *S. cerevisiae* (0.65 ± 0.02 g/L) and lastly *L. thermotolerans* Concerto (producing only 0.41 ± 0.01 g/L).

Table 3.11 Metabolites, above quantification/detection limits, present in wines following the completion of monoculture and co-culture synthetic grape juice-like and Muscat grape must fermentations by *S. cerevisiae* and *Lachancea* spp.

Synthetic grape juice-like fermentations							
Yeast species	**Sc VIN13 Mono**	**Lt Y940 Co**	**Lt Concerto Co**	**Ll CBS Co**	**Ll Y992 Co**	**Lf Y515 Co**	
Compound (g/L)							
Citric acid	0.41 ± 0.02^{a}	0.26 ± 0.01^{b}	0.36 ± 0.02^{a}	0.24 ± 0.03^{b}	0.25 ± 0.03^{b}	0.20 ± 0.01^{b}	
Malic acid	0.87 ± 0.08^{a}	0.83 ± 0.05^{a}	1.02 ± 0.09^{a}	0.83 ± 0.08^{a}	0.83 ± 0.08^{a}	0.89 ± 0.04^{a}	
Succinic acid	1.41 ± 0.16^{a}	1.27 ± 0.19^{ab}	1.52 ± 0.18^{a}	1.22 ± 0.21^{ab}	1.27 ± 0.12^{ab}	1.01 ± 0.13^{b}	
Acetic acid	0.63 ± 0.01^{b}	0.24 ± 0.02^{e}	0.24 ± 0.02^{e}	0.51 ± 0.02^{c}	0.42 ± 0.01^{d}	1.06 ± 0.06^{a}	
Glycerol	8.58 ± 1.26^{ab}	8.08 ± 0.32^{b}	9.36 ± 1.13^{ab}	9.87 ± 1.07^{ab}	8.92 ± 1.16^{ab}	9.43 ± 0.56^{a}	
Muscat grape must fermentations							
Compound (g/L)	**Sc VIN13 Mono**	**Lt Y940 Co**	**Lt Concerto Co**	**Ll CBS Co**	**Ll Y992 Co**	**Lf Y515 Mono**	**Lf Y515 Co**
Citric acid	0.40 ± 0.02^{b}	0.40 ± 0.04^{b}	0.49 ± 0.03^{b}	0.78 ± 0.06^{a}	0.83 ± 0.06^{a}	0.19 ± 0.04^{c}	0.25 ± 0.05^{c}
Malic acid	2.06 ± 0.11^{cd}	2.22 ± 0.12^{bc}	2.27 ± 0.17^{abc}	2.45 ± 0.17^{ab}	2.52 ± 0.11^{a}	1.66 ± 0.14^{b}	1.93 ± 0.07^{d}

Succinic acid	0.56 ± 0.13^{cd}	0.69 ± 0.08^{abc}	0.63 ± 0.09^{bcd}	0.83 ± 0.06^{a}	0.87 ± 0.07^{a}	0.43 ± 0.06^{d}	0.70 ± 0.05^{abc}
Acetic acid	0.65 ± 0.02^{c}	0.7 ± 0.01^{b}	0.41 ±0.01^{d}	0.86 ± 0.02^{ab}	0.75 ± 0.03^{b}	0.93 ± 0.09^{a}	0.91 ± 0.04^{a}
Glycerol	8.94 ± 0.48^{c}	9.27 ± 1.38^{bc}	9.45 ± 1.58^{bc}	11.13 ± 0.69^{ab}	11.65 ± 0.60^{a}	8.22 ± 0.77^{c}	10.14 ± 0.88^{abc}

Mono: monoculture; Co: co-culture fermentations inoculated with Sc VIN13: *S. cerevisiae* VIN13; Lt Y940: *L. thermotolerans* Y940; Lt Concerto: *L. thermotolerans* Concerto; Ll CBS: *L. lanzarotensis* CBS 12615; Ll Y992: *L. lanzarotensis* Y992; Lf Y515: *L. fermentati* Y515. Statistically significant differences for particular compounds are illustrated by letters, according to Tukey's test ($p<0.05$).

3.3.7.2 Higher alcohols

Analysis of various major volatiles in the resulting wines allowed investigation into the impact that the different inoculants had on the wine flavour profiles (**Table 3.12**). Regarding the total higher alcohol production, *S. cerevisiae* produced 274.53 ± 3.02 mg/L, while *L. thermotolerans* Concerto (313.65 ± 5.02 mg/L), *L. lanzarotensis* CBS 12615 (305.73 ± 8.11 mg/L) and *L. fermentati* Y515 (291.06 ± 10.11 mg/L) co-culture fermentations were all higher. In contrast to *S. cerevisiae*, the co-culture fermentations produced quantifiable levels of hexanol, however, all were under the odour threshold for this compound. Concentrations of butanol were also significantly higher but under the odour threshold, while *L. fermentati* Y515 was the only fermentation to produce levels of isobutanol and propanol higher than the respective thresholds.

An overall increase in higher alcohols was evident in wines produced from Muscat grape must, where both *L. thermotolerans* strains in co-culture fermentations as well as *L. fermentati* Y515 in monoculture and co-culture fermentations produced higher levels than *S. cerevisiae*. *L. thermotolerans* Y940 and *L. thermotolerans* Concerto produced 404.03 ± 12.36 mg/L and 409.49 ± 10.61 mg/L respectively; while *L. fermentati* Y515 monoculture and co-culture fermentations produced 531.64 ± 8.62 mg/L and 490.48 ± 7.66 mg/L respectively. Increase in the production of isobutanol, propanol and isoamyl alcohol was the major contributor to the heightened higher alcohol content in both the synthetic and Muscat wine. *S. cerevisiae* (357.36 ± 6.33 mg/L), *L. lanzarotensis* CBS 12615 (352.20 ± 10.66 mg/L) and Y992 (370.71 ± 9.53 mg/L) fermentations produced similar higher alcohol concentrations. In contrast to the fermentations completed in the synthetic juice, all the Muscat fermentations produced levels of isobutanol above the odour threshold and *L. fermentati* was unable to produce propanol above the threshold.

3.3.7.3 Esters

Similar levels of acetate and ethyl esters were detected in the synthetic wine for all species and strains (**Table 3.12**), with all compounds generally over their respective odour thresholds. However, an increase of ethyl acetate in *L. thermotolerans* Y940 (72.48 ± 3.70

mg/L) and *L. fermentati* Y515 (127.69 ± 1.22 mg/L) co-culture fermentations led to an increase in overall ester content in these wines, with the highest determined in *L. fermentati*. Similar and lower levels of esters were produced in the remaining fermentations, ranging from 28.51 to 33.19 mg/L. Ethyl phenylacetate was noticeably only produced in the co-culture fermentations.

