Screening and characterisation of wine-related enzymes produced by wine-associated lactic acid bacteria

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Phillip Senzo Mtshali **Date**

SUMMARY

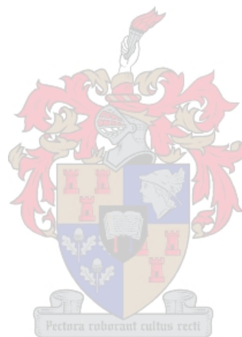
Among the factors contributing to wine complexity and quality, wine aroma is one of the most important factors. Wine aroma is the outcome of interaction among different compounds produced from the grapes, during fermentation as well as during the ageing process. Apart from its origin from grapes, fungi and yeasts, wine aroma can also be derived from the metabolic activity of wine lactic acid bacteria (LAB). These microorganisms are usually associated with malolactic fermentation (MLF) which normally occurs after alcoholic fermentation. MLF is beneficial to wine due to its contribution to deacidification, microbiological stabilisation and wine aroma formation, with the latter being the most important area of interest in our study. The production of volatile aromatic components in wine can, in part, be achieved through the hydrolytic action of enzymes produced by LAB associated with wine. These enzymes include β-glucosidase, protease, esterase, lipase and glucanase. Most of the work done on bacterial enzymes has been on LAB from food sources other than wine, in which these enzymes contribute to the flavour development of some cheeses, yoghurt and other fermented foods. The activity of these enzymes during wine fermentation has mostly been concerned with β-glucosidase from *Oenococcus oeni*. Only in recent years has there been a renewed interest in evaluating the activity of β-glucosidase in other genera of wine LAB.

The overriding goal of this study was to screen and characterise wine-related enzymes produced by LAB associated with wine. All the LAB isolates tested in this study were obtained from IWBT culture collection and were previously isolated from five different wineries situated in the Western Cape region, South Africa. We first screened isolates using classical methods. The isolates were grown on agar medium supplemented with appropriate substrate analogues in order to evaluate the activity of enzymes (i.e. β-glucosidase, glucanase, lipase and esterase). The colonies exhibiting enzymatic activity were identified by media colouration around the bacterial growth.

The second objective was to screen enzymes using molecular techniques. Bacterial colonies from MRS agar plates were applied directly to PCR in order to detect the presence of genes encoding different enzymes. The gene nucleotide sequences retrieved from the Integrated Microbial Genome database were employed to design enzyme-specific amplification primers for the detection of different enzyme genes from different species of LAB. The primers amplified single gene products with expected sizes corresponding to respective enzyme genes (i.e. protease, β-glucosidase, esterase and malolactic enzyme). Lipase gene-specific primer set gave PCR products with non-specific bands while glucanase primers did not yield any PCR product. Besides evaluating the presence of different enzymes from the bacterial isolates using both plate assay and PCR detection technique, 11 isolates were selected from which genomic DNA was extracted and used as template for amplifying the coding regions of different enzyme genes by means of PCR. The selected isolates possessed all four enzyme genes. Purified amplicons were cloned into pGEM-T easy vector and sequenced. Analysis of sequences revealed that gene

sequences are highly conserved between the species. These gene sequences also exhibited 99 - 100% homology with nucleotide sequences available in GenBank database.

The agar plate method for the determination of β-glucosidase activity using arbutin as a substrate only provided a qualitative estimation of enzyme activity. A quantitative assay using the β-glucoside analogue, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), was therefore developed and employed to quantify the amount of enzyme released from the selected isolates. β-Glucosidase was tested for activity under various physicochemical conditions simulating those of winemaking in order to investigate the influence of the combined parameters on the activity of the enzyme. The enzyme was active against *p*NPG although it was competitively inhibited by glucose.



OPSOMMING

Wynaroma is een van die belangrikste faktore wat tot die kompleksiteit en kwaliteit van wyn bydra. Wynaroma is die resultaat van interaksies tussen verskillende verbindings wat deur die druiwe, tydens gisting en tydens die verouderingsproses geproduseer word. Buiten sy herkoms uit druiwe, swamme en giste kan wynaroma ook van die metaboliese aktiwiteit van melksuurbakterieë (MSB) in die wyn afkomstig wees. Hierdie mikroörganismes hou gewoonlik verband met appelmelksuurgisting (AMG), wat gewoonlik ná alkoholiese gisting plaasvind. AMG is voordelig vir die wyn as gevolg van sy bydrae tot ontsuring, mikrobiologiese stabilisering en die vorming van wynaroma, met laasgenoemde wat van die grootste belang vir ons studie is. Die produksie van vlugtige aromatiese bestanddele in wyn kan gedeeltelik behaal word deur die hidrolitiese aksie van ensieme wat deur die MSB wat met wyn verband hou, geproduseer word. Hierdie ensieme sluit in β-glukosidase, protease, esterase, lipase en glukanase. Die oorgrote meerderheid werk wat op bakteriese ensieme gedoen is, was op MSB vanaf voedselbronne buiten wyn, waarin hierdie ensieme bydra tot die geurontwikkeling van sommige soorte kaas, jogurt en ander gegiste kossoorte. Studies van die aktiwiteit van hierdie ensieme tydens wyngisting was hoofsaaklik gemoeid met β-glukosidase afkomstig van *Oenococcus oeni*. Dit was slegs meer onlangs dat daar hernieude belangstelling in die evaluering van die aktiwiteit van β-glukosidase in ander genera van wyn-MSB was.

Die oorkoepelende doelwit van hierdie studie was om wynverwante ensieme wat geproduseer word deur MSB wat met wyn verband hou, te sif en te karakteriseer. Al die MSB-isolate wat in hierdie studie getoets is, was afkomstig van die IWBT-kultuurversameling en is vroeër vanaf vyf verskillende wynkelders in die Wes-Kaap streek van Suid-Afrika geïsoleer. Ons het eers die isolate gesif deur van klassieke metodes gebruik te maak. Die isolate is op agarmedium wat met die gepaste substraatanaloë aangevul is, gegroei om die aktiwiteit van die ensieme te evalueer (m.a.w. β-glukosidase, glukanase, lipase en esterase). Die kolonies wat ensimatiese aktiwiteit getoon het, is geïdentifiseer op grond van die verkleuring van die media om die bakteriese groei.

Die tweede doelwit was om ensieme deur middel van molekulêre tegnieke te sif. Bakteriële kolonies afkomstig van MRS-agarplate is direk aan PKR blootgestel om die teenwoordigheid van gene wat verskillende ensieme enkodeer, op te spoor. Die geen-nukleotiedsekwense wat van die *Integrated Microbial Genome Database* verkry is, is gebruik om ensiemspesifieke versterkte voorvoerders te ontwerp vir die opsporing van verskillende ensiemgene van verskillende spesies van MSB. Die voorvoerders het enkel geenprodukte met die verwagte grootte versterk in ooreenstemming met die onderskeie ensiemgene (d.i. protease, β-glukosidase, esterase en melksuurensiem). Die stel voorvoerders wat spesifiek was vir die lipasegeen het PKR-produkte opgelewer met nie-spesifieke bande, terwyl die glukanase-voorvoerders geen PKR-produkte opgelewer het nie. Buiten die evaluering van die teenwoordigheid van verskillende ensieme afkomstig van die bakteriële isolate met behulp van beide plaatbepaling en die PKR-opsporingstegniek is 11 isolate gekies waarvan die genomiese DNA geëkstraheer is en as

templaat vir die versterking van die enkoderende gebiede van die verskillende ensiemgene deur middel van PKR gebruik is. Die gekose isolate het gene van al vier ensieme bevat. Gesuiwerde amplikone is in *pGEM-T easy vector* gekloneer en gesekwenseer. ‘n Analise van die opeenvolging het getoon dat die geenvolgorde hoogs bewaar was tussen die spesies. Hierdie geenvolgordes het ook 99 tot 100% homologie getoon met nukleotiedvolgordes wat in die GenBank-databasis beskikbaar is.

Die agarplaatmetode vir die bepaling van β-glukosidase-aktiwiteit met behulp van arbutien as substraat het slegs ‘n kwalitatiewe skatting van ensiemaktiwiteit verskaf. ‘n Kwantitatiewe bepaling deur middel van die β-glukoside-analoog, *p*-nitrofeniel-β-D-glikopiranosied (*p*NPG) is dus ontwikkel en gebruik om die hoeveelheid ensiem wat uit die geselekteerde isolate vrygestel is, te kwantifiseer. β-Glukosidase is onder verskillende fisies-chemiese toestande, wat dié van wynbereiding gesimuleer het, vir aktiwiteit getoets om die invloed van die gesamentlike parameters op die aktiwiteit van die ensiem te ondersoek. Die ensiem was aktief teenoor *p*NPG, hoewel dit mededingend deur glukose geïnhibeer is.

BIOGRAPHICAL SKETCH

Phillip Senzo Mtshali was born in KwaZulu Natal, South Africa on 07 February 1983. He attended Kwethu Lower Primary School, Mhongozini Combined Primary School and matriculated in 2000 at Bantubaningi High School. He enrolled at the University of Zululand in 2001 and obtained a BSc (Biological Science) degree in 2004, majoring in Zoology and Botany. In 2004, he enrolled for BSc Honours at the same institution and obtained a degree in Zoology in the year 2005. In 2005, he also enrolled for an MSc degree in Wine Biotechnology at Stellenbosch University.

This thesis is dedicated to my family for their continuous support and enthusiasm

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Hierdie tesis is opgedra aan my gesin vir hulle volgehoue ondersteuning en entoesiasme

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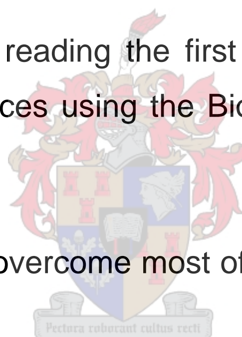

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PREFACE

This thesis is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the style of the *International Journal of Food Microbiology*, to which Chapters 3 and 5 will be submitted for publication.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**
Influence of wine-related enzymes on the sensory properties of wines during malolactic fermentation

Chapter 3 **Research Results**
Screening and genetic characterisation of certain wine aroma enzymes in lactic acid bacteria isolated from South African wines

Chapter 4 **General Discussion and Conclusions**

Chapter 5 **Addendum**
Partial characterisation of β-glucosidase from certain wine lactic acid bacteria isolated from South African wines

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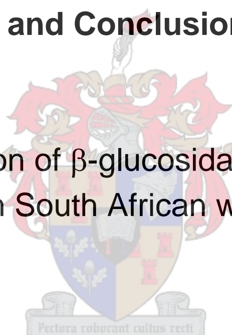

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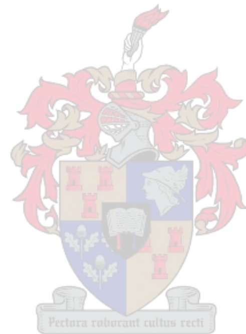
Chapter 1



GENERAL INTRODUCTION AND PROJECT AIMS

CHAPTER 1

1.1 INTRODUCTION

During winemaking two main fermentation processes take place. Alcoholic fermentation, which is conducted by yeasts, is the primary fermentation process that involves the conversion of grape sugars into ethanol and carbon dioxide (CO_2). Malolactic fermentation (MLF) is the secondary process which is conducted by the lactic acid bacteria (LAB). This process usually occurs after alcoholic fermentation but may also occur during alcoholic fermentation. It involves the decarboxylation of L-malic acid (malate) to L-lactic acid (lactate) and CO_2. This results in the concomitant increase in pH accompanied by the disappearance of harsh malate sensation (Wibowo *et al.*, 1985). MLF makes considerable contribution to wine with regard to deacidification, microbial stabilisation and enhancement of wine aroma. However, the latter has not been well characterised. A decrease in wine acidity is beneficial in cool-climate regions such as Canada, New Zealand and Europe where wines tend to have a high acid content and low pH. Nevertheless, MLF is also desired in warm-climate regions in which flavour changes associated with the growth of LAB are often considered beneficial to wine quality (Henick-Kling, 1993).

Due to the highly selective environment of different juices and wines, only very few types of LAB can be detected in wine (Wibowo *et al.*, 1985). The four genera to which the wine LAB species belong include *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus* (Lonvaud-Funel, 1999). Amongst the LAB species commonly found in wine during MLF, *Oenococcus oeni* is the most beneficial and probably the most frequently occurring species of LAB in wine. This is largely due to its tolerance in harsh physicochemical conditions of high acidity, nutrient depletion and high alcohol content present in wine after alcoholic fermentation (Wibowo *et al.*, 1985). *O. oeni* generally predominates in wines with pH values below 3.5, while in wines above pH 3.5, species of *Lactobacillus* and *Pediococcus* often predominate (Henick-Kling, 1993).

Under certain conditions, MLF can increase the microbiological stability of the wine. During their growth in wine, LAB consume nutrients such as amino acids, nitrogen bases and vitamins. The reduction in the availability of these nutrients has been thought to increase microbiological stability by limiting the potential growth of spoilage microorganisms. However, wines which have completed MLF can still support the growth of *O. oeni*, *Lactobacillus* and *Pediococcus* species (Costello *et al.*, 1983).

Beyond wine deacidification, which is the most well-known result of the growth of LAB in wine, the action of LAB can also influence wine aroma and flavour by various mechanisms. These mechanisms include the production of volatile secondary metabolites and the modification of grape and yeast-derived metabolites (Davis *et al.*, 1985, 1988; Henick-Kling, 1993). The products formed are a result of LAB activity and can either be

beneficial or detrimental to wine quality. This is largely dependent on the species predominantly involved during MLF. Undesirable odours brought about by MLF are usually associated with pediococci or lactobacilli, or can originate from MLF occurring above pH 3.5. In contrast, *O. oeni* is more desirable and is less likely to produce unpleasant aromas and flavours during MLF at pH below 3.5 (Du Toit and Pretorius, 2000; Jackson, 1994).

Wine aroma is the outcome of interaction amongst different substances produced from the grapes (pre-fermentative aroma), during fermentation (fermentative aroma) and those arising as a result of wine ageing either in barrels or bottles (post-fermentative aroma). Therefore, the production of specific compounds by wine LAB has a considerable impact on wine aroma, specifically involving fermentative aroma. According to Henick-Kling (1993) and Henick-Kling *et al.* (1994), MLF enhances the fruity aroma. The enrichment of fruitiness may be ascribed to the formation of esters by wine LAB, while an increase in buttery character may be as a result of diacetyl produced from citrate metabolism by wine LAB (Liu, 2002). However, the contribution of MLF on wine aroma varies with wine variety and LAB strain involved. Besides aroma, MLF is also believed to enhance the body and mouthfeel of wine and give a longer after-taste (Henick-Kling *et al.*, 1994).

Amongst different compounds produced by wine LAB during MLF, diacetyl has predominantly been implicated in distinguishing between wines which have undergone MLF and those which have not. Fornachon and Lloyd (1965) showed that wines having undergone MLF contained significantly more diacetyl than wines that had not. At low concentrations (1-4 mg/L) diacetyl imparts a desirable buttery or butterscotch flavour character. When present at high concentrations exceeding 5-7 mg/L diacetyl is considered a spoilage character (Davis *et al.*, 1986) as it imparts a rancid butter-like character which can easily dominate the wine. The sensory threshold of diacetyl in wine is generally dependent upon the style and type of wine (Rankine *et al.*, 1969; Martineau *et al.*, 1995).

Diacetyl is formed as an intermediate in the reductive decarboxylation of pyruvic acid to 2,3-butanediol (Ramos *et al.*, 1995). Apart from its formation from pyruvic acid, diacetyl production also results from the chemical oxidative decarboxylation of α-acetolactate (Hugenholtz and Starrenburg, 1992; Veringa *et al.*, 1984). Pyruvic acid arises from the metabolism of sugar and citric acid, and the formation of 2,3-butanediol may contribute to the redox balance of cellular metabolism (Bartowsky and Henschke, 2004). Yeasts are also able to contribute to the diacetyl content of wine. However, the concentration of diacetyl is usually below its sensory detection threshold due to the highly reductive conditions that exist at the end of alcoholic fermentation (Martineau *et al.*, 1995). This reduction of diacetyl to acetoin and 2,3-butanediol is beneficial for the yeast because the reduction products are less toxic than diacetyl and the reduction increases the levels of coenzymes NAD and NADP (De Revel and Bertrand, 1994).