For fermentations carried out in Muscat grape must, there was a general increase in ester production in comparison to those completed in synthetic juice (**Table 3.12**), including the production of ethyl phenylacetate by *S. cerevisiae.* Nevertheless, all fermentations carried out by *L. thermotolerans* and *L. lanzarotensis* strains produced higher levels of esters than *S. cerevisiae*. The highest levels were observed for the *L. lanzarotensis* CBS 12615 and Y992 co-culture fermentations, which produced 66.08 ± 2.79 and 63.99 ± 0.56 mg/L, respectively. *L. thermotolerans* Y940 (50.58 ± 1.9) and Concerto (57.98 ± 1.06) produced lower levels, followed by *S. cerevisiae* (39.46 ± 2.67). In contrast to the fermentations in synthetic juice, the *L. fermentati* co-culture fermentation was unable to produce quantifiable levels of ethyl acetate; this was also reflected in the *L. fermentati* monoculture fermentation. Due to this reduction in ester production, these fermentations produced the lowest levels of total esters (2.34 - 2.83 mg/L).

3.3.7.4 Volatile acids (excluding acetic acid)

The total volatile acids produced in the *L. fermentati* Y515 co-culture fermentation was the highest at 14.96 ± 2 mg/L, followed by the similar production by *L. thermotolerans* Concerto co-culture fermentation (9.20 ± 1.06 mg/L) and *S. cerevisiae* (11.10 ± 1.3 mg/L) (**Table 3.12**). The increase in volatile acidity by *L. fermentati* Y515 can be attributed to the increase in isobutyric acid (9.22 ± 0.57 mg/L), although still very much below its odour threshold. In contrast, volatile acids were lowest for the *L. thermotolerans* Y940, *L. lanzarotensis* CBS 12615 and *L. lanzarotensis* Y992; corresponding to 7.53 ± 1.06, 8.15 ± 0.16, 6.97 ± 0.43 mg/L respectively.

Fermentations completed in Muscat grape must resulted in the increased production of isobutyric, isovaleric and hexanoic acids in the co-culture fermentations (**Table 3.12**), resulting in the accumulated volatile acidity being higher in the co-culture fermentations in comparison to *S. cerevisiae*. The highest volatile acidity was determined in the *L. fermentati* monoculture fermentation (48.15 ± 1 mg/L), followed by the co-culture fermentation with this strain (22.88 ± 1.14 mg/L); attributed again to the increased production of isobutyric acid. Overall, strains of the same species possessed similar total volatile acids, *L. thermotolerans* Y940 and Concerto (16.02 ± 0.44 and 18.43 ± 0.12 mg/L, respectively) and *L. lanzarotensis*

CBS 12615 and Y992 (13.56 ± 1.22 and 13.84 ± 1.19 mg/L, respectively), all higher than that of *S. cerevisiae* (11.28 ± 1.11 mg/L).

Table 3.12 Major volatiles, above quantification/detection limits, present in wines following the completion of monoculture and co-culture synthetic grape juice-like and Muscat grape must fermentations by *S. cerevisiae* and *Lachancea* spp.

	Synthetic grape juice-like fermentations						Muscat must fermentations						
Yeast species	**Sc**	**Lt**		**Ll**		**Lf**	**Sc**	**Lt**		**Ll**		**Lf**	
	VIN13 Mono	**Y940 Co**	**Concerto Co**	**CBS 12615 Co**	**Y992 Co**	**Y515 Co**	**VIN13 Mono**	**Y940 Co**	**Concerto Co**	**CBS 12615 Co**	**Y992 Co**	**Y515 Mono**	**Y515 Co**
Compounds (mg/L)													
Esters													
Ethyl decanoate	1.06 ± 0.01^{a}	1.19 ± 0.05^{a}	0.93 ± 0.02^{a}	1.15 ± 0.01^{a}	1.06 ± 0.03^{a}	0.46 ± 0.02^{b}	0.21 ± 0.02^{d}	1.74 ± 0.98^{b}	2.33 ± 0.03^{a}	0.66 ± 0.02^{c}	1.18 ± 0.81^{b}	0.12 ± 0.02^{e}	0.23 ± 0.04^{d}
Ethyl phenylacetate	-	1.74 ± 0.18^{a}	1.41 ± 0.04^{b}	1.92 ± 0.32^{a}	1.40 ± 0.18^{b}	0.72 ± 0.06^{a}	1.06 ± 0.06^{c}	0.83 ± 0.02^{d}	3.78 ± 0.11^{a}	0.73 ± 0.02^{e}	0.67 ± 0.02^{e}	1.16 ± 0.17^{c}	1.62 ± 0.24^{b}
Ethyl acetate	26.49 ± 2.60^{a}	72.48 ± 3.70^{b}	30.60 ±1.59^{d}	26.99 ± 2.61^{e}	31.36 ± 1.01^{c}	127.69 ± 1.22^{a}	37.68 ± 1.24^{e}	46.86 ± 0.08^{d}	50.78 ± 0.38^{c}	63.53 ± 1.06^{a}	60.76 ± 0.52^{b}	-	-
Isoamyl acetate	0.45 ± 0.13^{b}	0.20 ± 0.08^{c}	0.24 ± 0.01^{bc}	0.23 ± 0.03^{c}	0.20 ± 0.01^{c}	1.00 ± 0.51^{a}	0.26 ± 0.02^{b}	0.64 ± 0.13^{a}	0.68 ± 0.08^{a}	0.62 ± 0.08^{a}	0.69 ± 0.16^{a}	0.63 ± 0.08^{a}	0.60 ± 0.13^{a}
2-Phenylethyl acetate	0.51 ± 0.01^{a}	0.47 ± 0.03^{b}	0.47 ± 0.01^{b}	0.51 ± 0.14^{ab}	0.58 ± 0.01^{ab}	0.69 ± 0.05^{a}	0.24 ± 0.02^{c}	0.51 ± 0.03^{ab}	0.41 ± 0.02^{bc}	0.54 ± 0.04^{ab}	0.68 ± 0.12^{a}	0.43 ± 0.01^{b}	0.38 ± 0.04^{bc}
Σ Esters	28.51 ± 3.19^{c}	74.30 ± 3.02^{b}	32.25 ± 5.72^{c}	28.87 ± 2.10^{c}	33.19 ± 1.01^{c}	129.84 ± 6.5^{a}	39.46 ± 2.67^{d}	50.58 ± 1.9^{c}	57.98 ± 1.06^{b}	66.08 ± 2.79^{a}	63.99 ± 0.56^{a}	2.34 ± 0.11^{f}	2.83 ± 0.05^{e}
Higher alcohols													
Isobutanol	23.66 ± 1.19^{d}	24.35 ± 0.29^{cd}	33.35 ± 0.4^{b}	32.36 ± 1.98^{b}	27.80 ± 2.34^{d}	54.64 ± 0.54^{a}	65.66 ± 0.68^{d}	74.97 ± 2.08^{c}	62.26 ± 0.48^{d}	57.66 ± 1.11^{f}	61.67 ± 2.76^{e}	183.41 ± 8.46^{a}	104.91 ± 1.52^{b}
Butanol	0.86 ± 0.09^{e}	5.81 ± 1.25^{a}	2.69 ± 0.28^{b}	1.46 ± 0.03^{c}	1.19 ± 0.03^{d}	0.52 ± 0.02^{f}	1.65 ± 0.01^{c}	2.31 ± 0.19^{a}	2.78 ± 0.03^{a}	1.74 ± 0.09^{c}	1.94 ± 0.09^{b}	0.59 ± 0.03^{e}	1.23 ± 0.06^{d}
Propanol	30.42 ± 1.79^{e}	33.81 ± 0.85^{d}	37.83 ± 1.46^{b}	34.64 ± 0.46^{c}	24.53 ± 4.75^{f}	53.79 ± 1.87^{a}	37.61 ± 3.79^{e}	48.28 ± 1.78^{a}	47.73 ± 1.65^{b}	37.05 ± 1.46^{e}	38.93 ± 2.59^{d}	33.73 ± 2.31^{f}	44.18 ± 1.71^{c}
Isoamyl alcohol	165.33 ± 2.31^{c}	135.93 ± 2.4^{e}	188.93 ± 1.13^{a}	187.62 ± 2^{a}	168.31 ± 2^{b}	159.42 ± 0.99^{d}	200.63 ± 2.02^{f}	226.20 ± 1.95^{c}	233.56 ± 6.56^{b}	203.70 ± 2.09^{e}	213.23 ± 2.06^{d}	283.66 ± 1.52^{a}	287.94 ± 1.16^{a}