The production of volatile aromatic components in wine can, in part, be achieved through the hydrolytic action of enzymes produced by LAB associated with MLF. These enzymes include β-glucosidase, protease, esterase, lipase and glucanase. Most of the

work done on bacterial enzymes has focused on LAB from food sources other than wine, in which these enzymes contribute to the flavour development of some cheeses, yoghurt and other fermented foods (Andersen *et al*., 1995; Magboul *et al*., 1997). The activity of these enzymes during wine fermentation has mostly been concerned with β-glucosidase from *O. oeni*. Only in recent years has there been a renewed interest in evaluating the activity of β-glucosidase in other genera of wine LAB.

1.2 PROJECT AIMS

Based on preliminary studies that assessed enzymes from the wine LAB, it is assumed that the LAB occurring in wine during MLF could be the potential source of enzymes that may synergistically affect wine aroma (Liu, 2002; Matthews *et al*., 2004). Therefore, the objective of this study was to screen and characterise wine-related enzymes produced by LAB associated with wine in order to elucidate the potential of LAB to positively alter the organoleptic quality of the wine.

The specific aims and approaches of this study were as follows:

(i) to screen bacterial isolates using classical methods by detecting enzyme activity on agar media supplemented with appropriate substrate analogues (protease, esterase, β-glucosidase, lipase and glucanase);

(ii) to PCR-screen isolates using enzyme-specific primers in order to detect the presence of β-glucosidase, esterase, protease and malolactic enzyme genes;

(iii) to sequence enzyme genes from the selected isolates and subsequently align gene sequences to determine homologies; and

(iv) to quantify the amount of β-glucosidase by partially characterising it under different physicochemical parameters such as temperature, pH, ethanol and glucose.

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Chapter 2

LITERATURE REVIEW

Influence of wine-related enzymes on the sensory properties of wines during malolactic fermentation

CHAPTER 2

2.1 THE AROMA OF WINE

There are various factors contributing to wine complexity, among which flavour is the most important. The flavour of wine is a complex interaction between aroma and taste components. The category of flavour components is composed of volatile compounds especially responsible for the odour of wine (alcohols, esters, aldehydes, ketones, hydrocarbons, etc.) as well as of non-volatile components particularly responsible for taste sensations such as sweetness, sourness, bitterness and saltiness. These flavour sensations are usually caused by compounds present in wine, including sugars, organic acids, phenolic compounds and mineral substances (Schreier, 1979). For these compounds to have an influence on the taste, they need to be present in levels of 1% or more.

The volatile compounds in wine can generally be perceived when present in much lower concentrations. This is because our sense of smell is extremely sensitive to certain aroma compounds. The perception thresholds of some compounds can vary between 10^{-4} and 10^{-12} g/L (Guadagni *et al*., 1963). As in many foods, the aroma of wine is caused by the interaction among several hundred different compounds. Because there is no real character impact compound, wine aroma is formed by the balance of all these compounds.

The development of flavour compounds in grapes and also during fermentation varies substantially due to the synergistic influence of various factors. These include environmental factors (climate, soil), grape cultivar, fruit condition (ripeness), numerous technological aspects (method of grape crushing, treatment of mash and must), fermentation conditions (pH, temperature, juice nutrients, microflora) as well as the various post-fermentation treatments such as ageing, blending, clarification and filtration (Rapp and Mandery, 1986).

Four major distinctions are made with regard to the formation of aroma in wine. The first is the aroma originating from the grapes. Wine aroma can also be derived from the components produced or changed due to the modifications caused by specific technological steps such as grape crushing and must treatment. The third is the aroma produced by substances which are formed or modified during fermentation, and lastly, the bouquet which results from the compounds originating during the ageing of wine through enzymatic or physicochemical actions in wood or in the bottle (Schreier, 1979).

2.1.1 Grape aroma

Owing to their occurrence in small quantities in grapes, only a few esters contribute to the aroma of *Vitis vinifera* varieties. These are mainly acetate esters of short chain alcohols. The acetates of some monoterpene alcohols and (E)-methyl geranoate are esters found in

Muscat type grape varieties. Esters contribute mainly to the intense and characteristic aroma of *V. labrusca* and *V. rotundifolia* varieties growing in the United States (Rapp and Mandery, 1986).

Interest in the monoterpenes originated due to their use in perfumes and as food flavours. Monoterpene alcohols and their derivatives play a crucial role in wine aroma, particularly for the aroma of Muscat cultivars (Gewürztraminer, Muscat blanc, Muscat d'Alexandrie) and aroma-related cultivars (Riesling, Scheurebe). To date more than 50 monoterpene compounds in grapes and wines are known (**Figure 2.1**). The most important monoterpene alcohols occurring in wine are linalool, geraniol, nerol, citronellol, α-terpineol and hotrienol. Ribéreau-Gayon *et al.* (1975) found that linalool and geraniol are the most aromatic within the terpene fraction. Geraniol and linalool play an important role in the aromas of grapes and wines as concentrations are often well above the olfactory perception thresholds. Nerol and α-terpineol have perception threshold values three or four times higher than linalool (100 μg/L).

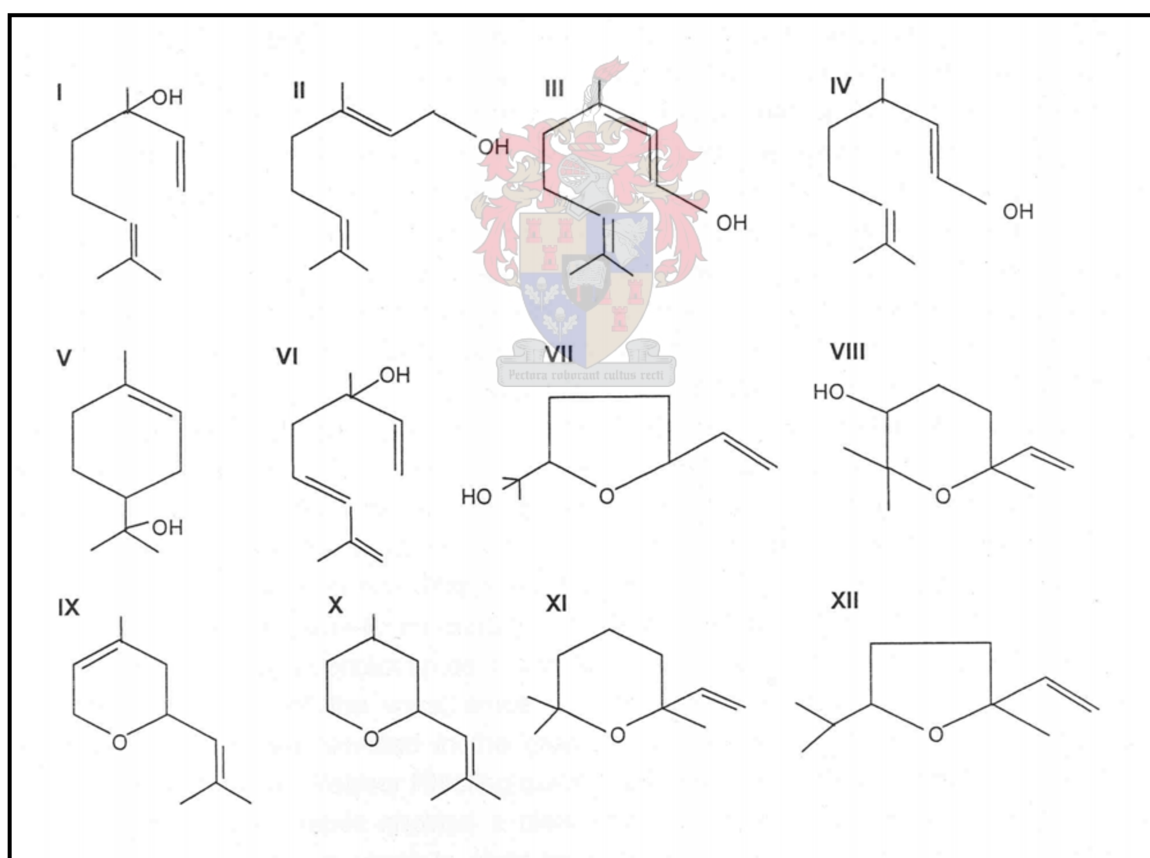

Figure 2.1 Volatile monoterpenes in wine. **I** - linalool, **II** - geraniol, **III** - nerol, **IV** - citronellol, **V** - α-terpineol, **VI** - hotrienol, **VII** & **VIII** - linalool oxides, **IX** - nerol oxide, **X** - rose oxide, **XI** & **XII** - ethers (Rapp and Mandery, 1986).

The terpenol content in grapes can be influenced by environmental factors among which the occurrence of *Botrytis cinerea* is prominent. This fungus causes the rotting of grapes but under special climatic conditions it is responsible for the noble rot. This rot is prerequisite for the production of botrytised wines having a distinct aroma. *B. cinerea* is incapable of producing terpenoids in grapes without terpenes, but transforms linalool which has been added to grape must into some other monoterpenes (Shimizu *et al*., 1982). With regard to the aroma composition of wines infected by *B. cinerea*, two compounds were found to be responsible for the flavour of these botrytised wines (Masuda *et al*., 1984). These compounds are ethyl-9-hydroxynonanoate and 4,5-dimethyl-3-hydroxy-2-(5H)-furanone (sotolone). The sotolone imparts a sweet, sugar- and caramel-like aroma, with a threshold value of 2-5 ppb. The concentration of this compound in botrytised wine is usually about 5-20 ppb. In normal wines made from uninfected grapes, the content of sotolone may be as low as below 1 ppb.

2.1.2 Fermentation aroma

The main part of wine aroma arises during yeast fermentation. Ethanol and glycerol are quantitatively the most dominant alcohols contributing to wine aroma. Following these alcohols are also diols, higher alcohols and esters. The latter group accounts for 0.2 - 1.2 g/L for white wines and 0.4 - 1.4 g/L for red wines. About 50% of these values are represented by n-propanol, n-butanol, 2-methylbutanol-1, 3-methylbutanol-1, phenylethanol, ethyl acetate and ethyl lactate. Apart from its distinctive smell, ethanol determines viscosity of wine, balances taste sensations and acts as a fixer for odours (Rapp and Mandery, 1986).

Higher alcohols are quantitatively the largest group of aroma compounds in alcoholic beverages. They are formed as secondary products of alcoholic fermentation. By definition, these alcohols refer to those possessing more than two carbon atoms. Higher alcohols, also known as fusel alcohols, can be recognised by their strong, pungent smell and taste. They can have a significant influence on the taste and character of wine (Lambrechts and Pretorius, 2000). Higher alcohols usually contribute to the desirable complexity of wine when present at concentrations below 300 mg/L. When their concentrations exceed 400 mg/L, the fusel alcohols are regarded as a negative influence on the quality of wine (Rapp and Mandery, 1986).

Aldehydes are the key compounds in the biochemical reaction involving the production of higher alcohols from amino acids and sugars by yeast. They contribute flavour characteristics ranging from ‘apple-like’ to ‘citrus-like’ to ‘nutty’, depending on the chemical structure. Because of their low sensory threshold values, aldehydes are important to the aroma and bouquet of wine. Among these, acetaldehyde is the major component contributing more than 90% of the total aldehyde content in wines and spirits (Lambrechts and Pretorius, 2000).

The volatile phenols are aromatic compounds that affect wine quality. These phenolic compounds usually originate from the metabolic activity of the wine spoilage yeasts, *Brettanomyces bruxellensis*. These yeasts can spoil wines by developing off-odours which have been described as mousy, wet wool, medicinal, smoky and spicy (Fugelsang and Zoecklein, 2003). The secondary metabolites of *B. bruxellensis* which are responsible for wine spoilage are 4-ethyphenol (4-EP) and 4-ethyguaiacol (4-EG). They are produced in a two-step mechanism from hydroxycinnamic acids, *p*-coumaric acid and ferulic acid respectively. During the first step, phenolic acids are directly decarboxylated to 4-vinylphenol and 4-vinylguaiacol by the enzyme cinnamate decarboxylase. In the second reaction, vinyphenol reductase converts 4-vinylphenol and 4-vinylguaiacol into 4-EP and 4-EG (Chatonnet *et al*., 1995). The precursors, *p*-coumaric acid and ferulic acid, are naturally present in must.

Volatile organic sulphur compounds make a considerable contribution to wine aroma because of their reactivity and extremely low threshold values. The most important sulphur-containing compound that predominantly occurs in wine is hydrogen sulphide (H_2S). The production of this compound has been the subject of many studies because of its occurrence in high amounts during the fermentation of grapes. H_2S has an unpleasant aroma with a low sensory threshold. It imparts an aroma which is reminiscent of rotten eggs (Rapp and Mandery, 1986). Recent studies show that high amounts of H_2S can also lead to the formation of other undesirable volatile sulphur compounds. In the past, one of the main sources of H_2S was the reduction of free elemental sulphur from residues originating with applications of dusting sulphur in the vineyard as fungicide. The formation of sulphur compounds is closely linked with yeast metabolism (Lambrechts and Pretorius, 2000).

Pectora roborant cultus recti

Esters are a group of volatile compounds present in wine, most of which are formed by yeasts during alcoholic fermentation. The concentration of esters usually found in wine is generally above their sensory threshold levels and they make up numerically the largest group of aroma compounds in alcoholic beverages. Esters mostly impart pleasant odours which are reminiscent of fruit (Lambrechts and Pretorius, 2000). Ethyl acetate is the main ester occurring in wine. Other esters also found in wine are those of fusel alcohols and short chain fatty acids. They are termed 'fruit esters' because of their pleasant, fruity aroma. Fatty acid ethyl esters are prominent for white wines in particular. These ethyl esters include ethyl butanoate, caproate, caprylate, caprate and laurate. Their amount is usually below 10 mg/L, but this value is approximately 10 times their perception threshold (Rapp and Mandery, 1986).

2.1.3 Wine bouquet

The bouquet of wine refers to more complex flavour compounds originating as a result of fermentation and ageing in barrels or bottles. During wine storage, several chemical

reactions pose a negative influence on the composition of volatile constituents in wine and subsequently transform the aroma into the bouquet. Wine bouquet can be derived from oxidation induced by the presence of aldehydes and acetals. It also arises as a result of reduction which is formed after ageing in bottles (Rapp and Mandery, 1986).

When the red wine is aged in wooden barrels it benefits from enhanced flavour arising from various aromatic components of wood extracted into the wine without becoming dominant in the final wine character. Phenolic compounds from lignin degradation were detected in wines which were aged in wooden casks, and also in whiskey and brandy (Rapp and Mandery, 1986). Apart from the extraction of wood elements and reactions of wood with the ageing wine, oxygen penetrates through the wood and causes drastic flavour changes.

In contrast to the bouquet of oxidation, acetals are relatively not important for the bouquet of reduction. Previous studies investigating changes in aroma substances of Riesling wines during storage in bottles showed that there is no rise in acetal concentration during bottle ageing. Contributing to the pleasant fruit-like aroma of new wines, the acetates are produced enzymatically in excess of their equilibrium concentrations. During storage they hydrolyse until they approach equilibrium with their corresponding acids and alcohols (Rapp and Mandery, 1986).

2.2 ENZYMES IN WINEMAKING

Over the past years, substantial progress has been made regarding the modification of wine flavour with the sole aim of improving wine aroma. Wine aroma can be derived from an interaction between aromas originating from different sources. Apart from aromas originating from the grapes and alcoholic fermentation, wine aroma can also be derived from metabolic activity of the lactic acid bacteria (LAB). These bacteria occur in wine during malolactic fermentation (MLF) which follows alcoholic fermentation. Although poorly understood, the metabolic potential of wine LAB is diverse and complex. A broad range of secondary modifications are of great importance for the taste and flavour improvement of wine (Liu and Pilone, 2000). These include amino acid metabolism, proteolysis and peptidolysis, ester synthesis and hydrolysis, metabolism of lipids, and hydrolysis of glycosides.