Hexanol	-	0.31 ± 0.01^{a}	0.33 ± 0.01^{a}	0.32 ± 0.01^{a}	0.32 ± 0.01^{a}	0.32 ± 0.02^{a}	0.73 ± 0.04^{a}	0.58 ± 0.01^{a}	0.58 ± 0.01^{a}	0.55 ± 0.02^{a}	0.55 ± 0.01^{a}	0.54 ± 0.04^{a}	0.61 ± 0.05^{a}
2-Phenylethanol	54.25 ± 1.01^{a}	41.93 ± 0.42^{e}	50.53 ± 1.14^{b}	49.33 ± 0.58^{c}	43.41 ± 1.75^{d}	22.35 ± 0.6^{f}	51.08 ± 1.59^{c}	51.69 ± 1.05^{c}	62.59 ± 1.19^{a}	51.51 ± 0.92^{c}	54.39 ± 1.69^{b}	29.71 ± 1.12^{d}	51.60 ± 1.3^{c}
Σ Higher alcohols	274.53 ± 3.02^{c}	242.14 ± 4.23^{d}	313.65 ± 5.02^{a}	305.73 ± 8.11^{b}	265.57 ± 7.98^{c}	291.06± 10.11^{b}	357.36 ± 6.33^{d}	404.03 ± 12.36^{c}	409.49 ± 10.61^{c}	352.20 ± 10.66^{d}	370.71 ± 9.53^{d}	531.64 ± 8.62^{a}	490.48 ± 7.66^{b}
Volatile Acids													
Propionic acid	2.57 ± 0.04B^{c}	2.35 ± 0.17^{cd}	2.19 ± 0.06^{e}	3.15 ± 0.01^{a}	2.64 ± 0.04^{b}	2.22 ± 0.09^{de}	3.68 ± 1.21^{bc}	5.52 ± 0.31^{a}	5.38 ± 0.09^{a}	3.71 ± 1.45^{bc}	4.35 ± 1.73^{ab}	2.53 ± 0.81^{c}	2.80 ± 0.34^{c}
Isobutyric acid	1.92 ± 0.07^{d}	2.57 ± 0.02^{c}	3.52 ± 0.05^{b}	1.88 ± 0.02^{d}	1.55 ± 0.01^{e}	9.22 ± 0.57^{a}	3.01 ± 0.23^{f}	5.52 ± 0.52^{e}	8.42 ± 0.16^{c}	4.82 ± 0.22^{e}	4.53 ± 0.83^{e}	41.07 ± 0.78^{a}	15.23 ± 1.24^{b}
Isovaleric acid	1.79 ± 0.11^{a}	0.84 ± 0.03^{d}	1.08 ± 0.04^{c}	1.20 ± 0.08^{bc}	0.99 ± 0.13^{cd}	1.32 ± 0.06^{b}	2.02 ± 0.22^{d}	2.40 ± 0.20^{b}	2.26 ± 0.05^{bc}	2.27 ± 0.07^{bc}	2.14 ± 0.28^{cd}	2.94 ± 0.37^{a}	2.73 ± 0.22^{a}
Hexanoic acid	1.53 ± 0.08^{a}	0.36 ± 0.07^{b}	0.31 ± 0.06^{b}	0.34 ± 0.02^{b}	-	0.32 ± 0.18^{b}	0.68 ± 0.03^{d}	1.00 ± 0.09^{ab}	0.89^{bc}	1.14 ± 0.03^{a}	1.12 ± 0.12^{a}	0.62 ± 0.01^{b}	0.76 ± 0.06^{cd}
Octanoic acid	2.04 ± 0.06^{a}	0.55 ± 0.05^{c}	0.73 ± 0.03^{bc}	0.66 ± 0.04^{bc}	0.68 ± 0.03^{bc}	0.78 ± 0.09^{b}	0.78 ± 0.02^{a}	0.88 ± 0.12^{ab}	-	0.96 ± 0.01^{ab}	1.00 ± 0.18^{a}	-	0.64 ± 0.01^{c}
Decanoic acid	1.24 ± 0.05^{ab}	0.87 ± 0.04^{d}	1.37 ± 0.02^{a}	0.92 ± 0.01^{cd}	0.93 ± 0.04^{cd}	1.09 ± 0.09^{bc}	1.11 ± 0.01^{a}	0.71 ± 0.03^{b}	0.72 ± 0.05^{b}	0.66 ± 0.03^{b}	0.70 ± 0.02^{b}	0.66^{c}	0.72 ± 0.01^{b}
Σ Volatile acids	11.10 ± 1.3^{b}	7.53 ± 1.06^{bc}	9.20 ± 1.06^{b}	8.15 ± 0.16^{bc}	6.97 ± 0.43^{c}	14.96 ± 2^{a}	11.28 ± 1.11^{f}	16.02 ± 0.44^{d}	18.43 ± 0.12^{c}	13.56 ± 1.22^{e}	13.84 ± 1.19^{e}	48.15 ± 1^{a}	22.88 ± 1.14^{b}

Data represented are that of completed fermentations: Co-culture (Co) fermentations carried out by 48 h sequential inoculation of Lt (*L. thermotolerans* Y940 and Concerto), Ll (*L. lanzarotensis* CBS12615 and Y992) and Lf (*L. fermentati* Y515) with Sc (*S. cerevisiae* VIN13). *S. cerevisiae* monoculture (Mono) fermentation acted as the control. Monoculture inoculation of *L. fermentati* Y515 was able to complete the fermentation of Muscat grape must and data for this fermentation is therefore illustrated. Statistically significant differences for particular compounds are illustrated by letters, according to Tukey's test ($p<0.05$). The values falling below limit of detection and/or quantification have been discarded

3.3.7.5 Principal component analysis

The above mentioned major volatiles were all analyzed by principal component analysis (PCA) and the discrimination between the volatiles from each completed fermentation presented in correlation biplot graphs. For the fermentations performed in synthetic juice (**Figure 3.6**), the first component (PC1), explaining 41.40% of the variation, grouped the *L. lanzarotensis* and *L. thermotolerans* co-culture fermentations as well as *S. cerevisiae* monoculture in the negative dimension, while *L. fermentati* co-culture fermentation was separated in the positive dimension. Explaining 30.99% of the variance, PC2 further separated *S. cerevisiae* and the *L. lanzarotensis* and *L. thermotolerans* fermentations. The *L. thermotolerans* and *L. lanzarotensis* grouped together and were differentiated from the other fermentations due to high production of ethyl decanoate, ethyl phenylacetate, hexanol, butanol, isoamyl alcohol and propionic acid. *L. fermentati* Y515 was differentiated due to high production of volatile acids (acetic acid and isobutyric acid), higher alcohols (isobutanol and propanol) and esters (2-phenylethyl acetate, isoamyl acetate and ethyl acetate). The *S. cerevisiae* monoculture fermentation was differentiated from the co-culture fermentations due to its increased volatile acid (decanoic, hexanoic, octanoic and isovaleric acids) levels.