The hydrolysis of compounds contributing to wine aroma is achieved through the action of enzymes. Enzymes play a crucial role in the process of winemaking. During winemaking, enzymes are desired as early as the pre-fermentation stage. Their activities originate not only from the grape itself but also from yeasts and other microorganisms, such as fungi and bacteria (Canal-Llauberés, 1993). Enzymes derived from yeasts and fungi are well documented (Mateo and Di Stefano, 1997; Spagna *et al*., 1998) while those of wine LAB are poorly understood. Most of the work done on LAB enzymes has been

concerned with characterising these enzymes in the dairy industry (Visser *et al*., 1986; Williams and Banks, 1997).

Besides inherent enzymes present in grapes, yeasts and bacteria, the winemakers supplement the action of these endogenous enzymes by using commercial enzyme preparations. Using additional enzymes in wine is a common practice that has become ubiquitous in most winemaking sectors. It should, however, be noted that adding commercial enzyme preparations to wine is an expensive practice although it does not jeopardise the integrity of the traditional methods that many winemakers have adhered to through the centuries. Moreover, this practice is viewed as an artificial or unnatural intervention by the winemaker. Nevertheless, added to grape must or wine, enzymes can hydrolyse the problematic high molecular weight substances such as pectin, protein and β-glucan, improving clarification and filtration. Furthermore, enzymes can allow for enhanced flavour development by converting tasteless components into valuable components such as terpenols (www.biocatalysts.com).

The rest of this chapter gives a review on the mechanism of wine-related enzymes produced by wine-associated microorganisms, as well as their use in winemaking to enhance the organoleptic quality of wine. Special attention will be given to enzymes produced by the LAB due to their potential to hydrolyse flavour components that positively influence wine aroma. However, other aspects will also be discussed, such as enzymes from sources other than wine LAB.

2.3 HYDROLYSIS OF GLYCOSIDES

Many aromatic compounds found in grapes, must and wines occur in two different forms: free and sugar-bound. The sugar-bound components are generally non-volatile and therefore do not contribute to wine aroma. One of the major aroma components which contribute to the varietal character of aromatic or floral varieties are known as terpenes (Marais, 1983). Terpenes are one of the most important groups of aroma compounds of grapes, must and wines. Depending on the number of carbon isoprene units, terpene compounds can be classified into various groups, including monoterpenes, sesquiterpenes, diterpenes, triterpenes and carotenoids.

The monoterpenes are natural aroma compounds with very low sensory thresholds and are trace constituents in grapes, particularly in aromatic cultivars such as Muscat, Gewürztraminer and Riesling (Günata *et al*., 1985; Delcroix *et al*., 1994). Non-aromatic cultivars such as Sauvignon blanc and Chardonnay also contain monoterpenes but at lower concentrations (Augustyn *et al*., 1982; Simpson and Miller, 1984). The occurrence of monoterpenes in grape varieties has been divided into three groups, including: (1) intensely flavoured Muscats with monoterpene concentrations as high as 6 mg/L; (2) aromatic non-Muscat varieties, such as Gewürztraminer, Riesling and others, with total

monoterpene concentration of 1-4 mg/L; and (3) more neutral varieties not dependent upon monoterpenes for their flavour (Mateo and Jiménez, 2000).

It has been shown that three forms of monoterpenes are present in grape juice and wines. These forms include free-, polyhydroxylated- and glycosidically bound monoterpenes. From these, only the free monoterpenes are odorous (Williams *et al*., 1981). The most important terpenols and their aromas associated with the hydrolytic action of glycosidases are linalool (citrus), nerol (fresh fruit) and geraniol (freshly cut grass). The majority of these compounds are localised in the grape skins (geraniol and nerol) and juice (linalool), with very little being found in the pulp. Amongst all the terpene compounds, linalool is the one in highest concentration in the Muscat group, and is generally always above its threshold value (Wilson *et al*., 1986).

2.3.1 Acidic hydrolysis

The glycosidic precursors which impart an important aroma in wines can be hydrolysed either enzymatically through glucosidases or via acid hydrolysis (Günata *et al*., 1988). Acid hydrolysis has been studied as a method for the release of bound aroma compounds, where samples are adjusted to lower pH levels to break glycosidic bonds (Williams *et al*., 1981). However, the drawback is that acidic hydrolysis of terpene glycosides can provoke a molecular rearrangement of monoterpenols and they can consequently be transformed into other compounds (Mateo and Di Stefano, 1997). Further, several authors have suggested that acidic wine conditions may cause denaturing of these enzymes and inhibition of their activity (McMahon *et al*., 1999; Pilatte *et al*., 2003; Ugliano *et al*., 2003). Therefore, abiotic stresses, such as low pH levels, may be considered a limiting factor in the commercial use of glycosidase enzymes (Spano *et al*., 2005). Nevertheless, this way to liberate terpenes simulates the reactions which take place during ageing of wines (Mateo and Jiménez, 2000).

2.3.2 Enzymatic hydrolysis

Wine aroma and flavour are determined primarily by the glycosidic compounds which are present in wine partly as free aglycones (flavour precursors) and largely as bound glycoconjugates (Abbott *et al*., 1993; Williams and Francis, 1996). Bound glycosides exist mainly as monoglucosides or disaccharides. The glycosides that are commonly found are 6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside, 6-O-α-L-arabinofuranosyl-β-D-glucopyranoside, 6-O-α-L-apiofuranosyl-β-D-glucopyranoside, or β-D-glucopyranoside (Günata *et al*., 1985; Salles *et al*., 1990).

Unlike acidic hydrolysis which can interfere with wine aroma, enzymatic hydrolysis is alternatively preferred for hydrolysing sugar-conjugated flavour precursors. Under the latter conditions, the changes in the natural monoterpenol distribution are minimal (Günata *et al*., 1988). Some aromatic aglycones may be released through the sequential hydrolytic

action of glycosidases. In general, the mechanism for enzymatic hydrolysis of glycosidic precursors occurs through two successive steps (**Figure 2.2**). In the first phase, the glucose is separated from the terminal sugars by a hydrolase group (α-L-arabinofuranosidase) before, in the second phase, β-D-glucosidase (also known as β-D-glucopyranosidase) breaks the bond between the aglycone and glucose (Günata *et al*., 1988; Spagna *et al*., 1998), hence liberating the volatile flavour precursor.

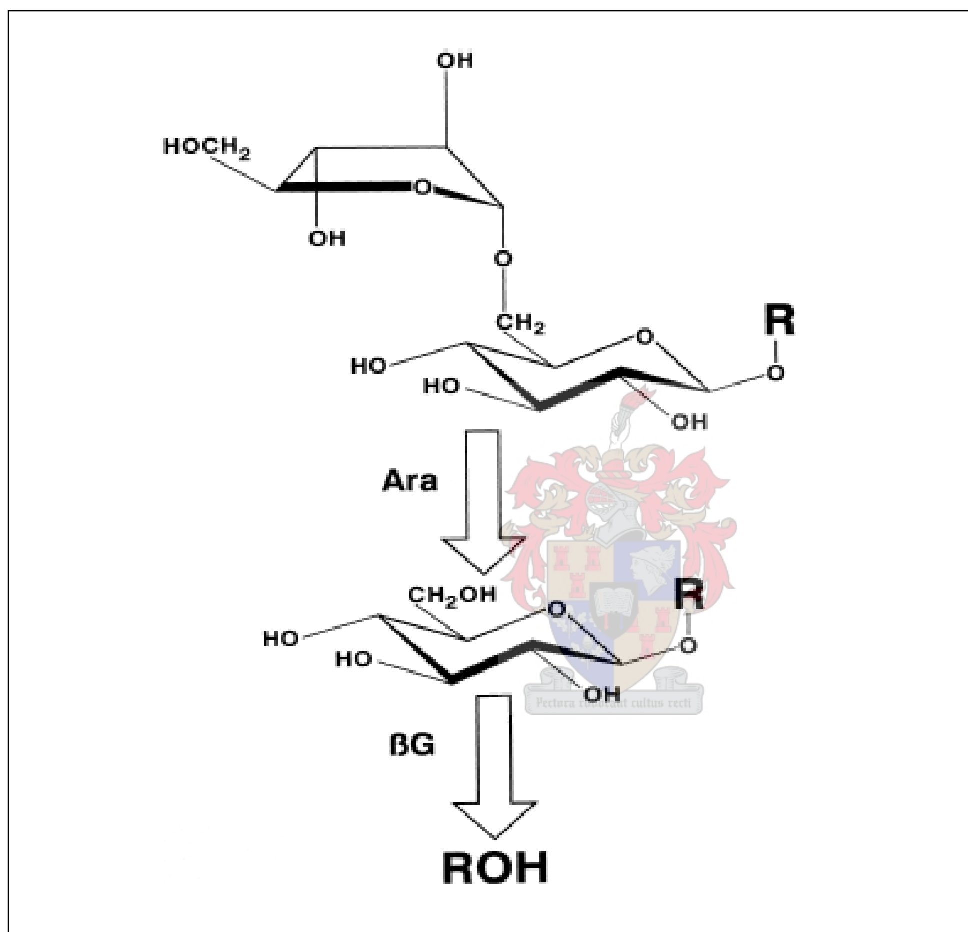

Figure 2.2 Mechanism of α-L-arabinofuranosidase (Ara) and β-D-glucosidase (βG) on glycosidic precursors. ROH represents the volatile aglycone such as monoterpenols and other alcohols (Spagna *et al*., 1998).

Collectively, glycoside hydrolases (glycosidases) refer to those enzymes that hydrolyse O-glucosyl compounds (Aryan *et al*., 1987). These enzymes cleave a linkage between the aglycone and glycone. If the carbohydrate residue is glucose then the resulting compound is a glucoside. Similarly, if the carbohydrate residue is glucose then the enzyme is glucosidase. Glycosidases generally act on glycosidic compounds containing a sugar and non-sugar residue in the same molecule. They then catalyse the

hydrolysis of an acetal linkage between a carbohydrate and a non-carbohydrate moiety. The sugar and non-sugar components are commonly referred to as glycones and aglycones, respectively. The non-carbohydrate residues may be methyl alcohol, glycerol, sterol, phenol, etc.

β-Glucosidases (β-D-glucoside glucohydrolases; EC 3.2.1.21) are enzymes that hydrolyse a bond between glucose and an aglycone, such as monoterpene, norisoprenoid or resveratrol (Czjzek *et al*., 1999). The use of commercial enzymes, such as β-glucosidases, has attracted much interest in commercial preparation of wine because of their ability to catalyse the hydrolysis of glycosidically bound components, thereby releasing volatile compounds which will enhance wine aroma. The sugar-conjugated compounds are generally non-volatile and they therefore do not contribute directly to wine aroma.

In general, the cleavage of glycosidic bonds by β-glucosidases is important for a number of biological pathways, such as cellular signalling, biosynthesis, degradation of structural and storage polysaccharides, and host-pathogen interactions (Czjzek *et al*., 1999). β-Glucosidases can be found in plants, yeasts, fungi and bacteria. It has been shown that these enzymes are most often associated with the cell wall in microorganisms, yet there is still some debate as to whether they remain associated with the cell wall or whether they are always free in the media (Darriet *et al*., 1988).

2.3.2.1 Grape glycosidases

Grapes have been shown to possess enzymes capable of hydrolysing aroma precursors and, more specifically, terpenyl glycosides. These glycosides are responsible for the varietal character of many grapes (Marais, 1983; Rapp and Mandery, 1986). However, only low activities of α-rhamnosidase, α-arabinosidase or β-apiosidase have been detected (Günata *et al*., 1990*b*). β-Glucosidases originating from the grapes have been shown to have optimal activity at pH 5.0 and are inhibited by glucose. Moreover, grape glycosidases are not able to hydrolyse sugar conjugates of tertiary alcohols such as linalool; they exhibit specificity with respect to aglycone hydrolysis (Aryan *et al*., 1987). Further studies on the properties of grape glycosidases have reported that grape β-glucosidases are relatively unstable with low activities at grape juice or wine pH values (Lecas *et al*., 1991). Collectively, these results suggest that inherent glycosidases of the grape are hardly suitable for liberating glycosidically bound conjugates able to enhance wine aroma.

2.3.2.2 Exogenous glycosidases

Several grapevine fungal pathogens, such as *Aspergillus* and *Botrytis*, produce large quantities of glycosidase activities that have high level of specificity to purified wine glycosides (Manzanares *et al*., 2000). *Aspergillus*, mainly *Aspergillus niger*, is a common

source of commercial enzyme preparations with “GRAS” (Generally Regarded As Safe) status. Glycosidases produced by *Aspergillus* have been shown to increase the amounts of terpenols in a model wine solution (Spagna *et al*., 1998). The most suitable enzymic preparations that are used during the winemaking process are those which possess all glycosydic activities (Cordonnier *et al*., 1989). However, the enzymes produced by fungi are often impure and require purification before characterisation in the laboratory (Spagna *et al*., 1998). They also pose undesirable effects on the wine (Abbott *et al*., 1991). More importantly, the enzymes of fungi are frequently ineffective in wine (Aryan *et al*., 1987). Results found by Aryan *et al*. (1987) concerning the inhibition of fungal β-glucosidase activity by glucose suggest that fungal glycosidases are hardly effective in cleaving sugar-bound components contributing to wine aroma.

2.3.2.3 Yeast glycosidases

Among the yeasts, a strain of *Hansenula* species isolated from fermenting must was reported to have β-glucosidase activity (Grossmann *et al*., 1987). This enzyme, although able to liberate aroma substances in wine, seemed to be less effective in must; it was inhibited by glucose. β-Glucosidases of *Candida molischiana* (Gonde *et al*., 1985) and *C. wickerhamii* (Leclerc *et al*., 1984) have also been shown to possess activities towards various β-glucosides. These were, however, little influenced by the nature of aglycone (Günata *et al*., 1990*a*).

Glycosidase activities have also been studied in yeasts of oenological interest, with much attention devoted to *Saccharomyces cerevisiae*. Darriet *et al*. (1988) located *S. cerevisiae* β-glucosidase in the periplasmic space of yeast cells. It was also shown that the activity of this enzyme was glucose independent. This is in contrast to what has been found for β-glucosidase from grape (Lecas *et al*., 1991) and fungal origin (Aryan *et al*., 1987). Further studies (Delcroix *et al*., 1994; Mateo and Di Stefano, 1997) have confirmed that β-glucosidase from *S. cerevisiae* is weakly sensitive to the presence of sugar.

Based on the results obtained thus far regarding β-glucosidase activity in wine yeasts, it is now possible to conclude that yeast β-glucosidases can be used as a way to hydrolyse glucosidase precursors of the terpenes in grape juice (Mateo and Di Stefano, 1997). This is largely due to their enzymatic activity in contrast to currently available commercial enzymes whose activity is barely inhibited by glucose.

2.3.2.4 Bacterial glycosidases

Although glycosidase activities have been investigated from sources other than LAB, little is known about the potential of wine LAB to possess glycosidase activities. Preliminary studies done on LAB β-glucosidase have focused on evaluating the activity of this enzyme mainly in *Oenococcus oeni*. However, the research is now directed towards evaluating glycosidase activities of other genera of wine LAB.

The results reported on the ability of wine LAB to hydrolyse glycoconjugates are contradictory. β-Glucosidase activity in wine LAB (mainly *O. oeni*) was observed in a synthetic media by Guilloux-Benatier *et al*. (1993). This was further confirmed by Grimaldi *et al*. (2000) who found readily detectable activity of β-glucosidase in 11 commercial preparation of *O. oeni*. Further studies (Mansfield *et al*., 2002) detected the production of β-glucosidase enzymes in strains of *O. oeni*, although cultures of the same strains failed to hydrolyse native grape glycosides. In contrast, McMahon *et al*. (1999) observed no enzymatic activity in commercial strains of *O. oeni* against arbutin, an artificial glycosidic substrate.

These findings suggest that even wine LAB have the potential to hydrolyse glycoconjugates consequently affecting wine aroma and colour. However, β-glucosidase enzymes in yeasts and bacteria are usually inhibited by winemaking parameters such as pH, ethanol and sugars (Delcroix *et al*., 1994; McMahon *et al*., 1999; Grimaldi *et al*., 2000). The acidic conditions in wine may result in denaturing and/or inhibition of enzymatic hydrolysis, although strains of *O. oeni* may retain 80% of maximum β-glucosidase activity at pH 3.5 (Grimaldi *et al*., 2000). It is therefore crucial to understand if and how β-glucosidase enzymes are regulated by abiotic stresses. This will enable the selection of starter cultures able to positively alter the wine volatile fraction throughout the liberation of glycosidically bound aroma components (Spano *et al*., 2005).

Although many studies have focused on evaluating β-glucosidase activity from the malolactic bacteria, *O. oeni*, a recent study (Spano *et al*., 2005) has further evaluated this enzyme by determining specific probes of β-glucosidase genes from *Lactobacillus plantarum* and *O. oeni*. In this study, the authors compared amino acid sequences of β-glucosidase proteins from different LAB species such as *Lb. plantarum*, *O. oeni*, *Pediococcus damnosus*, *Lb. paraplantarum* and *Lb. pentosus*. From these results, it is probable that wine LAB can impart desirable characteristics in the flavour composition of wine.

2.4 HYDROLYSIS OF LIPIDS

Lipases (triacyglycerol acylhydrolases; EC 3.1.1.3) are enzymes hydrolysing tri-, di- and monoglycerides at the interface of a heterogeneous system. They are widespread in nature and have been found in microorganisms, animals and higher plants. The initial step in the hydrolysis is the splitting of the fatty acids esterified to the primary hydroxyls of glycerol (Jaeger *et al*., 1994).

The systematic name of lipase is given as glycerol-ester hydrolase. This definition does not separate the action of a lipase clearly enough from that of an esterase. A lipase and esterase may act on the same substrate, depending on the physical nature of the substrate. For example, triacetin is hydrolysed by an esterase when the former is present

in water-soluble form, but once the aqueous phase is supersaturated and a heterogeneous system is formed, this substrate is hydrolysed by lipase. It follows that the reaction rate of a lipase is a function of the total surface area of the interface, rather than of the substrate concentration as such in the assay system (Hübscher, 1970). Microbial lipases are of great interest to the industry due to their substrate specificity and ability to remain active in organic solvents (Sharon *et al*., 1998). Applications of microbial lipolytic enzymes are widely found in food, detergent, pharmaceutical and chemical industries (Godfrey, 1995; Sharon *et al*., 1998).

Lipases belong to the class of serine hydrolases and do not require any cofactor. The natural substrates of lipases are triacylglycerols, which have very low solubility in water. With regard to their mechanism of action, lipases act on the carboxyl ester bonds of triacyglycerols at the interface between aqueous and organic phases containing substrate, thereby liberating organic acids and glycerol (**Figure 2.3**). Under certain experimental conditions, such as in the presence of traces of water, lipases are capable of reversing the reaction. The reverse reaction leads to esterification and formation of glycerides from fatty acids and glycerol (Ghosh *et al*., 1996).

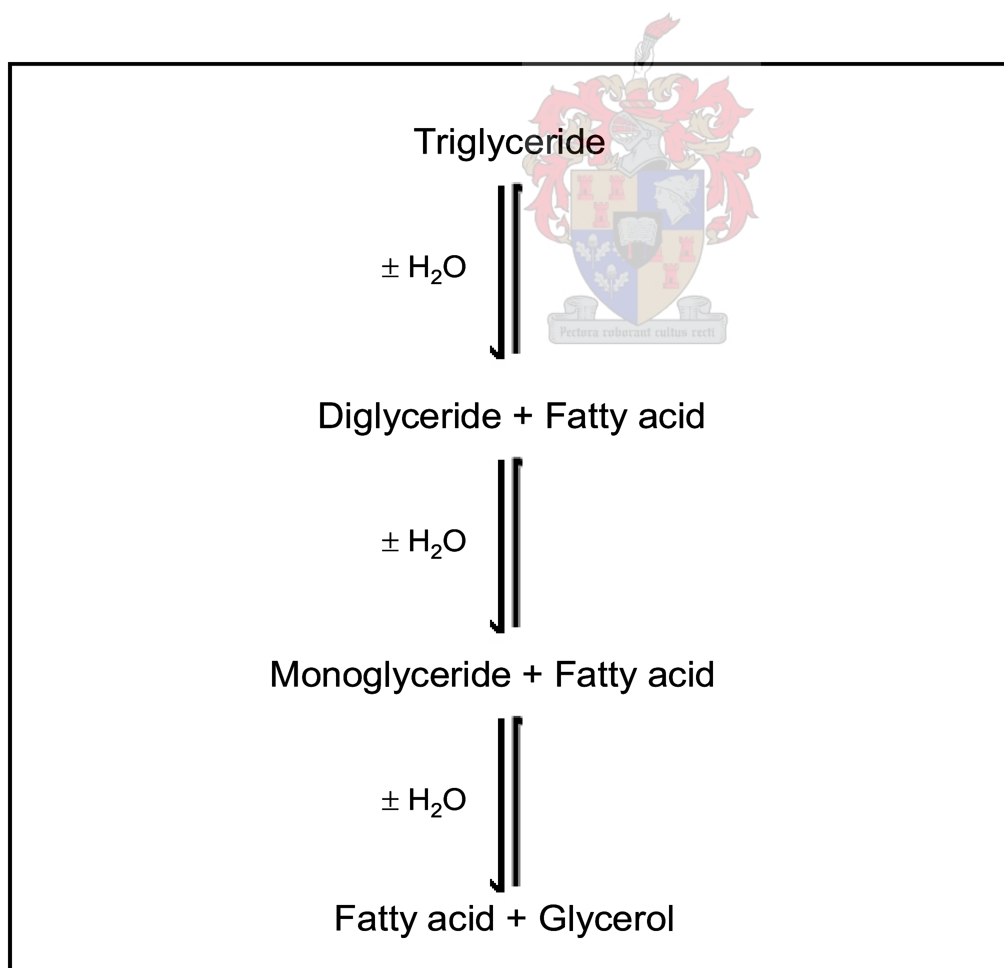

Figure 2.3 Enzymatic reaction of a lipase (Ghosh *et al*., 1996).

Lipolytic activity has been found in *Lactococcus* species (Kamaly *et al*., 1990; Lawrence *et al*., 1967; Umemoto and Sato, 1978). Fryer *et al*. (1967) found tributyrin lipase activity in strains of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*. The lipase was found to be most active towards lactococcal neutral lipids (Umemoto and Sato, 1978). This implies the primary role of the enzyme in meeting the physiological and metabolic functions of the organisms rather than the hydrolysis of exogenous triacyglycerol substrates (Holland and Coolbear, 1996). In addition, Holland and Coolbear (1996) demonstrated that lipolytic activity levels of lactococcal strains are low in comparison to organisms such as pseudomonads and other typically lipolytic dairy spoilage microbes.

2.4.1 Lipase assay systems

A variety of techniques to determine lipolytic activity have been developed. Some of these techniques are employed for the determination of lipolytic activity from lactic acid bacteria (Jaeger *et al*., 1994). A summary of currently used techniques for the determination of lipase activity is given in **Table 2.1**. Plate assays have been described to screen for lipase-producing microorganisms. Lipase-producing colonies can be identified on agar medium containing indicator dyes such as Victoria blue, Methyl red, Phenol red or Rhodamine B (Converse *et al*., 1981; Kouer and Jaeger, 1987; Samad *et al*., 1989). The indicator dyes will react with the free fatty acids released via the hydrolysis of triacyglycerides (Meyers *et al*., 1996). Substrate hydrolysis results to the formation of colour or fluorescent halos around bacterial colonies upon exposure to UV illumination (Jaeger *et al*., 1994).

In a colorimetric assay using long-chain fatty acid 1,2-diglycerides, the lipase produces a 2-monoglyceride from which glycerol is released by the action of a 2-monoglyceride lipase. The glycerol concentration is determined by a sequence of enzymatic reactions with glycerol kinase, glycerol phosphate oxidase and peroxidase. All of these produce a violet quinone monoamine dye with a peak absorption at 550 nm (Fossati *et al*., 1992). Another technique involves a series of coupled enzymatic reactions which use the oxidation of NADH as the final step (Woollett *et al*., 1984). Rhodamine 6G is used for forming a complex with free fatty acids liberated during lipolysis. A pink colour appears and absorbance is measured at 513 nm (van Autrye *et al*., 1991). Enzymatic activity can also be measured using chromogenic substrates, such as para-nitrophenyl-esters or β-naphthyl esters. However, these compounds are not suitable for specific lipase assays because they can also be hydrolysed by esterases (Miles *et al*., 1992; Stuer *et al*., 1986).

Another useful technique to assess lipolytic activity is the pH-stat method (Lee and Rhee, 1993), which uses triacyglycerides as well as natural complex substrates, such as butter oil (olive oil). The lipolytic reaction liberates an acid which can be assayed titrimetrically. Since the pH is an important parameter for enzyme catalysis, it should be kept constant by continuously adding NaOH solution (Erlanson and Borgström, 1970; Gargouri *et al*., 1986).

Table 2.1 Currently used assay systems for the detection of lipolytic microorganisms (adapted from Jaeger *et al*., 1994)

PLATE ASSAYS				
Substrate	*Reaction product*	*Method*		
Glycerides[1]	FFA[2]	Coloured indicators (Victoria blue, rhodamine blue, phenol red, etc.)		
SPECTROSCOPIC				
Substrate	*Reaction product*	*Method*	*Final product*	*Wavelength*
1,2-diglycerides	Glycerol	Enzymatic conversion	Quinone	550 nm
Glycerides[1]	FFA	Enzymatic conversion	NAD	340 nm
Glycerides	FFA	Complex formation	Rhodamine 6G	513 nm
Glycerides[1]	FFA	Negative charge	Safranine	520 / 560 nm
Glycerides	FFA	Complex formation	Cu(II) salt	715 nm
*p*NP esters	*p*-nitrophenol	Product is coloured		410 nm
FLUORESCENCE				
Substrate	*Reaction product*	*Method*	*Final product*	*Wavelength*
Glycerides[1]	FFA	Complex formation	11-undecanoic acid	ex. 350 nm, em. 500 nm
Glycerides[3]	FFA analogues	Fluorescence shift	FFA analogues	ex. 340 nm, em. 400 nm
TITRIMETRIC				
Substrate	*Reaction product*	*Method*		
Glycerides[4]	FFA	pH - determination		
SURFACE PRESSURE				
Substrate	*Reaction product*	*Method*		
Dicaprin	FFA	Measurement of barrier movement		
Triglycerides[5]	FFA	Measurement of drop volume or decrease in surface tension		

[1] Triolein
[2] Free fatty acids
[3] Glycerides with pyrene ring
[4] Tributyrin
[5] Long chain triglycerides

2.4.2 Lipolysis in wine LAB

The lipolytic system of LAB under the winemaking conditions has not been given thorough attention. Much of the work undertaken in assessing lipolytic activity has been focused on the LAB lipases from the dairy industry. Preliminary study that was done by Davis *et al*. (1988) found that several strains of *O. oeni* and one species of *Lactobacillus* exhibited lipolytic activity. In contrast, a more recent study failed to find any lipolytic activity in wine isolates comprising 32 *Lactobacillus* strains, two *Leuconostoc* strains and three

Lactococcus strains (Herrero *et al*., 1996). This follows that LAB are acknowledged for being weakly lipolytic in comparison to other groups of bacteria such as *Pseudomonas*, *Aeromonas*, *Acinetobacter* and *Flavobacterium* (Kalogridou-Vassiliadou, 1984).

Wine lipids can originate from a number of sources, including grape berries (Gallander and Peng, 1980; Miele *et al*., 1993) and yeast autolysis (Pueyo *et al*., 2000). Within the berry, grape lipids can be derived from skin, seeds and berry pulp. The grape lipid profile varies with grape maturation (Bauman *et al*., 1977), climate (Izzo and Muratore, 1993) and variety (Gallander and Peng, 1980). Red wines tend to have greater total lipid contents than white varieties. In addition, variation is also observed with respect to the concentration and fatty acid composition of neutral lipids, glycolipids and phospholipids (Miele *et al*., 1993).

During yeast autolysis which occurs after fermentation, many different types of lipids are liberated, including tri-, di-, and monoacyglycerols and sterols. However, these lipids are produced in amounts and proportions which vary with respect to the yeast strain, and they have been shown to have an influence on the sensory properties of sparkling wine (Pueyo *et al*., 2000). The breakdown of triacylglycerols to fatty acids and glycerol plays a major role in the development of flavours. Microorganisms produce a wide spectrum of lipases with variations in substrate specificity, reaction rate, thermal stability, optimum pH, etc. (Lee and Rhee, 1993).

2.5 SYNTHESIS AND HYDROLYSIS OF ESTERS

A large number of volatile compounds have been identified in wine, with esters being prominent in determining wine aroma and flavour. Esters are a large group of volatile compounds occurring in wine as secondary products of sugar metabolism by yeasts during alcoholic fermentation. They are usually present in wine at concentrations above their sensory threshold (Matthews *et al*., 2004). Esters can be derived from grapes (Rapp and Mandery, 1986), chemical esterification of alcohols and carboxylic acids (Etievant, 1991), or through an enzyme-catalysed esterification of a fatty acid to an alcohol (Nordström, 1961).

Esters have the ability to alter the organoleptic quality of wine by imparting a fruity character. However, they can have a negative influence at concentrations beyond their threshold levels. The most important wine esters and their aromas are isoamyl acetate (banana), ethyl hexanoate (fruity, violets), ethyl octanoate (pineapple, pear) and ethyl decanoate (floral) (Lambrechts and Pretorius, 2000). During winemaking, the presence of esterolytic activity could result in either the increase or decrease in wine organoleptic quality, depending on the ester involved (Davis *et al*., 1988). Further, the compounds produced as a result of esterolytic activity could also enhance wine aroma (Etievant, 1991; Lambrechts and Pretorius, 2000).

2.5.1 General properties of esterases

Esterases (acetyl ester hydrolases; EC 3.1.1.6) are enzymes capable of hydrolyzing esters into corresponding alcohols and carboxylic acids. They therefore determine the final levels of esters present during wine fermentation.

Esters can be classified in accordance with their substrate specificity. For example, the group of carboxyl esterases preferably hydrolyse short-chain fatty acid esters as their substrates, particularly the six-carbon fatty acid esters. Additionally, these esterases have a broad range of substrate specificity and are thus called non-specific esterases (Parkkinen and Suomalainen, 1982). Carboxyl esterases can further be sub-classified into phenolic acid esterases, which act on esterified phenolic acids, and acetyl esterases, which are involved in cell wall degradation. Other types of esterases incorporate acetylcholine esterases, cholesterol esterases and thio-esterases (Kroon *et al*., 1997).

2.5.2 Esterolytic activity of bacteria

Esterolytic activities have been reported for several dairy LAB (Lee and Lee, 1990) and they are usually higher in lactobacilli than in lactococci. Based on biochemical data, esterases are highly active over a broad range of pH and temperature values. With regard to their substrate specificity, esterases prefer β-naphthyl esters containing short-chain fatty acids (C_4-C_8) and remarkable activity on tributyrin has also been reported. Activity declines with medium- and long-chain fatty acid substrates. Further, the kinetic studies of an esterase enzyme from *Lb. casei* subsp. *casei* IFPL731 showed high affinity for the substrates *p*-nitrophenyl butyrate and *p*-nitrophenyl caprylate (Castillo *et al*., 1999).

Esterases from several LAB strains have been shown to be strongly inhibited by phenylmethylsulphonyl fluoride (PMSF) (Castillo *et al*., 1999) and this suggests that a serine residue might be involved in the catalytic mechanism of the enzyme. It has been recognised that most of the proteins in the family of esterases and lipases have a Ser-Asp-His catalytic triad, similar to that observed in serine proteinases (Drablos and Petersen, 1997). In addition, inactivation of esterase by PMSF could be an indication for essential OH groups in its active site. Di-isopropyl fluorophosphate (DFP), which has a similar inhibitory effect as PMSF, could not inactivate the esterase and this might be due to its greater steric demand (Tsakalidou and Kalantzopoulos, 1992).