For the fermentations performed in Muscat must (**Figure 3.7**), the first and second components (PC1 and PC2) explained 44.65% and 22.70% of the variance, respectively. PC1 grouped the *L. fermentati* monoculture and co-culture fermentations in the negative dimension and the *L. thermotolerans* and *L. lanzarotensis* in the positive dimension. PC2 separated the *S. cerevisiae* fermentation from both the *L. fermentati* and the *L. thermotolerans* and *L. lanzarotensis* fermentations. A grouping of the *L. thermotolerans* and *L. lanzarotensis* strains and a separation of *L. fermentati* and the *S. cerevisiae* control was once again observed. *L. fermentati* monoculture and co-culture fermentations were distinguished due to increased production of isovaleric acid, isoamyl alcohol and once again increases in acetic acid, isobutyric acid and isobutanol. The *L. thermotolerans* and *L. lanzarotensis* strains grouped together due to the combined increase in a variety of compounds, while *S. cerevisiae* was differentiated from the other fermentations again due to increased decanoic acid and hexanol production.

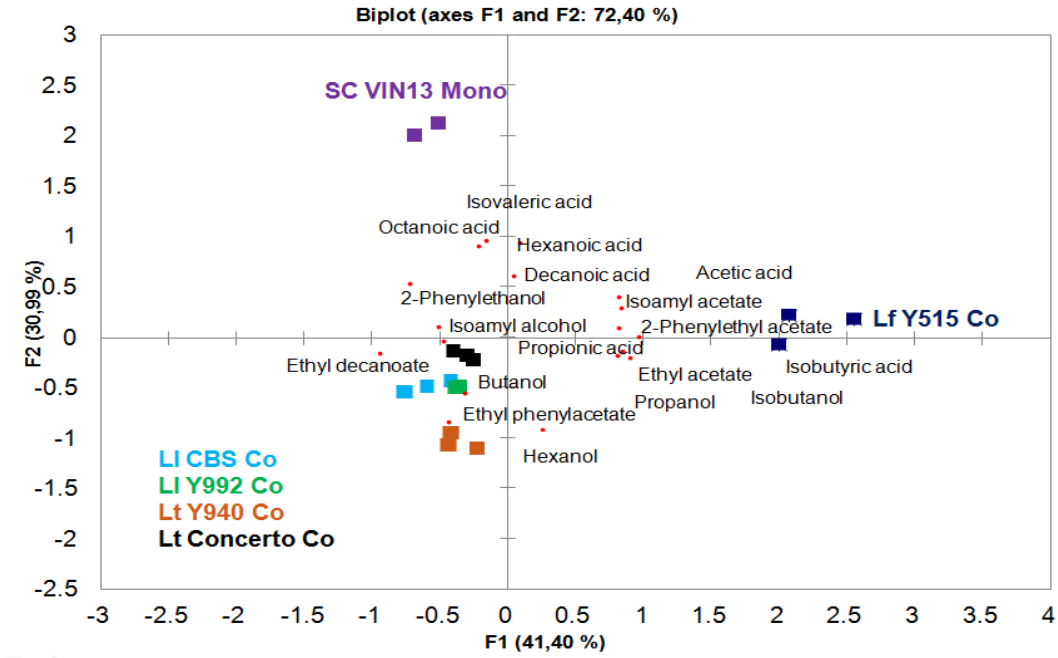


Figure 3.6 Biplot of the first and second components obtained from Principal Component Analysis (PCA) of the major volatile compounds present in the fermentation of synthetic grape juice-like media through *S. cerevisiae* inoculated (Sc VIN13 Mono) and 48h sequential co-culture fermentations inoculated with *L. thermotolerans* Y940 and Concerto (Lt Y940 Co and Lt Concerto Co), *L. lanzarotensis* CBS 12615 and Y992 (LI CBS Co and LI Y992 Co) and *L. fermentati* Y515 (Lf Y515) together with *S. cerevisiae*

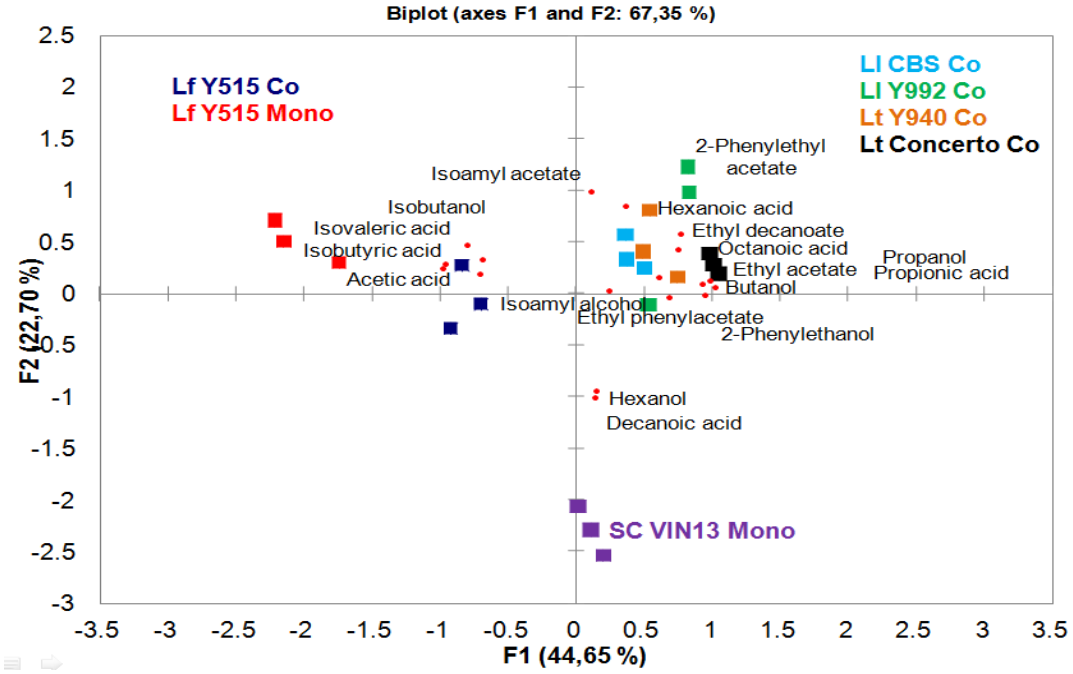


Figure 3.7 Biplot of the first and second components obtained from Principal Component Analysis (PCA) of the major volatile compounds present in the fermentation of Muscat grape must through *S. cerevisiae* (Sc VIN13 Mono) and *L. fermentati* Y515 (Lf Y515 Mono) inoculated fermentation as well as 48h sequential co-culture fermentations inoculated with *L. thermotolerans* Y940 and Concerto (Lt Y940 Co and Lt Concerto Co), *L. lanzarotensis* CBS 12615 and Y992 (LI CBS Co and LI Y992 Co) and *L. fermentati* Y515 (Lf Y515) together with *S. cerevisiae*