Regarding inhibition of enzyme by metal ions, previous studies have reported a strong inhibition of esterase by Hg^{2+} and Ag^{+}, and a moderate stimulation by Ca^{2+}, Mg^{2+} and Mn^{2+} (Lee and Lee, 1990). The stimulatory effect of Ca^{2+} may be attributed to better alignment of the enzyme on the substrate molecule and to the neutralisation of fatty acids liberated from the substrate. Inhibition by the Hg^{2+} may be due to its binding to the thiol groups of the enzyme. Inhibition by the Ag^{+} may be attributed to a reaction with a histidine residue in the enzyme (Chopra *et al*., 1982; Lee and Lee, 1990).

The current knowledge of LAB esterases is based primarily on work done in the dairy industry, in which these enzymes are directly involved in the flavour development of cheeses (Hosono *et al*., 1974). Most of this work has focused on the metabolism of esters by LAB, and it is now suspected that esterases have the ability to both synthesise and hydrolyse esters (Liu, 2002). In a preliminary study aimed at evaluating esterolytic activity of the wine LAB, Davis *et al*. (1988) found that the majority of LAB strains were able to hydrolyse an ester substrate, although these enzymes (esterases) were not further characterised or evaluated for their ability to synthesise esters. Zeeman *et al*. (1982) reported a small decrease in the levels of some esters following MLF. This could be attributed to the activity of esterases. These results suggest that like the dairy LAB isolates, esterases of wine LAB are also involved in both the synthesis and hydrolysis of esters.

2.5.3 Yeast esterases

During alcoholic fermentation by yeast, esters are produced in a reaction between alcohol and acyl CoA molecules, which are primarily the key intermediates in the production of free organic acids (Berry, 1995). In *S. cerevisiae*, esters are usually hydrolysed by the action of esterases. Schermers *et al*. (1976) found a positive correlation between esterase activity and the level of acetate esters in *S. cerevisiae*. However, Suomalainen (1981) suggested that esterase enzymes could also produce esters by the reverse reaction in the absence of acetyl-CoA, although this reaction proceeds very slowly.

Besides ester formation by *S. cerevisiae*, a number of yeasts have also been reported to synthesise esters to contribute to flavour development. It was previously shown that *H. anomala* and *C. krusei* yeasts produce less esters than, for example, *S. pombe* (Suomalainen and Lehtonen, 1979). These authors also showed that *S. cerevisiae* produces significantly more isoamyl acetate, ethyl caproate, ethyl caprylate and ethyl caprate than does *S. uvarum*.

2.6 PROTEOLYSIS AND PEPTIDOLYSIS

To date, numerous strains of LAB have been shown to possess proteolytic activities that allow them to degrade caseins (Magboul *et al*., 1997; Monnet *et al*., 1989; Sasaki *et al*., 1995). However, more studies have focussed on characterising the proteolytic systems of LAB in the dairy industry in which these enzymes are directly involved in the development of flavour (Visser *et al*., 1986; Williams and Banks, 1997). Although there are pronounced variations in LAB with respect to their proteolytic activity, some LAB are known to contain proteolytic systems that allow them to grow on protein-rich substrates. There are two traits that differentiate these LAB from many other proteolytic microorganisms (Kok and De Vos, 1993). Firstly, LAB are fastidious organisms with multiple amino acid requirements.

Consequently their growth is critically dependent on efficient systems for the degradation of proteins and the transport of amino acids and small peptides. Secondly, several LAB contain a proteolytic system that is highly specific and results in the production of unique peptides (Kok and De Vos, 1993).

LAB are a group of organisms that are used as starter cultures in the manufacture of dairy products. Among these, *Lactococcus* species are the dominant organisms (Hugenholtz, 1986). The degradation of milk proteins (caseins) by lactococci yields peptides and amino acids that are the sources of essential amino acids stimulating their growth (Chopin, 1993). On the other hand, the products resulting from casein degradation also play a critical role in the flavour development of dairy products (Poolman *et al*., 1998). However, other undesirable bitter-tasting peptides can also be produced thereby leading to the development of off-flavours (Kunji *et al*., 1996).

The sections that follow give a review about kinetics of proteolytic systems of the LAB isolated from dairy food products. Special attention will also be focused on the proteolysis of wine LAB. Other topics will also be covered, including the classification and location of proteinases, and occurrence of proteinases in LAB genera other than *Lc. lactis* which is well known for possessing proteolytic enzymes.

2.6.1 The proteolytic system

Caseins constitute about 80% of all proteins present in bovine milk and serve as the major organic nitrogen source for the growth of starter cultures in milk fermentations (Exterkate and de Veer, 1987*a*; Mills and Thomas, 1981). The four different types of caseins found in milk are α_{s1}-, α_{s2}-, β- and κ-caseins. They are organised in micelles to form soluble complexes (Schmidt, 1982). The enzymes which are active in casein degradation by lactococci can be divided into five groups: (i) a cell wall-associated caseinolytic proteinase, (ii) an extracellular peptidase, (iii) amino acid transport systems, (iv) peptide transport systems, and (v) intracellular peptidases. All these enzymes together form the proteolytic system of lactococci (Smid *et al*., 1991).

2.6.2 General properties of proteinases

Based on biochemical studies, lactococcal proteinases are very large proteins with molecular weights ranging from 80-145 kDA, pH optimum around 5.5-6.5, isoelectric points of 4.40-4.55 and can either be activated or stabilised by Ca^{2+}-ions. In addition, lactococcal proteinases are serine-type proteinases since they are inhibited by the typical serine proteinase inhibitors, PMSF and DFP. On the basis of location, it is generally agreed that proteinases are located primarily in the cell wall (Kok, 1990; Pritchard and Coolbear, 1993).

2.6.3 Classification of proteinases

A number of lactococcal strains has been classified based on the differences in their proteinase activity, -specificity and -immunology (Visser *et al*., 1986; Hugenholtz *et al*., 1984; Laan *et al*., 1988). Based on the degradation patterns of α_{s1}-, β- and κ-caseins, two proteinase specificity-classes have been identified in lactococci. They are generally referred to as P_I and P_{III} (Visser *et al*., 1986). The primary substrate of P_I-type enzymes is β-casein although κ-casein is also degraded, while P_{III}-type enzymes degrade α_{s1}-, β- and κ-caseins (Pritchard and Coolbear, 1993). Although both types of enzymes attack β-casein, their degradation patterns and specificities differ (Smid *et al*., 1991).

2.6.4 The proteolytic pathway of *Lactococcus lactis*

The proteolytic system is composed of three components: (i) proteinases which initially cleave caseins to peptides, (ii) peptidases which cleave the peptides and amino acids, and (iii) transport systems involved in the cellular uptake of small peptides and amino acids (Law and Haandrikman, 1997). LAB that are used in the dairy industry for the production of food products have an active proteolytic system that is involved in the degradation of caseins (Poolman *et al*., 1998).

The hydrolysis of milk proteins (caseins) generates peptides and amino acids that can subsequently be taken up by the cells. Proteolysis is initiated by a single cell-wall bound extracellular proteinase (PrtP) (Figure 2.4). However, not all the dairy LAB strains contain such an extracellular proteinase. Therefore, strains not possessing this enzyme are mainly dependent on other strains in the starter culture for the production of peptides and amino acids. Several of these peptides are released from the C-terminal part of proteinase (Kunji *et al*., 1996).

The uptake of peptides by the cells occurs via oligopeptide transport system (Opp) and di-tripeptide transport system (DtpT), although the latter transport system plays a minor role in the uptake of essential amino acids in the form of peptides. Other peptides do accumulate in the medium in spite of a functional Opp system. The fact that large peptides accumulate in the medium is a result of the size-exclusion restrictions of the oligopeptide transporter. In addition, certain peptides may not be transported due to the competition of peptides for the oligopeptide binding protein, although the lactococcal oligopeptide transport system has a broad substrate specificity (Kunji *et al*., 1996; Poolman *et al*., 1998).

Following uptake, the peptides are hydrolysed intracellularly by a variety of peptidases that have extensively been studied in both lactococci and lactobacilli. These peptidases of LAB can be divided into endopeptidases, aminopeptidases, di-tripeptidases and proline-specific peptidases (Kunji *et al*., 1996). Biochemical and genetic studies suggest that these enzymes are located intracellularly and therefore play no role in the extracellular degradation of casein-derived peptides (Poolman *et al*., 1998).

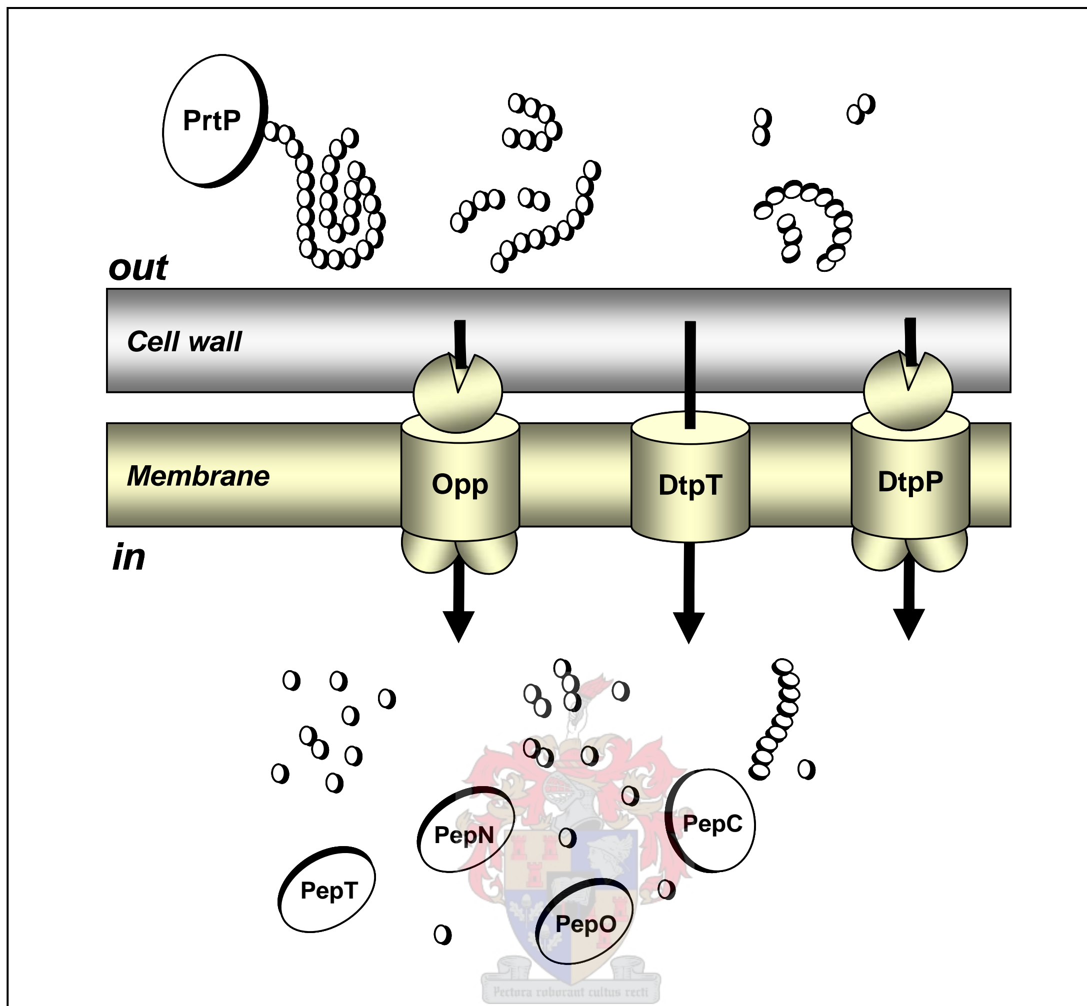

Figure 2.4 The proposed pathway for the proteolytic degradation of caseins by *Lactococcus lactis*. PrtP - extracellular proteinase enzyme; Opp - oligopeptide transport system; DtpT - di-tripeptide transport system for hydrophilic substrates; DtpP - di-tripeptide transport system for hydrophobic substrates; PepN, PepT, PepC and PepO are various intracellular peptidases (Kunji *et al*., 1996).

2.6.5 Localisation of proteolytic enzymes

An analysis of products from casein degradation has indicated that most peptides released from casein by the proteinase are too large to be taken up by the cell (Law, 1978; Rice *et al*., 1978). Therefore, extracellular peptidase activity is needed in order to obtain peptides that can be transported across the cytoplasmic membrane (Tan *et al*., 1992) to fulfil the needs of *Lc. lactis* for essential and growth-stimulating amino acids (Smid *et al*., 1991).

An extracellular location of lactococcal peptidases has been proposed because of their presumed role in casein degradation. However, PrtP, the lactococcal proteinase, remains the only proteolytic enzyme whose extracellular location is certain (Law and Haandrikman,

1997). Based on the assumption that PrtP-generated casein degradation products are too big to traverse the membrane, extracellular peptidases have been implicated in the proteolytic pathway (Exterkate and de Veer, 1987*c*; Law, 1979; Smid *et al*., 1989).

However, several peptidases from lactococci have previously been characterised biochemically, immunologically and genetically. The data summarised in **Table 2.2** strongly suggest that lactococcal peptidases are located intracellularly (Baankreis, 1992; Tan *et al*., 1992).

Table 2.2. Well-characterised proteolytic enzymes of *Lactococcus lactis*

Enzyme	*Mw (kDA)*	*Class*	*Substrate*	*Leader peptide*	*Location*	*References*
PrtP	200	Serine	casein	Yes	envelope	(a)
Neutral proteinase	93	Metallo	β-casein		intracellular	(b)
NisP	54	Serine	nisin precursor	Yes		(c)
PepA	43	Metallo	Glu/Asp-pNA	No	intracellular	(d)
PepC	50	Thiol	Leu/Lys-pNA	No	intracellular	(e)
PepN	95	Metallo	Leu/Lys-pNA	No	intracellular	(e)
PepXP	90	Serine	X-Pro-pNA	No	intracellular	(e)
PCP	25	Serine	Pyr-pNA	No	intracellular	(d)
Dipeptidase	49	Metallo	Leu-Leu		intracellular	(f)
PepT	52	Metallo	tripeptides	No	intracellular	(e)
Prolidase	43	Metallo	X-Pro		intracellular	(g)
Imino peptidase	50	Metallo	Pro-X-(Y)		intracellular	(d)
LEPI/MEP	98	Metallo	αs1-CN(f1-23)		intracellular	(h)
LEPII	40	Metallo	αs1-CN(f1-23)		intracellular	(i)
PepO/NOP	70	Metallo	αs1-CN(f1-23)	No	intracellular	(e)

(a) Hugenholtz *et al*., 1984
(b) Musset *et al*., 1989
(c) van der Meer *et al*., 1993
(d) Baankreis, 1992
(e) Tan *et al*., 1992
(f) van Boven *et al*., 1988
(g) Booth *et al*., 1990
(h) Yan *et al*., 1987*a*
(i) Yan *et al*., 1987*b*

2.6.6 Other bacterial proteinases

As a dominant species, *Lc. lactis* has been given much attention with regard to its ability to possess proteolytic activities necessary for casein degradation. Less is known regarding the proteinases of lactobacilli, and more research is now increasingly directed to other genera of LAB which also ought to possess proteolytic activities.

Proteinases from LAB species other than *Lc. lactis* have been purified and characterised at biochemical and genetic levels (El Soda *et al*., 1986; Ezzat *et al*., 1985,

1987). The results clearly show that the proteolytic systems observed in other LAB, particularly lactobacilli, have similarities with those known for *Lc. lactis*. For instance, the serine proteinase produced by *Lb. casei* HN14 is similar to the lactococcal PrtP with regard to its extracellular location and is released in a calcium-free buffer. Substrate specificity data revealed that this proteinase cleaves only β-casein and thus resemble the P_I-type proteinase of *Lactococcus* (Law and Haandrikman, 1997). The same is also true for *Lb. plantarum* and *Lb. acidophilus*, which were shown to have proteinases that can be removed from the cell wall by repeated washing of whole cells in a Ca^{2+}-free buffer. This could indicate the role of Ca^{2+}-ions in maintaining the attachment of proteinase to the cell wall. Further, both *Lactobacillus* enzymes were found to be serine-type proteinases with regard to their inhibition by typical serine proteinase inhibitors and it was estimated that *Lb. acidophilus* proteinase had a molecular size of 145 kDA (Kok, 1990). This size falls within the range of the molecular weight for lactococcal proteinases.

2.6.7 Effect of metal ions and inhibitors

It is now well established that the presence of certain metal ions in the media has either a stimulatory or an inhibitory effect on proteolytic enzymes. Based on biochemical data, the proteolytic enzyme from *Lb. plantarum* DPC2739 was strongly inhibited by Cu^{2+} and Zn^{2+}, weakly by Co^{2+} and was stimulated by Ca^{2+}, Mg^{2+}, Ba^{2+} and Mn^{2+} (Magboul *et al*., 1997). Similar observations were reported for proteinases from other *Lactobacillus* species (Naés *et al*., 1991). An activating effect of Ca^{2+} was also reported for proteinase from *Streptococcus lactis* NCDO 763 (Monnet *et al*., 1987) and *S. cremoris* (Geis *et al*., 1985). According to Exterkate and de Veer (1987*b*), some cations have a structural function and stabilise the enzyme molecules in an active configuration. In contrast, the inhibitory effect of Zn^{2+} and Cu^{2+}-ions reducing the initial activity of the *Lb. casei* may be due to the precipitation of the substrate by these ions (Naés *et al*., 1991).

2.6.8 Proteolytic activity of wine LAB

Many studies have shown that the development of flavour in dairy products is mainly linked to enzymatic activities of the LAB involved (Huggins, 1984; McKay, 1985). The proteolytic system of dairy LAB is well documented. In contrast, the metabolism of proteins by wine LAB remains poorly understood. Since wines also contain proteins, there is a possibility that wine LAB could degrade these compounds through the action of proteases and peptidases. The metabolism of proteins by these enzymes will generate peptides and amino acids to impact on wine aroma. Although preliminary study has been done on proteolytic systems of wine LAB (Davis *et al*., 1988), the results showed that none of the LAB strains tested gave a positive reaction for protease production. Nevertheless, it is significant that the concentrations of some amino acids increase during MLF (Davis *et al*., 1986; Wibowo *et al*., 1985). Besides their contribution to wine aroma, amino acids are also

important for the growth of *O. oeni* strains as well as other wine LAB, both as nitrogen and carbon sources (Amoroso *et al*., 1993).

Recent studies have, however, detected the production of exocellular proteases by strains of *O. oeni* (Rollan *et al*., 1993). These oenococcal proteases have also been partially characterised (Rollan *et al*., 1995; Farias *et al*., 1996). This suggests that wine LAB have the ability to positively alter wine quality through their metabolic activity. More detailed studies are required to elucidate the contribution of proteolysis and peptidolysis on wine aroma during MLF.

2.7 HYDROLYSIS OF POLYSACCHARIDES

β-Glucans are the major polysaccharide components of plant cell walls. They include β-1,4-glucans (cellulose), β-1,3-glucans (callose) and β-1,3-1,4-glucans (cereal β-glucans). The cell walls of certain groups of fungi have β-1,3-1,6-glucans (Bacic *et al*., 1988). Most of the work investigating β-glucans and their degradation has been concerned with cellulose. This is due to its abundance and importance. However, many other β-glucans are produced by both microbial and non-microbial sources (Pitson *et al*., 1993).

The sections below provide detailed information about the structure of β-glucans and the mechanism of action of enzymes involved in the degradation of polysaccharides. Particular attention will also be paid to the involvement of β-glucans in wine processing.

2.7.1 The structure and hydrolysis of glucans

β-Glucans are homopolymers of D-glucose linked in a β-configuration. Some are relatively simple molecules comprising linear chains of glucosyl residues joined by a single linkage type. Others are more complex and can consist of a variety of linkages in either linear or branched chains. Linkage groups incorporate β-1,3-; β-1,4-; β-1,6-; β-1,3-1,4-; β-1,3-1,6- and β-1,2-1,4- (reviewed by Pitson *et al*., 1993).

The production of β-glucan-degrading enzymes is a characteristic attributed to a wide variety of organisms, although the fungi are the most predominant producers of these enzymes. Many β-glucan hydrolysing enzymes are classified according to the type of β-glucosidic linkage(s) they cleave and their mechanism of substrate attack (Pitson *et al*., 1993). A summary of different β-glucan-hydrolysing enzymes is outlined in **Table 2.3**.

Cellulases are the most widely found β-glucanases in fungi. This is attributed to the wide occurrence of cellulose in nature. These enzymes hydrolyse the β-1,4-glucan, cellulose (Pitson *et al*., 1993). Cellulases often comprise endo-glucanases, exo-glucanases and cellobiases that act in a stepwise and synergistic process to achieve efficient hydrolysis of cellulose (**Figure 2.5**). The major end product of endo-glucanase and exo-glucanase activity is cellobiose, which is subsequently hydrolysed to glucose by

cellobiases. Further details can be obtained in a review by van Rensburg and Pretorius (2000).

Table 2.3 Nomenclature and action of β-glucan-degrading enzymes (adapted from Pitson *et al*., 1993).

EC number	Common name	Systematic name	Action
3.2.1.4	Cellulase	1,4-(1,3;1,4)-β-D-Glucan 4-glucanohydrolase	Endohydrolysis of 1,4-linkages in cellulose and β-D-glucans containing 1,3- and 1,4-linkages
3.2.1.6	Laminarinase	1,4-(1,3;1,4)-β-D-Glucan 3(4)-glucanohydrolase	Endohydrolysis of 1,3- or 1,4- linkages in β-D-glucans when the glucose residue whose reducing group is involved in the linkage to be hydrolysed is itself substituted at C-3
3.2.1.21	β-Glucosidase	β-D-Glucoside glucohydrolase	Hydrolysis of terminal non-reducing β-D-glucosyl residues with the release of β-D-glucose
3.2.1.39	Endo-1,3-β-glucanase	1,3-β-D-Glucan glucohydrolase	Endohydrolysis of 1,3-linkages in 1,3-β-D-glucans
3.2.1.58	Exo-1,3-β-glucanase	1,3-β-D-Glucan glucohydrolase	Exohydrolysis of 1,3-linkages in 1,3-β-D-glucans with the release of α-glucose
3.2.1.71	Endo-1,2-β-glucanase	1,2-β-D-Glucan glucanohydrolase	Endohydrolysis of 1,2-linkages in 1,2-β-D-glucans
3.2.1.73	Lichenase	1,3-1,4-β-D-Glucan 4-glucanohydrolase	Endohydrolysis of 1,4-linkages in β-D-glucans containing 1,3- and 1,4-linkages
3.2.1.74	Exo-1,4-β-glucanase	1,4-β-D-Glucan glucohydrolase	Exohydrolysis of 1,4-linkages in 1,4-β-D-glucans
3.2.1.75	Endo-1,6-β-glucanase	1,6-β-D-Glucan 4-glucanohydrolase	Endohydrolysis of 1,6-linkages in 1,6-β-glucans

Pectora roborant cultus recti

β-1,3-Glucanases are widely distributed among bacteria, fungi and higher plants. They are classified as exo-β-1,3-glucanases (EC 3.2.1.58) and endo-β-1,3-glucanases (EC 3.2.1.6 and EC 3.2.1.39). β-1,3-Glucanases catalyse the hydrolysis of β-1,3-glucosidic linkages in β-1,3-glucan. This polymer is a major component of fungal cell walls and a major structural and storage polysaccharide (Hong *et al*., 2002).

The physiological functions of β-1,3-glucanases are distinct and depend on their source. In plants, involvement in cell differentiation and defence against pathogenic fungi has been proposed (Castresana *et al*., 1990). In fungi, β-1,3-glucanases seem to have different functions in development and differentiation, β-glucan mobilisation and interactions of plant fungal pathogens (De la Cruz *et al*., 1995). In bacteria, the enzymes are released to break down fungal cell walls to allow them to be used as a food source

(Watanabe *et al*., 1992). Although they have the same hydrolytic activity, the bacterial enzymes are classified into GH-16 (glycosyl hydrolase family 16), whereas most plant and fungal enzymes are grouped into GH-17, on the basis of differences in their amino acid sequences (Henrissat and Bairoch, 1993).

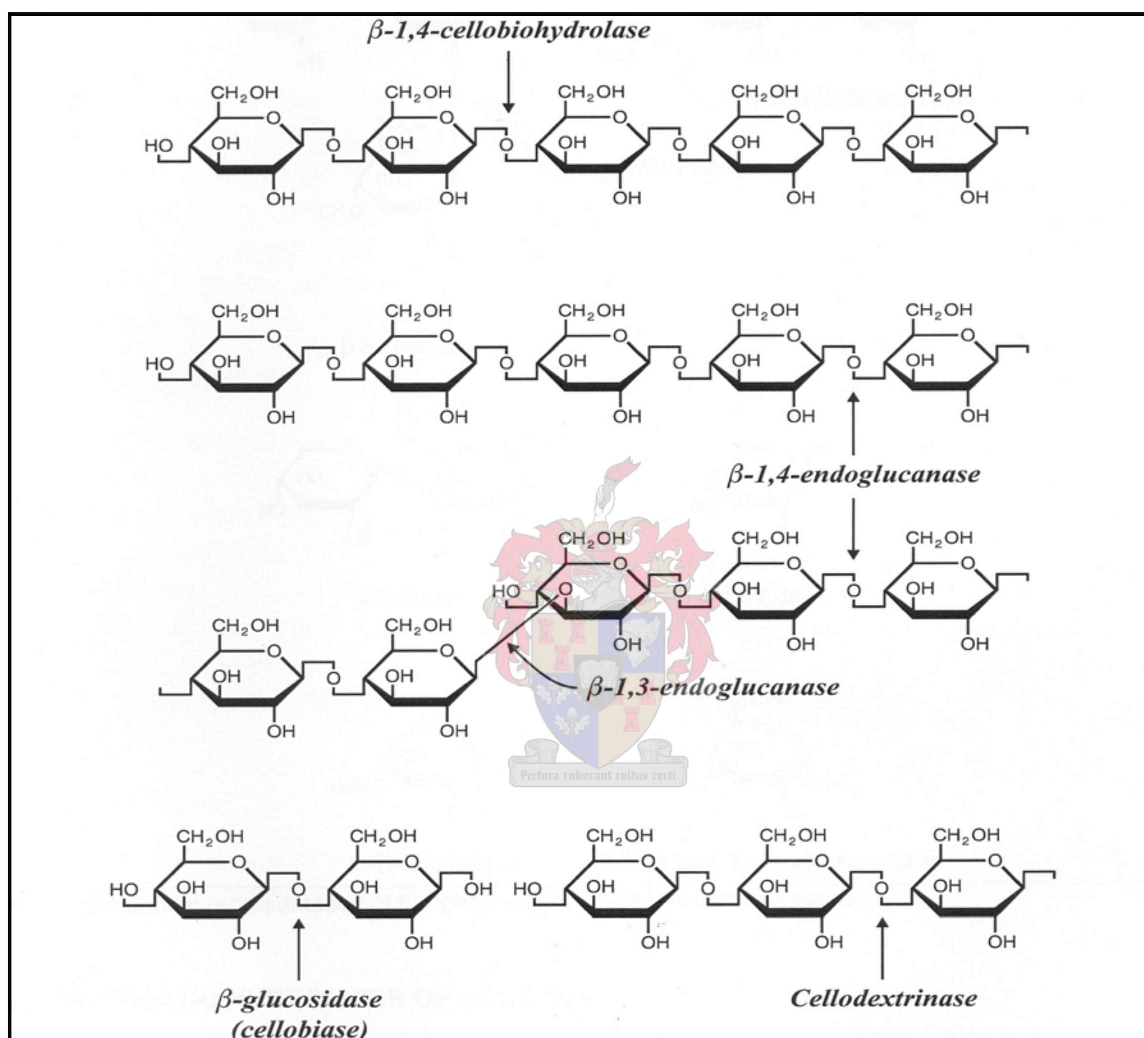

Figure 2.5 Schematic representation of the enzymatic degradation of glucan and cellulose (van Rensburg and Pretorius, 2000).

β-1,3-1,4-Glucans are linear polysaccharides found in the cell walls of higher plants such as cereals. These polymers play the role of storage polysaccharides and are most abundant in the endosperm of barley, oat, rye, rice, sorghum and wheat grain (Stone and Clarke, 1992). They comprise a mixture of both β-1,3- and β-1,4-glucoside linkages (Parrish *et al*., 1960). A similar type of β-glucan called lichenan has been found in lichens.

Lichenan from *Cetraria islandica*, moss starch polyglucan, is a linear polysaccharide structure composed of mix-linked β-1,3- and β-1,4-glycosidic bonds (Anderson and Stone, 1975).

The biodegradation of β-1,3-1,4-glucans in nature is catalysed by glycoside hydrolases differing in their substrate specificity (Grishutin *et al*., 2006). Cellulases (EC 3.2.1.4) are able to split internal β-1,4-linkages in β-glucans, thus displaying β-glucanase activity (Wilhelmi and Morgan, 2001). Like cellulases, lichenases (EC 3.2.1.73) are enzymes acting specifically on β-glucans. 1,3-1,4-β-Glucanase (lichenase) is an endo-β-glucanase that specifically hydrolyses β-1,4-glycosidic bonds adjacent to β-1,3-glycosidic linkages in mix-linked β-glucans. Hydrolysis of lichenan by this enzymes yields mainly 82% cellotriose and 9.5% cellopentaose, while hydrolysis of barley β-glucans yields 63.5% cellotriose and 29.5% cellotetraose as the major products (Erfle *et al*., 1988). However, lichenases have no activity against true β-1,4-glucans such as cellulose and carboxymethylcellulose (CMC) (Pitson *et al.*, 1993).

2.7.2 Glucanases in wine clarification and processing

Glucanase preparations for winemaking were developed and tested in the 1980s (Dubourdieu *et al*., 1985). The only specific industrial enzyme preparation available is derived from the culture of a selected strain of *Trichoderma* species. It was developed to solve clarification and filtration problems with juices extracted from grapes infected with *Botrytis cinerea* (Canal-Llaubéres, 1993). The origin of this problem is usually β-glucan, a polymer of glucose synthesised by this fungus.

Polysaccharides serve two important functions. They are either structural in nature (cellulose, pectin) or energy reserves (starch). These macromolecules occur in wine as a carryover from juice extraction. They can also result from microbial activity (Zoecklein *et al*., 1995). Because of their size and colloidal nature, polysaccharides can present problems in clarification and filtration; they are responsible for turbidity, viscosity and filter stoppages (Pretorius, 2000). Cellulose and hemicellulose are the primary structural polysaccharides of the plant cell wall (Zoecklein *et al*., 1995) and form the largest reservoir of fixed carbon in nature (van Rensburg and Pretorius, 2000). Upon acid hydrolysis, a portion of polysaccharides may be released into the wine.

Of all polysaccharides, the β-glucans produced by *B. cinerea* in botrytised grape juice can be regarded as the strongest influence on the clarification and stabilisation of must and wine (van Rensburg and Pretorius, 2000). Processing difficulties arise because of a high molecular weight polysaccharide produced by this fungus. The structure of this macromolecule has been identified as β-1,3-1,6-glucan and comprise a β-D-1,3-linked backbone with very short β-D-1,6-linked side chains (Dubourdieu *et al*., 1981; Villettaz *et al*., 1984).

During grape processing, the glucose polymer produced by *B. cinerea* is released into the grape juice and later found in wine. The degradation of this polymer by exogenous enzymes has appeared to be the unique solution (Canal-Llaubéres, 1993). This is simply because the removal of glucose polymer through hydrolysis with endogenous enzymes or by conventional treatment has proven impossible. Conventional treatments, such as fining and centrifugation, will force the sedimentation of the cloud particles in the grape must but will not remove the glucan, and filtration problems remain (van Rensburg and Pretorius, 2000).

2.7.3 Bacterial glucanases

The glucanase activities of LAB have largely been studied in fermentation processes other than winemaking. Little is known about the formation of extracellular polysaccharides by wine LAB. Some wine LAB are known to induce ropiness in wine (Liu, 2002). For instance, *P. damnosus* isolated from a ropy wine produced a β-D-glucan composed of a trisaccharide repeating unit of D-glucose (Canal-Llaubères *et al*., 1990; Lonvaud-Funel *et al*., 1993).