3.3.7.6 Monoterpenes

Monoterpene levels were evaluated in the monoculture and co-culture fermentations carried out in Muscat grape must. Although the monoculture fermentations with *Lachancea* spp., were unable to complete to dryness, if the total free monoterpene levels (**Table 3.13**) were normalized to the amount of sugar these strains were able to ferment (their relative growth), the *L. lanzarotensis* strains and *L. thermotolerans* Y940 were able to release the highest levels of monoterpenes (1.97 – 2.07 µg/L monoterpenes/g of sugar) followed by *L. thermotolerans* Concerto (1.79 µg/L monoterpenes/g of sugar), *S. cerevisiae* (1.52 µg/L monoterpenes/g of sugar) and lastly *L. fermentati* (1.27 µg/L monoterpenes/g of sugar). The highest levels of free monoterpenes were accounted by linalool, α-terpineol and geraniol, where much lower levels of eucalyptol, nerol and citronellol were present.

Comparing the total monoterpene levels present following the monoculture fermentations, the *L. thermotolerans* fermentation contained similar levels to *S. cerevisiae* (**Table 3.13**). *L. thermotolerans* Y940 did however possess higher concentrations of eucalyptol, while both *L. thermotolerans* fermentations possessed higher levels of geraniol. In contrast, the total monoterpene levels were lower for the monoculture fermentations with the *L. lanzarotensis* and *L. fermentati* strains. When comparing the completed (co-culture and *L. fermentati* Y515 monoculture) fermentations to the *S. cerevisiae* control, the total monoterpene levels were lower for all except the *L. fermentati* co-culture fermentation. The *L. fermentati* co-culture fermentation produced similar concentrations of linalool, α-terpineol and citronellol while producing higher levels of geraniol.

Table 3.13 Concentrations of a selected subset of monoterpenes at the end of Muscat grape juice monoculture and co-culture fermentations by *Lachancea* spp. and *S. cerevisiae*

Compound (µg/L)	Sc VIN13	Lt Y940		Lt Concerto		Ll CBS		Ll Y992		Lf Y515	
	Mono	Mono	Co	Mono	Co	Mono	Co	Mono	Co	Mono	Co
Eucalyptol	0.67 ± 0.02b[a]	2.89 ± 0.82a	0.78 ± 0.10[a]	1.83 ± 0.65b	-	1.48 ± 0.62b	-	1.87 ± 0.73b	-	0.87 ± 0.57b	-
Linalool	145.74 ± 3.13a[a]	137.86 ± 1.96ab	102.37 ± 3.13[b]	130.91 ± 0.93b	101.40 ± 5.82[b]	109.43 ± 1.74c	101.76 ± 5.41[b]	111.77 ± 1.76c	98.27 ± 6.86[b]	114.41 ± 3.68c	135.33 ± 3.41[a]
α-Terpineol	85.88 ± 0.72a[a]	75.79 ± 1.29b	52.83 ± 0.81[b]	74.12 ± 2.24b	55.17 ± 0.31[b]	63.71 ± 0.23c	54.77 ± 1.38[b]	70.18 ± 3.68b	54.07 ± 2.82[b]	71.35 ± 2.10b	84.31 ± 3.05[a]
Nerol	9.97 ± 0.26a[a]	9.54 ± 0.18a	7.25 ± 0.56[b]	8.36 ± 1.10ab	7.85 ± 0.23[b]	7.24 ± 1.02bc	7.63 ± 0.27[b]	8.39 ± 0.07ab	7.18 ± 0.43[b]	5.68 ± 0.35c	6.90 ± 0.13[b]
Citronellol	9.10 ± 0.24a[a]	8.79 ± 0.12a	5.16 ± 1.17[d]	7.67 ± 0.95ab	5.62 ± 0.24[cd]	6.66 ± 0.93bc	7.18 ± 0.15[abc]	7.87 ± 0.13ab	6.91 ± 0.54[bcd]	5.08 ± 0.33c	7.75 ± 0.03[ab]
Geraniol	33.39 ± 1.60c[b]	65.78 ± 2.51a	32.91 ± 0.81[b]	61.85 ± 0.94a	37.56 ± 0.15[a]	49.52 ± 1.59b	35.65 ± 1.31[ab]	29.75 ± 0.55c	35.18 ± 1.29[ab]	34.29 ± 2.79c	36.76 ± 0.48[a]
Σ Terpenes	284.75 ± 1.77a[a]	300.65 ± 4.3a	201.3 ± 2.90[c]	284.74 ± 4.92a	207.6 ± 5.66[c]	238.03 ± 2.57b	206.99 ± 8.51[c]	229.83 ± 3.46b	201.61 ± 11.77[c]	231.68 ± 1.92b[b]	271.05 ± 6.72[a]

Mono: monoculture; Co: 48h sequential co-culture fermentations inoculated with Sc VIN13: *S. cerevisiae* VIN13; Lt Y940 and Concerto: *L. thermotolerans* Y940 and Concerto; Ll CBS and Y992: *L. lanzarotensis* CBS 12615 and Y992 and Lf Y515: *L. fermentati* Y515. Statistically significant differences for particular compounds are illustrated by letters, according to Tukey's test ($p<0.05$), with the differences between the monoculture fermentations in regular font and that between the completed fermentations (Sc VIN13, *L. fermentati* Y515 monoculture and co-culture fermentations) illustrated in superscript.

3.4 Discussion

In order to evaluate the oenological potential of wine-related *Lachancea* species, several strains of *L. thermotolerans*, *L. lanzarotensis* and *L. fermentati* were screened for enzyme activities of oenological interest as well as H_2S production and tolerance to ethanol and SO_2. Using the resulting profiles, strains showing beneficial characteristics were selected and their respective fermentation capabilities as well as enzyme expression during fermentations were evaluated. Regarding the initial enzymatic screening, β-glucosidase, β-xylosidase, protease and pectinase activity was evaluated. None of the *Lachancea* strains illustrated protease or pectinase activity, while all exhibited β-xylosidase activity (with the exception of *L. lanzarotensis*), β-glucosidase activity and produced varying levels of H_2S. *L. thermotolerans* and *L. fermentati* seemed to be more adapted to the increasing ethanol and SO_2 exposure, respectively, while *L. lanzarotensis* illustrated increased sensitivity when exposed to these inhibitory compounds.