Wine LAB may be able to hydrolyse polysaccharide components besides biosynthesis. An extracellular β-1,3-glucanase activity has been demonstrated in *O. oeni* (Guilloux-Benatier *et al*., 2000). This enzyme was found capable of degrading yeast cell wall macromolecules. Thus, it was proposed that the enzyme plays a role in yeast cell autolysis following alcoholic fermentation. This report provides the first evidence that *O. oeni* has the ability to hydrolyse polysaccharides, such as β-glucans. Clearly, further work is required to confirm the significance of this activity together with the activity of other polysaccharide-hydrolysing enzymes in a wide range of wine LAB.

In general, polysaccharides can affect wine processing due to the increased viscosity. These macromolecules reduce juice extraction and are primarily responsible for fouling of filters during clarification steps (Pretorius, 2000). Polysaccharides may also affect sensory properties of wine through changes in clarity. The effect of viscosity may influence mouthfeel and body. Excessive levels of polysaccharides in wine are undesirable in terms of inducing ropiness. However, moderate levels of polysaccharides may add complexity to wine (Liu, 2002). Further studies are required to elucidate the potential of wine LAB to hydrolyse polysaccharides.

2.8 CONCLUSIONS AND PERSPECTIVES

A considerable amount of research has been done to assess the potential of LAB to possess enzymatic activities able to contribute to the development of flavour. A great deal of studies have characterised the enzymes from the LAB isolated from food sources other than wine. Nevertheless, preliminary studies done to assess enzymatic activities of wine

LAB suggest that similar trends also exist in wine. Based on the mechanism of action of these enzymes, it is apparent that wine aroma can substantially benefit from the hydrolytic action of enzymes. However, many questions still need to be answered as some aspects of enzymes from the LAB remain poorly understood. It is therefore important to understand the metabolic activity of LAB in order to get a better understanding of the contribution of enzymes towards wine aroma.

Besides wine deacidification as the most well known outcome of LAB activity, wines undergoing MLF can benefit from the metabolic action of wine LAB. Further studies are required to give insight into the metabolic activities of wine LAB. These include amino acid metabolism, proteolysis and peptidolysis, ester synthesis and hydrolysis, lipid metabolism, metabolism of polysaccharides, and hydrolysis of glycosides. Apart from that, it should also be noted that most of the screening systems are based on laboratory media. Apparently the activity of enzymes from the LAB may change in the actual winemaking. This stems from the fact that wine is a complex medium encompassing various compounds, such as phenols, anthocyanins and tannins. These compounds may pose an inhibitory effect on the activity of enzymes.

In most winemaking environments, adding commercial enzyme preparations to wine is a common practice. The addition of these enzymes, most of which are of fungal origin, has beneficial effects in wine. In general commercial enzymes are added in wine to assist in settling and clarifying must, improve the varietal aroma of certain wines, improve the colour of red wine and improve the filterability of wine. However, it should be noted that the exploitation of commercial enzymes during winemaking is an expensive practice. As an alternative to the addition of exogenous enzymes, more detailed studies are required in quest of LAB strains with desired enzymatic activities of interest in winemaking. This will enable winemakers to select the best and suitable LAB strains producing desired compounds through their enzymatic activities without the development of off-flavours.

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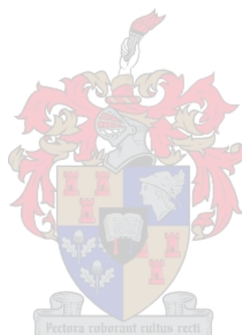

Chapter 3

RESEARCH RESULTS

Screening and genetic characterisation of certain wine aroma enzymes in lactic acid bacteria isolated from South African wines

CHAPTER 3

Screening and genetic characterisation of certain wine aroma enzymes in lactic acid bacteria isolated from South African wines

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ABSTRACT

Malolactic fermentation (MLF) is the secondary fermentation process which is normally conducted by lactic acid bacteria (LAB) present in wine after alcoholic fermentation. The former fermentation process involves the conversion of a dicarboxylic acid (malate) to a monocarboxylic acid (lactate) and carbon dioxide (CO_2). MLF is beneficial to wine due to its contribution to deacidification, microbial stabilisation and wine aroma formation, with the latter being an important part of research in this study. LAB isolated from South African wines during spontaneous MLF were screened for different enzymes of interest in winemaking using both classical and molecular techniques. The results obtained from screening have shown that LAB possess different combinations of enzymes such as β-glucosidase, protease, esterase, glucanase and lipase. Isolates were screened for β-glucosidase and glucanase with plate assays. Results obtained showed that 40% were positive for β-glucosidase, 80% for glucanase tested with carboxymethylcellulose, and 65% for glucanase tested with lichenan. Isolates were also screened using molecular techniques. The enzyme-specific primers used for PCR amplifications were deduced from nucleotide sequences previously identified in putative genes from *Lactobacillus plantarum* WCFS1 strain. These primers amplified genes with sizes corresponding to 1392 bp (β-glucosidase), 1263 bp (protease), 1020 bp (esterase) and 1644 bp (malolactic enzyme). Of all the isolates tested, 40% were positive for β-glucosidase, 35% for serine protease, 42% for esterase and 36% for malolactic enzyme. Among the isolates that possessed all four enzyme genes, 11 were selected from which genomic DNA was extracted and used as template to amplify the coding regions of the respective genes. Purified amplicons generated with enzyme-specific primers were further cloned into pGEM-T easy vector and sequenced. Analysis of sequences revealed that gene sequences were highly conserved between species, and these sequences exhibited significant homology with nucleotide sequences available from GenBank database. These results suggest that wine LAB can be the potential source of enzymes for use in vinification.

Keywords: Enzymes, malolactic fermentation, lactic acid bacteria, screening, gene

3.1 INTRODUCTION

During winemaking two main fermentation processes take place. Alcoholic fermentation, which is conducted by yeasts, is the primary fermentation process that involves the conversion of grape sugars into ethanol and carbon dioxide (CO_2). Malolactic fermentation (MLF) is the secondary process which is conducted by the lactic acid bacteria (LAB). This process usually occurs after alcoholic fermentation but may also occur during alcoholic fermentation. It involves the decarboxylation of L-malic acid (malate) to L-lactic acid (lactate) and CO_2. This results in the concomitant increase in pH accompanied by the disappearance of harsh malate sensation (Wibowo *et al.*, 1985). MLF makes considerable contribution to wine with regard to deacidification, microbial stabilisation and enhancement of wine aroma. However, the latter has not been well characterised. A decrease in wine acidity is beneficial in cool-climate regions such as Canada, New Zealand and Europe where wines tend to have a high acid content and low pH. Nevertheless, MLF is also desired in warm-climate regions in which flavour changes associated with the growth of LAB are often considered beneficial to wine quality (Henick-Kling, 1993).

Beyond wine deacidification, which is the most well-known result of the growth of LAB in wine, the action of LAB can also influence wine aroma and flavour by various mechanisms. These mechanisms include the production of volatile secondary metabolites and the modification of grape and yeast-derived metabolites (Davis *et al.*, 1985, 1988; Henick-Kling, 1993). The products formed are a result of LAB activity and can either be beneficial or detrimental to wine quality. This is largely dependent on the species predominantly involved during MLF. Undesirable odours brought about by MLF are usually associated with pediococci and lactobacilli, or can originate from MLF occurring above pH 3.5. In contrast, *Oenococcus oeni* is more desirable and is less likely to produce unpleasant aromas and flavours during MLF at pH below 3.5 (Du Toit and Pretorius, 2000; Jackson, 1994).

The production of volatile aromatic components in wine can, in part, be achieved through the hydrolytic action of enzymes produced by LAB associated with MLF. These enzymes include β-glucosidase, protease, esterase, lipase and glucanase. Most of the work done on bacterial enzymes has been on LAB from food sources other than wine, in which these enzymes contribute to the flavour development of some cheeses, yoghurt and other fermented foods (Andersen *et al.*, 1995; Magboul *et al.*, 1997). The activity of these enzymes during wine fermentation has mostly been concerned with β-glucosidase from *O. oeni* (Grimaldi *et al.*, 2000; Mansfield *et al.*, 2002). Only in recent years has there been a renewed interest in evaluating the activity of β-glucosidase in other genera of wine LAB.

The overriding goal of this study was therefore to screen and genetically characterise wine-related enzymes produced by LAB associated with wine during MLF. We investigated different species of LAB to detect the presence of certain wine aroma enzymes such as protease, esterase, β-glucosidase, lipase and glucanase. Bacterial

isolates were also evaluated for the presence of malolactic enzyme. Isolates were first tested for β-glucosidase, glucanase, esterase and lipase on agar plates supplemented with appropriate substrate analogues and subsequently screened with colony PCR using enzyme-specific primers for amplifying β-glucosidase, esterase, protease and malolactic enzyme genes. Among the isolates that possessed all four enzyme genes, 11 were selected from which genomic DNA was extracted and used as template to amplify the coding sequences of respective genes. Purified amplicons were cloned into pGEM-T easy vector and sequenced.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial isolates, media and culture conditions

All bacterial isolates used in this study are listed in **Tables 3.3** and **3.4**. These isolates form part of culture collection of the Institute for Wine Biotechnology and were collected from five different commercial wineries situated in the Western Cape region, South Africa. Most of these isolates were identified to the species level by means of colony PCR with different species-specific primers (Krieling, 2003). All bacterial isolates were grown at 30°C in MRS (Biolab) medium. *Escherichia coli* cells were grown in Luria-Bertani (Biolab) broth at 37°C (Sambrook *et al.*, 1989). Solid media contained 2% agar (Biolab).

3.2.2 Classical screening method

Screening of isolates for different enzymes was carried out on agar plates supplemented with appropriate substrate analogues to ascertain the potential of LAB to possess β-glucosidase, glucanase and lipolytic activities. The cells were prepared by inoculating a loopful of cells from MRS agar plates into 5 ml of MRS broth. Unless otherwise stated, after 24 h incubation at 30°C, 10 μl of the liquid culture were spotted onto the surface of indicator plates.

3.2.2.1 β-glucosidase activity

Bacterial isolates were screened for β-glucosidase activity using indicator agar plates containing arbutin, a β-glucoside analogue (Rosi *et al.*, 1994). The medium consisted of (per litre): 50 g MRS broth, 5 g arbutin (Sigma-Aldrich) and 20 g agar. The pH of the medium was adjusted to 5.5 and the medium was sterilised by autoclaving at 121°C for 15 min. After autoclaving, 0.02% (w/v) of filter-sterilised ammonium ferric citrate solution was added to the medium. The medium was poured into Petri dishes and allowed to solidify. To evaluate β-glucosidase activity, each plate was inoculated with six different cultures and incubated at 30°C for 8-10 days. Isolates with β-glucosidase activity hydrolyse the substrate and a dark brown colour develops in the agar.

3.2.2.2 Glucanase production

Glucanase activity was tested on PHB agar medium as described by Heng *et al*. (1997). The medium contained (per litre): 12 g Todd-Hewitt broth (Sigma-Aldrich), 3 g MRS broth, 0.1% (w/v) lichenan or carboxymethylcellulose (CMC) (Sigma-Aldrich) and 15 g agar. After spotting cultures on agar medium, the plates were incubated at 30°C for 24 h, after which the colonies were washed off the plates followed by flooding the surface of the plates with 0.1% (w/v) solution of Congo red (dissolved in 20% ethanol). Dark colouration around the bacterial growth was an indication of enzyme activity.

3.2.2.3 Lipolytic activity

The production of lipolytic activity was detected on tributyrin agar plates supplemented with or without rhodamine B, an indicator dye. Tributyrin was used as a substrate. This agar plate method was employed to determine true lipase activity (Lee and Rhee, 1993), because substrate hydrolysis induces the formation of orange fluorescent halos around bacterial colonies visible upon irradiating the plates under UV illumination at 350 nm. The medium consisted of (per litre): 8 g W.L. nutrient medium (Biolab), 4 g NaCl and 10 g tributyrin agar (Fluka). The pH of the medium was adjusted to 7.0 using NaOH and the medium was autoclaved at 121°C for 15 min. After sterilisation, 0.25% (v/v) tributyrin (Sigma-Aldrich) and 0.001% (w/v) filter-sterilised rhodamine B (Sigma-Aldrich) were added to the medium with vigorous stirring and emulsified by mixing for 5 min using a Waring blender. After this medium was allowed to stand for 10 min at 55°C to reduce foaming, 20 ml of medium were poured into each Petri dish. For the detection of enzyme activity, 20 μl of overnight culture were spotted onto the agar surface and the plates were maintained at 30°C for analysis of enzyme activity after 24 and 48 h.

3.2.3 PCR detection and sequence analysis

In order to detect the presence of different enzyme genes from different LAB species, all the isolates were screened using colony PCR. Bacterial isolates were first grown on MRS agar plates prior to screening. After 3 - 5 days of incubation at 30°C, one colony from each plate was applied directly to PCR with specific primers relevant to the different genes. A list of primer sequences used for PCR amplifications is presented in **Table 3.1**. All gene sequences from which the primers were designed were extracted from the Integrated Microbial Genome database (http://img.jgi.doe.gov) and the primers were synthesised by Inqaba Biotechnical Industries (Pretoria, South Africa).

For PCR experiments, each colony was added to a 50 μl PCR mixture containing 0.025 U *Supertherm Taq* (Southern Cross Biotechnology), 0.4 μM each primer, 1.5 mM $MgCl_2$, 0.25 mM dNTP mix and 1x PCR buffer. The reaction mixtures were cycled using T3 Thermocycler (Whatman Biometra® GmbH, Germany) through the temperature profiles

indicated in **Table 3.2**. PCR products were analysed by gel electrophoresis in 1% (w/v) agarose (Whitehead Scientific) gels containing ethidium bromide (Sigma-Aldrich) at a final concentration of 0.2 μg/ml. Gels were run for *ca* 60 min at 80 V in 1x TAE (100 mM Tris-Cl, 1 mM EDTA, pH 8.0, 20 mM acetate) buffer. DNA fragments were visualised by UV transillumination and documented with Alpha Imager (Alpha Innotech Corporation, San Leandro, Calif.). Lambda DNA digested with *Eco*RI and *Hind*III (Fermentas) was used as the standard molecular weight marker.

3.2.3.1 DNA preparation

Genomic DNA was extracted from the selected LAB isolates that possessed genes for all four enzymes (i.e. β-glucosidase, protease, esterase and malolactic enzyme). Preparation of chromosomal DNA was performed by phenol extraction using the method modified from Vaquero *et al*. (2004). Bacterial isolates were pre-cultured in 5 ml MRS broth and then inoculated (5% v/v) in 10 ml MRS broth and grown to an optical density of 1-1.6 at 600 nm. The cells were pelleted by centrifugation, washed twice with distilled water and resuspended in 1 ml of solution A (10 mM Tris-HCl, 10 mM EDTA, pH 8.0, 50 mM NaCl, 20% w/v sucrose) containing 10 mg/ml lysozyme (Sigma-Aldrich). The cells were lysed by adding 50 μl of 10% (w/v) SDS and 40 μl proteinase K (20 mg/ml) (Roche). Crude DNA preparations were purified by performing two phenol/chloroform/isoamylalcohol (25:24:1) and one chloroform/isoamylalcohol (24:1) extractions. Chromosomal DNA was precipitated by adding one tenth volume of 3 M sodium acetate and two volumes of prechilled 100% ethanol. The pellet was washed with 70% ethanol, dried in a speedy vacuum and resuspended in 100 μl of 1x TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer containing 5 μl of RNase (10 mg/ml; Sigma-Aldrich). The sample was then incubated at 65°C for 4 min before storage at -20°C.

The quality of the extracted DNA was monitored by gel electrophoresis on a 1% (w/v) agarose gel using 1x TAE buffer containing 0.2 μg/ml ethidium bromide (Sigma-Aldrich). The lambda DNA cleaved with *Eco*RI and *Hind*III was used as the standard molecular weight marker. The quantification of DNA was performed spectrophotometrically using a NanoDrop® ND-1000 (NanoDrop Technologies, Inc., Wilmington, USA).