Studies screening for oenologically relevant enzyme activities are lacking and limited in literature for *L. lanzarotensis* and *L. fermentati*, respectively, while various reports exist for *L. thermotolerans* (Rosi et al., 1994*;* Mostert 2013; Romo-Sánchez *et al.,* 2010; Comitini *et al.,* 2011; Cordero-Bueso *et al.,* 2013; Belda *et al.,* 2016). Similar to our current findings, the majority of previous studies found no pectinase and protease activity for *L. thermotolerans* and no pectinase activity for *L. fermentati*, reports of protease activity by *L. fermentati* is lacking in literature (Sakai *et al.,* 1984; Schwan *et al.,* 1997; Romo-Sánchez *et al.,* 2010; Alimardani-Theuil *et al.,* 2011; Comitini *et al.,* 2011; Cordero-Bueso *et al.,* 2013; Mostert, 2013; Belda *et al.,* 2016). Previous studies evaluating β-glucosidase activity have made use of arbutin as a substrate and have shown strain differentiation for *L. thermotolerans* and *L. fermentati* (Rosi *et al.,* 1994; Mostert, 2013; Romo-Sánchez *et al.,* 2010; Comitini *et al.,* 2011; Cordero-Bueso *et al.,* 2013; Belda *et al.,* 2016), however, in the current study all the strains demonstrated the ability to hydrolyse arbutin. The reported strain variability could be attributable to different β-glucosidases having different affinities for substrates, which can explain *L. lanzarotensis* illustrating β-glucosidase activity against arbutin but not 4-MUG (van Rooyen *et al.,* 2005). These assays are qualitative in nature or semi-quantitative at best, and are performed under conditions dissimilar to that of a fermentation. Rosi *et al.* (1994) in fact reported that high glucose and low pH (parameters common in wine fermentations) can have a repressive effect on yeast β-glucosidase activity. Despite the limitations of these assays, the resulting profiles such as higher tolerance to ethanol and SO_2 and positive β-glucosidase activity aided in selecting potential, oenologically beneficial, strains from each *Lachancea* species. Based on the positive correlation of β-glucosidase activity and *Lachancea* spp., as well as the availability of comparative data in literature, this enzyme activity was chosen to

analyze during fermentation, to investigate whether this activity is in fact present under winemaking conditions.

Synthetic juice was used to analyze the fermentation capability of the selected strains; *L. thermotolerans* Y940 and Concerto, *L. lanzarotensis* CBS 12615 and Y992 and *L. fermentati* Y515 in a medium of defined composition. The *L. thermotolerans* and *L. fermentati* strains illustrated faster fermentation vigour, with the commercial *L. thermotolerans* Concerto being the fastest. In contrast, slower fermentation rates were demonstrated by the *L. lanzarotensis* strains, with the isolate Y992 being the slowest, however, none of the *Lachancea* strains could ferment to dryness. The faster fermentation rates correspond to investigations previously performed for *L. thermotolerans* and *L. fermentati* (Mora *et al.,* 1990; Ciani *et al.,* 2006; Cordero-Bueso *et al.,* 2013). In contrast, there is currently a gap in literature regarding the fermentative capabilities of *L. lanzarotensis*. In the synthetic medium, *L. fermentati* did not perform as well as *L. thermotolerans*, which contradicts the report by Cordero-Bueso *et al.* (2013), which saw *L. fermentati* to demonstrate a faster fermentation tempo; these fermentations were however carried out in real grape must (Malvar white variety). Indeed, when validating the *Lachancea* spp. fermentations in real (Muscat d'Alexandrie) grape must, *L. fermentati* demonstrated a higher fermentation tempo, comparable to that of *S. cerevisiae*, suggesting that beyond the difference in strain between this study and that of Cordero-Bueso *et al.* (2013), the behaviour of this species is dependent on the composition of the grape must. Aside from *L. fermentati*, the trends remained similar to what was observed in the synthetic medium, highlighting the synthetic medium to be a good representation of the real grape must. The *L. thermotolerans* and *L. lanzarotensis* strains therefore required the inoculation of *S. cerevisiae* to complete the fermentation. While these co-culture fermentations were able to ferment to dryness, the fermentation rate was slower than that of *S. cerevisiae*, which corresponds to reports for *L. thermotolerans* (Kapsopoulou *et al.,* 2007; Gobbi *et al.,* 2013; Balikci *et al.,* 2016). Until now, *L. fermentati* had not been reported in co-culture fermentations, so like for *L. lanzarotensis*, this information is novel.

Furthermore, during the synthetic and Muscat juice fermentations, the production of β-glucosidases as well as the cellular localization of these enzymes was monitored. The enzyme activity accumulated at the beginning stages of the fermentations, linked to the exponential phase of growth or high metabolic activity of the yeasts after which a general decrease was observed; a pattern also reported by Fia *et al.* (2005) for other non-*Saccharomyces* yeasts. This decrease in activity could potentially be due to the inhibition of enzyme expression or activity by inhibitory compounds, such as the increasing ethanol levels (Mateo & Di Stefano, 1997; Maturano *et al*., 2012). Most of the enzyme activity was located in the cell wall while none was detected extracellularly, which corresponded to

previous studies on other species (Rosi *et al.,* 1994; Mateo & Di Stefano, 1997; Manzanares *et al.,* 2000; Cordero Otero *et al.,* 2003; Fia *et al.,* 2005). The strong association to the cell wall suggests the enzyme to play a role in cell wall structure and maintenance. Indeed, glucosidases have been linked to the biosynthesis of cell wall β-1,6-glucan in *S. cerevisiae* (Abeijon & Chen, 1998). Intracellular enzyme activity was also present, albeit at lower levels. Nevertheless, intracellular enzymes have previously been shown to be able to hydrolyze natural glucosides (such as nerol, α-terpineol and geraniol) and therefore, following yeast cell lysis, their contribution cannot be disregarded (Rosi *et al.,* 1994).

During fermentation in the synthetic medium glycosidically bound complexes, that are natural substrates for β-glucosidase activity, were absent, nevertheless activity was present in all *Lachancea* spp., suggesting this enzyme to be constitutively expressed in these yeasts. Indeed, similar enzyme levels were determined throughout the synthetic and Muscat grape must fermentations, wherein the glycosidically bound complexes were present, confirming the constitutive expression observed. Previous studies have also observed constitutive expression of β-glucosidase activity by yeasts such as *S. cerevisiae*; however, the investigators have also proposed that the observed activity could possibly be due to exo-glucanases, which are able to hydrolyze the pNP substrate, and therefore result in false constitutive activity (Mateo & Di Stefano, 1997; Rodríguez *et al.,* 2003). In both grape matrices, *L. thermotolerans* Y940 consistently showed high enzyme activity in comparison to the other *Lachancea* spp. and *S. cerevisiae*. *L. thermotolerans* Concerto and *L. fermentati* behaved similarly, followed by the *L. lanzarotensis* strains. Co-culture fermentations all resulted in increased enzyme activity compared to the *S. cerevisiae* fermentation, suggesting a compound/cumulative effect of both the *Lachancea* spp. and *S. cerevisiae*, which highlights the advantage of inoculating the *Lachancea* spp. Nevertheless, similar total monoterpene levels were present in the fermentations by *L. thermotolerans* Y940, *L. thermotolerans* Concerto and *S. cerevisiae*. Therefore, even though *L. thermotolerans* Y940 exhibited significantly higher β-glucosidase activity, this did not translate to significantly higher monoterpene levels. Most of the naturally present monoterpenol complexes exist as diglycosides as opposed to monoglucosides, and therefore require the action of additional enzymes, namely α-L-arabinofuranosidase, α-L-rhamnopyranosidase or β-D-apiofuranosidase prior to the action of β-glucosidase (Günata *et al.,* 1988; Rosi *et al.,* 1995). The absence of such enzyme activity would therefore hinder the ability for the β-glucosidase enzymes to release monoterpenols from a large portion of the available glycoside complexes. Indeed, Belda *et al.* (2016) reported the majority of *L. thermotolerans* strains, of which 88 strains were screened, and all the *S. cerevisiae* (11 strains), to lack α-L-arabinofuranosidase activity. This lack in activity could account for the reduced monoterpene accumulation, which has been reported before (Rosi *et al.,* 1995). When analyzing these

results, it is also important to recognize the limitations of making use of an artificial substrate (nitrophenyl-glycosides), due to β-glucosidase activity previously being shown to have less activity against the natural glucoside substrates (Margolles-Clark *et al.,* 1996). Following the co-culture fermentations, lower levels and less variation were surprisingly observed for the monoterpene content, corresponding to literature (Garcia *et al.,* 2002; Cordero Otero *et al.,* 2003), despite the higher enzymatic activity measured during these fermentations. This could be due to the increased fermentation vigour of the co-culture fermentations, leading to increased evaporation of the volatile monoterpenes due to enhanced CO_2 production, which has been hypothesised before in literature (Günata *et al.,* 1986). Additionally, during the co-culture fermentations, the *Lachancea* spp. declined below detection much earlier than in the monoculture fermentations and upon lysis of the cells the released enzymes could have resulted in the hydrolysis of the monoterpenes earlier in the fermentation process, subsequently leading to the biotransformation of the monoterpenes, into less fragrant compounds (such as monoterpene oxides and diols), not measured in this study (Günata *et al.,* 1986; Vaudano *et al.,* 2004). While this could also corroborate the decrease in total monoterpene content in the co-culture fermentations, further investigation is required.

In addition to the monoterpene production, the chemical profiles of the completed fermentations demonstrated interesting results. Fermentations that ran to dryness included the co-culture fermentations, *S. cerevisiae* monoculture fermentations and *L. fermentati* monoculture fermentation in Muscat grape must. Co-culture fermentations, while fermenting at a slower rate than *S. cerevisiae*, resulted in minimal residual sugar and comparable ethanol levels to *S. cerevisiae*. The resulting acetic acid:ethanol ratio in the co-culture fermentations were however better than *S. cerevisiae*, with the exception of *L. fermentati*. While the *L. thermotolerans* and *L. lanzarotensis* co-culture fermentation led to a reduction in the acetic acid levels, the levels produced during monoculture and co-culture fermentations with *L. fermentati* were above what is considered acceptable in wine (0.7 g/L). This overproduction of acetic acid highlights the *L. fermentati* strain tested in this study to potentially generate spoilt wine; however, further investigation is required through sensory analysis. Regarding the volatile compounds formed in the fermentations, in both matrices, increased acetic acid, isobutyric acid and isobutanol production led to the differentiation of *L. fermentati* from the other co-culture fermentations. Interestingly, isobutanol was much higher in the Muscat grape must in comparison to that measured in the synthetic juice, which corresponds to a previous report on this species (Romano & Suzzi, 1993). The differentiation in the volatile compound profiles for the different yeasts could allude to potential differences in their respective metabolism and amino acid uptake. For instance, the increase in isobutanol and isobutyric acid could be due to better or preferential uptake of valine by *L. fermentati*, leading to the production of these compounds. Alternatively, it has recently been

shown in *S. cerevisiae* that an increased flux from pyruvate can also account for the increase in isobutanol (Rollero *et al.,* 2017) Co-culture fermentations with *L. thermotolerans* and *L. lanzarotensis* strains also led to wines distinguishable from *S. cerevisiae*, due to increased production of various esters and higher alcohols, including butanol, 2-phenylethanol and phenylethyl aceate, features previously reported for *L. thermotolerans* (Comitini *et al.,* 2011; Gobbi *et al.,* 2013; Beckner Whitener, 2015; Benito *et al.,* 2016). The increased production of 2-phenylethanol and phenylethyl aceate suggests to the increased uptake of phenylalanine by these species. An increase in phenylethanol can also result from sugars via the pentose phosphate pathway (Rollero *et al.,* 2017). Sensory analysis of the resulting wines utilizing these *Lachancea* species will further elucidate whether, like the chemical profiles suggested, significant differences occurred compared to wines produced from *S. cerevisiae* alone.

The current study provided novel information regarding the fermentative capability and oenological characteristics of *L. fermentati* and *L. lanzarotensis*, while increasing our understanding of the oenological potential of *L. thermotolerans*. Our data, revealed that although *L. fermentati* had higher fermentation vigour in real grape must, this was also accompanied by heavy flocculation and high production of acetic acid, possibly identifying this strain as a wine spoilage yeast, or as a result on strain-specific behaviour. *L. lanzarotensis*, while fermenting at a slower rate and therefore requiring inoculation with *S. cerevisiae*, was able to produce wines with significantly different chemical composition to *S. cerevisiae*. Both these species exhibited β-glucosidase activity during fermentations. In contrast, *L. thermotolerans* Y940 demonstrated significantly higher β-glucosidase activity under the harsh winemaking conditions, and subsequently resulted in the accumulation of monoterpenes comparable to *S. cerevisiae*, despite this strain not being able to persist until the end of fermentation. Inoculation of the *Lachancea* spp. into mixed culture fermentations with *S. cerevisiae* resulted in complete fermentations with dissimilar chemical profiles, highlighting the suitability of *Lachancea* spp., to bring about diverse wine products in the winemaking industry.

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Chapter 4

General discussion and conclusions

Chapter 4 – General discussion and conclusions

4.1 Concluding remarks and future prospects

Various investigations into non-*Saccharomyces* yeasts have revealed diverse characteristics that may be exploited in mixed culture fermentations with *Saccharomyces cerevisiae* in order to bring about fermentation completion while enhancing certain properties of the final wine, such as the production of secondary metabolites (Ciani & Comitini, 2011; Padilla *et al.,* 2016; Varela, 2016). Indeed, several non-*Saccharomyces* yeasts have been commercialized for regular use in winemaking, including *Lachancea thermotolerans* Concerto™ released in 2012 by Chr Hansen. This yeast strain is advertised to help increase the total acidity of wines produced in warmer climates. While *Lachancea fermentati* and *Lachancea lanzarotensis* have also been isolated in the wine fermentation environment and are members of the same genus, they have not received the same focus in research as *L. thermotolerans*. The current study sought out to further explore the oenological potential of these *Lachancea* spp., by evaluating the oenological potential of strains isolated naturally from grape must and comparing their behaviours to those of the commercial *L. thermotolerans* Concerto.

In comparison to the other yeast strains, *L. thermotolerans* Concerto demonstrated the fastest growth and fermentation vigour in the initial stages of the fermentation, corresponding to what has been reported for this strain (Benito *et al.,* 2015; Beckner Whitner *et al.,* 2015, 2016, 2017). Lower fermentation vigour was demonstrated by the remaining *L. thermotolerans* and *L. lanzarotensis* strains. In contrast, the behaviour of *L. fermentati* seemed to be grape matrix dependent, where a fermentation rate comparable to *S. cerevisiae* was illustrated in the Muscat d'Alexandrie grape must and a lower rate during the fermentation of synthetic medium. In contrast to the other *Lachancea* spp., *L. fermentati* demonstrated strong flocculation behaviour. *L. fermentati* has previously been reported to flocculate and has been determined to be mannose-sensitive, where the addition of mannose prevented the flocculation of this species (Suzzi *et al.* 1992). This type of flocculation is referred to as Flo1-type (Govender *et al.,* 2011). Flocculation of yeast cells following alcoholic fermentation can aid in reducing the necessity of time-consuming and expensive wine clarification and fining practices (Bauer *et al.,* 2010; Govender *et al.,* 2011). It can therefore be considered as a positive feature. Further investigation into the flocculation of *L. fermentati* could provide insight into its specific flocculation behaviour and whether co-flocculation with *S. cerevisiae*'s cells occurred during co-culture fermentations. In stark contrast to the positive characteristic of flocculation, the strong fermentative behaviour of *L. fermentati* led to the overproduction of acetic acid and higher alcohols, potentially spoiling the wine. These characteristics flag this species as a potential spoilage yeast. However, a limitation of this study was the investigation of only one strain, therefore this behaviour could in fact be strain specific and not a reflection of the *L. fermentati*

species as a whole. Limited reports evaluating *L. fermentati* (with co-culture fermentations lacking in literature), have not reported the same high levels of acetic acid and higher alcohol production as seen for *L. fermentati* Y515 in this study (Romano & Suzzi, 1993; Romano *et al.,* 1997; Cordero-Bueso *et al.,* 2013). Cordero-Bueso *et al.* (2013) did however report high residual sugar levels indicating the occurrence of a stuck fermentation when using *L. fermentati* in monoculture, while the other reports did not specify whether the fermentation ran to completion or not. The high levels found in this study could therefore have been due to the increased fermentation capabilities of this strain or to the specific conditions of the fermentation. For instance, higher levels of glycerol were also noticeable for *L. fermentati* fermentations, therefore the higher sugar levels in the grape must could account for the increase in glycerol and acetic acid. Increased carbon utilizing for glycerol production and away from ethanol production, would have enhanced the need to regenerate NADH; accounted for by oxidizing acetaldehyde, leading to an increase in acetic acid (Eglinton *et al.,* 2002). Future investigations with *L. fermentati* could explore whether the overproduction of acetic acid is in fact grape must composition dependent. Further research into adapting the inoculation dosage and/or the inoculation strategy could provide a means to reduce the growth of this yeast while still exploiting its higher alcohol production and flocculation characteristics, while negating the overproduction of volatile compounds, such as acetic acid.

Similarity in the levels of β-glucosidase activity during fermentations of synthetic and Muscat grape juice, irrespective of the presence or absence of glycosidic complexes, suggested constitutive expression of β-glucosidase by the *Lachancea* spp. However, a more reliable comparison would involve fermentations in grape juice of the same composition, with the only difference being the presence or absence of these complexes, as well as the same protein extraction method being utilized. Future analysis could therefore elucidate on this constitutive expression. Nevertheless, within the *Lachancea* strains analyzed, *L. thermotolerans* Y940 demonstrated unrivalled high levels of cell wall associated and intracellular β-glucosidase activity throughout monoculture fermentations of Muscat grape must. This was accompanied by significantly higher levels of eucalyptol and geraniol production, both over their respective odour threshold levels and would therefore be able to impart eucalyptus and freshly geraniol/rose aromas to the wine (Francis & Newton, 2005; Saliba *et al.,* 2009). The total monoterpene levels produced by this strain were comparable to those of *S. cerevisiae*. Unlike *L. fermentati*, this yeast strain could not ferment to dryness and therefore required the inoculation of *S. cerevisiae*; however, in co-culture fermentation the same high monoterpene content was not achieved. This could be due to the earlier death of *L. thermotolerans* Y940 in co-culture fermentations or to the increased CO_2 production inducing the increased loss of volatiles. This finding suggests this strain would not be suitable for industrial winemaking, since its contribution is significantly curtailed in mixed fermentations, which are typical in this industry. The high expression of β-glucosidase observed for *L. thermotolerans* Y940, under the harsh conditions of a wine

fermentation can however be further explored. *L. thermotolerans* has been shown to perform better with the introduction of oxygen during the fermentation (Shekhawat *et al.,* 2017). Implementing oxygen during fermentations in future studies could therefore impact the persistence of *L. thermotolerans* Y940 and perhaps lead to the increased impact on monoterpene levels, which is also perhaps applicable for the other *Lachancea* spp. as well. Increasing the investigation of β-glucosidase expression by wine yeasts, such as *L. thermotolerans* Y940, could potentially lead to the isolation of an enzyme with reliable and stable activity when exposed to conditions common for wine fermentations, such as high sugar and ethanol concentrations and low pH. A limitation of this study was the utilization of an artificial substrate to measure the β-glucosidase activity, which could lead to an overestimation of activity against the natural glucosidases present in wine (Margolles-Clark *et al.,* 1996; de klerk, 2009). Further investigations could make use of terpene bound glucosidases from grape must, to not only investigate the direct potential of utilizing *Lachancea* spp. for increasing levels of free monoterpenes but also to more directly compare the overall accuracy of using synthetic substrates to measure β-glucosidase activity.

This study provided novel information on the relatively uninvestigated fermentation-associated *Lachancea* spp. The oenological potential of these yeasts was highlighted, regarding their fermentation capabilities, enzyme expression during fermentation and overall impact on the organoleptic quality of the resulting wines, producing wines significantly different from those produced by *S. cerevisiae*. However, with sensory analysis, whether these significantly different wines were in fact preferred could be determined. Further exploiting what is known about how oxygen addition into the fermentation enhances *L. thermotolerans* growth, can also be implemented to potentially further enhance the contribution from *Lachancea* spp. to the organoleptic quality of the wine (Shekhawat *et al.,* 2017). Fermentations utilizing varying grape cultivars, environmental conditions and inoculation strategies/dosages could identify the optimal conditions for these *Lachancea* spp., in which they are able to impart beneficial wine attributes. Increased exploration into wine-related non-*Saccharomyces* yeast species, such as those of the *Lachancea* genus, could potentially lead to an increase in the commercialization and usage of these yeasts in industrial winemaking, providing umpteen opportunities for better quality and more diverse wine products.

4.2 Literature cited

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