3.2.3.2 PCR generation of gene sequences

The same primers and PCR programmes were used as described in section 3.2.3, except that 10 ng of template DNA and 0.025 U of *TaKaRa Ex Taq* (Separations) were used. Amplification products were separated by gel electrophoresis on a 1% (w/v) agarose gel. DNA fragments were visualised under UV light and documented with Alpha Imager. The lambda DNA cleaved with *Eco*RI and *Hind*III served as the standard molecular weight marker. PCR amplifications were purified with QIAquick PCR Purification Kit (Qiagen) and

cloned into pGEM-T easy vector (Promega) according to the specifications of the supplier. All the sequencing reactions were performed by Inqaba Biotechnical Industries.

3.2.3.3 DNA sequencing

DNA sequencing was performed on both strands by using universal primers (T7 and SP6). In the case of malolactic enzyme gene sequences (1644 bp), internal primers were designed and used to obtain full gene sequences. Nucleotide sequence data were assembled and the analysis was carried out with the Biological sequence alignment editor (Ibis Therapeutics, Carlsbad). The Basic local alignment search tool (Altschul *et al*., 1990) of the National Center of Biotechnology Information (NCBI) was used for searching homologous nucleotide sequences.

3.3 RESULTS

3.3.1 Screening

LAB isolates were screened for β-glucosidase activity using indicator agar plates supplemented with a β-glucoside analogue (arbutin) as the sole carbon source. Isolates hydrolysing this compound were considered positive and this resulted to the media discolouration to a dark brown colour accompanied by the formation of a dark halo around enzyme-producing isolates (**Figure 3.1A**).

Of all the isolates tested, *Lactobacillus plantarum* was predominantly the most abundant species (**Tables 3.3 and 3.4**). For all bacterial species tested for β-glucosidase activity on plates, 40% were found positive. Four *Leuconostoc mesenteroides* species tested did not possess β-glucosidase activity. Of the four *Pediococcus* spp. tested, none was positive whereas only one *P. acidilactici* strain showed enzyme activity.

Bacterial isolates possessing glucanase activity were tested on PHB agar plates supplemented with lichenan or CMC. The hydrolysis of these substrates caused the formation of dark colouration visible upon washing the colonies off the plates followed by flooding the plates with Congo red solution. Glucanase activity was therefore observed by a detectable dark colouration around the bacterial growth on an otherwise red-coloured plate (**Figure 3.1B**). Of the isolates tested for enzyme activity, 80% possessed glucanase activity when tested on CMC and 65% on lichenan, although activity was very low in some of the isolates especially on lichenan.

The extracellular lipase activity was evaluated on tributyrin agar plates. Two media, one with rhodamine B dye and the other without, were employed to test isolates for the presence of lipase activity. As shown in **Figure 3.1C**, the formation of yellow colour zones around colonies indicated the presence of lipolytic activity. However, tributyrin is not a suitable substrate for detecting a true lipase activity because it can also be hydrolysed by

esterase. This could therefore be overcome by using a lipase-specific dye method in order to determine the true lipase activity. This assay system is based on the incorporation of a fluorescent dye, such as rhodamine B, in the medium. Lipase activity would therefore be detected by the formation of orange fluorescence around the colonies visible upon irradiating the plates under UV illumination at 350 nm. **Figure 3.1D** shows the rhodamine B agar plate inoculated with six different bacterial cultures. The colonies induced the formation of yellow colour zonation and no orange fluorescence was observed after exposing the plates under UV light, which means no true lipase activity was observed.

3.3.2 Molecular detection of genes

The gene nucleotide sequences retrieved from the Integrated Microbial Genome database were employed to design enzyme-specific amplification primers for the detection of different enzyme genes from different species of LAB. *Lb. plantarum* WCFS1 strain was employed as the basis for designing primers for amplifying the coding regions of protease, β-glucosidase, esterase and malolactic enzyme genes. Using the genomic DNA extracted from the selected isolates belonging to *Lb. plantarum*, *Lb. paracasei*, *Lb. hilgardii*, *Lb. brevis* and *Lb. pentosus*, the primer sets were tested for PCR amplifications of the latter enzyme genes.

The primers amplified single products of 1392 bp (β-glucosidase gene), 1020 bp (esterase gene), 1263 bp (protease gene) and 1644 bp (malolactic gene) (**Figure 3.2**). No amplification products were observed for Gluc-1/Gluc-2 and Lip-1/Lip-2 primer sets. The nucleotide sequences from which these primer pairs were designed were of putative glucanase and lipase genes identified on *Lb. acidophilus*, respectively. This *Lactobacillus* species does not occur in wine and this could explain why no PCR amplifications were observed. Of all the isolates screened for the different enzyme genes using PCR, 40% were found positive for β-glucosidase, 36% for malolactic enzyme, 35% for protease and 42% for esterase (**Table 3.4**). Of all the positive isolates, 24 possessed all four enzyme genes evaluated in this study. Among these, 11 isolates were selected and used to obtain nucleotide sequences for different enzyme genes. Purified amplicons were cloned into pGEM-T easy vector and sequenced.

All the gene sequences were assembled and aligned in order to study their homology patterns and compare them to those available in GenBank database. Sequence analyses of cloned genes revealed that nucleotide gene sequences are highly conserved between the species. These nucleotide sequences also showed 99 - 100% homology to gene sequences of *Lb. plantarum* WCFS1 available in GenBank database (Benson *et al.*, 1999).

3.3.3 Analysis of gene sequences

From the analysis of nucleotide sequences, it was interesting to note that gene sequences are highly conserved between the species, and that these sequences are closely related

to the nucleotide gene sequences of *Lb. plantarum* WCFS1 strain available in GenBank database. This was the case for malolactic enzyme gene sequences of Lpar 83.1 and Lbrev 116.3 which showed a close genetic homology with that of *Lb. plantarum* WCFS1 strain from which amplification primer sequences were designed (**Figure 3.3**). It was also noteworthy that malolactic enzyme genes from Lbrev 117.2, Lplant 40.3, Lplant 69 and Lhil 87.1 were genetically similar at nucleotide position 105 (*nt* 105) whereas *Lb. plantarum* WCFS1, Lpar 83.1, Lbrev 116.3, Lhil 3 and Lbrev 81.1 showed nucleotide similarity in the same nucleotide position. This may be an indication that, even though gene sequences possess highly conserved regions between species, there is a possibility that gene sequences may differ with few nucleotides at certain positions within the open reading frame (ORF). On the other hand, minor differences in nucleotides may arise from point mutations or sequencing errors, and this could be resolved by re-sequencing the genes.

Analysis of nucleotide sequences of β-glucosidase genes from Lhil 87.1, Lplant 69 and Lplant 40.3 revealed that these genes are homologous at *nt* positions 41, 112 and 1366 (**Figure 3.4**). This trend of nucleotide homology is similar to that observed for malolactic enzyme genes from the latter species. From these results, it is therefore noteworthy that these species share similar features which can further be investigated in future studies. Moreover, gene sequences of Lplant 113.1 and Lbrev 116.3 share homologies at *nt* positions 328, 717, 1319, 1341, 1344 and 1368. Some of these homologies are similar to that of *Lb. plantarum* WCFS1 and *O. oeni* β-glucosidase genes (Spano *et al.*, 2005), particularly at *nt* positions 1319, 1341, 1344 and 1368. In addition, Lplant 113.1 and Lbrev 116.3 do not possess certain portion of the sequence from *nt* position 967 to 1050 of the ORF. These isolates also possessed fragments with lower band sizes (data not shown) during PCR detection of genes using primers specific for β-glucosidase.

Esterase gene sequences of Lhil 3, Lpar 79, Lplant 69, Lplant 40.3 and Lbrev 117.2 were highly conserved between the species. These genes also exhibited a significant homology with putative esterase gene of *Lb. plantarum* WCFS1, with minor differences observed in few nucleotides (**Figure 3.5**). Similar trend of nucleotide sequence homology was also observed for serine protease HtrA genes of Lhil 87.1, Lbrev 117.2, Lpar 79, Lpar 83.1, Lplant 40.3, Lplant 69, Lbrev 81.1, Lhil 3 and Lpent 79.2, with minor differences also observed in few nucleotides (**Figure 3.6**).

3.4 DISCUSSION

3.4.1 Enzyme activity

Most of the work done on bacterial enzymes has been on LAB from food sources other than wine, in which these enzymes contribute to the flavour development of some cheeses, yoghurt and other fermented foods (Andersen *et al.*, 1995; Magboul *et al.*, 1997).

The activity of these enzymes during wine fermentation has mostly been concerned with β-glucosidase from *O. oeni* (Grimaldi *et al*., 2000; Mansfield *et al*., 2002). In this study we have identified, using both plate assay and PCR detection technique, some of the hydrolytic enzymes produced by wine LAB associated with MLF. Bacterial isolates were first screened on agar media supplemented with appropriate substrate analogues. From the results obtained, it was noteworthy that the isolates possessed different combinations of the enzymes investigated in this study. Although β-glucosidase activity was tested on plates, activity of the enzyme was not characterised as strong, moderate or weak according to the colour intensity of the halo.

To date, the results reported on the ability of wine LAB to hydrolyse glyco-conjugates are contradictory. β-Glucosidase activity in wine LAB (mainly *O. oeni*) was detected in a synthetic media (Guilloux-Benatier *et al*., 1993). This was further confirmed by Grimaldi *et al*. (2000) who found readily detectable activity of β-glucosidase in 11 commercial preparation of *O. oeni*. Further studies (Mansfield *et al*., 2002) detected the production of β-glucosidase enzymes in strains of *O. oeni*, although cultures of the same strains failed to hydrolyse native grape glycosides. In contrast, McMahon *et al*. (1999) observed no enzymatic activity in commercial strains of *O. oeni* against arbutin, an artificial glycosidic substrate.

These findings suggest that even wine LAB have the potential to hydrolyse glyco-conjugates consequently affecting wine aroma and colour. However, β-glucosidase enzymes in yeasts and bacteria are usually inhibited by winemaking conditions such as pH, ethanol and sugars (Delcroix *et al*., 1994; McMahon *et al*., 1999; Grimaldi *et al*., 2000). The acidic conditions in wine may result in denaturing and/or inhibition of enzymatic hydrolysis, although strains of *O. oeni* may retain 80% of maximum β-glucosidase activity at pH 3.5 (Grimaldi *et al*., 2000). It is therefore crucial to understand if and how β-glucosidase enzymes are regulated by abiotic stresses. This will enable the selection of starter cultures able to positively alter the wine volatile fraction throughout the liberation of glycosidically bound aroma components (Spano *et al*., 2005).

Apart from evaluating β-glucosidase activity on the plates, the presence of glucanase activity was tested on CMC and lichenan as substrates. Bacterial isolates tested seemed to show high affinity for CMC in comparison to lichenan. Nevertheless, more than 50% isolates tested exhibited glucanase activity. From these results, it is now probable that wine LAB have the ability to improve wine clarification through the degradation of polysaccharides that can present problems in clarification and filtration. These macromolecules are responsible for turbidity, viscosity and filter stoppages (Pretorius, 2000). The effect of viscosity may influence mouthfeel and body and excessive levels of these polysaccharide molecules in wine are undesirable in terms of inducing ropiness. However, moderate levels of polysaccharides may add complexity to wine (Liu, 2002).

The plate assay for detecting a true lipase enzyme proved unsuccessful. Tributyrin was used as the substrate even though it does not clearly differentiate between esterase

and lipase. Lipase and esterase may act on the same substrate, depending on the physical nature of the substrate. It is suggested that tributyrin can only be hydrolysed by lipase if the aqueous phase is supersaturated and a heterogeneous system is formed but once the substrate is present in water-soluble form, tributyrin can then be hydrolysed by esterase (Hübscher, 1970). To overcome this, true lipase activity can be detected by using lipase-specific dye technique that incorporates rhodamine B for the formation of orange fluorescence around bacterial colonies exhibiting lipase activity. Most of the isolates tested induced the formation of yellow colour zones around bacterial colonies and this indicated the presence of esterase activity (Singh *et al.*, 2006). It was also observed that the colour zonations increased during prolonged incubation periods and this problem could be solved by incorporating $CaCl_2$ in the medium in order to quench the spread of fatty acids. However, the plates were exposed under UV light at a wavelength of 365 nm instead of 350 nm, and this could explain why the lipase activity was not observed.

3.4.2 Analyses of bacterial sequences

All gene sequences from the selected isolates were aligned using the biological sequence alignment editor in order to study the homology patterns between gene sequences from different LAB species. The results showed that gene sequences are highly conserved between the species. Bacterial gene sequences also exhibited significant similarities with gene sequences available in GenBank database (Benson *et al.*, 1999). β-Glucosidase genes from all the sequenced bacterial clones yielded significant alignments with *O. oeni* and *Lb. plantarum* WCFS1 strain β-glucosidase genes previously identified by Spano *et al.* (2005). Gene sequences of *Lb. plantarum* WCFS1 strain were employed as the basis for designing enzyme-specific primers to amplify the coding regions of different enzyme genes (i.e. β-glucosidase, protease, esterase and malolactic enzyme). Similar results were also observed for esterase and serine protease genes which showed significant homologies with nucleotide sequences of esterase and serine protease HtrA genes from *Lb. plantarum* WCFS1 strain, respectively. Finally, malolactic enzyme gene sequences were highly homologous to different malolactic enzyme genes of different genera of LAB. These results suggest that malolactic enzyme gene is widely distributed across different species of LAB. Other primer pairs, such as Est-1/Est-2 and Prt-1/Prt-2, yielded gene sequences which were only homologous to bacterial gene sequences of *Lb. plantarum* WCFS1 strain esterase and serine protease genes, respectively. This might be because of a limited number of gene sequences readily available in GenBank database. To our knowledge, this is the first time that esterase and protease genes from wine LAB have been reported. An expansion of research as an endeavour to increase knowledge on the genetic data of wine LAB is therefore crucial to better understand their metabolic action to positively influence wine aroma.

Although a limited number of isolates were genetically tested through PCR detection and subsequently sequenced, the results of gene alignments indicate a very close genetic similarity among different species of *Lactobacillus*. These results support the findings of Spano *et al*. (2005) who first reported the similarity in amino acid sequences of β-glucosidase genes from *Lb. plantarum*, *O. oeni*, *Lb. paraplantarum* and *P. damnosus*. For the purpose of our study, we cloned different genes from the selected bacterial isolates. These isolates were selected based on the fact that they possessed β-glucosidase, protease, esterase and malolactic enzyme genes that were investigated in this study. Attempts to amplify other enzyme genes such as lipase and glucanase genes did not prove successful. The primer sequences to amplify the latter genes were designed from putative lipase and glucanase genes previously identified on *Lb. acidophilus* NCFM strain. However, *Lb. acidophilus* does not occur in wine and this could explain why there were no PCR amplifications. Moreover, a second set of lipase primers (Lipdel-1/Lipdel-2) from *Lb. delbrueckii* gave non-specific bands and several attempts of troubleshooting proved unsuccessful.

3.5 CONCLUSIONS

In conclusion, the results reported in this study give an indication that wine LAB which are normally encountered in wine during MLF can be the potential source of enzymes for use in vinification. In general, MLF is well known for its ability to induce wine deacidification, microbial stabilisation and wine aroma formation. The latter has not previously been well exploited by looking at the expression levels of different genes at a molecular level. This study therefore forms the basis for future studies, including the characterisation of these enzymes under different physicochemical conditions simulating those of winemaking. Besides using classical methods that most researchers have adhered to in evaluating the activity of enzymes, we have shown using molecular techniques that bacterial isolates from wine also possess genes encoding different enzymes of interest in winemaking. For future studies, it would also be interesting to evaluate all the enzymes investigated in this study for their ability to influence wine aroma.

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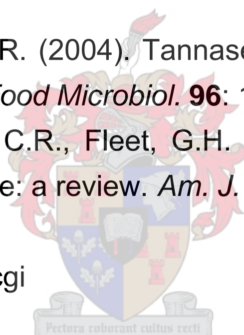
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Table 3.1 The list of primers used in this study

Primer name	***Primer sequence (5` - 3`)***	***Application***	***Reference***
BGL-1	GTGACTATGGTAGAGTTTCC - fwd	β-Glucosidase gene	Spano *et al*., 2005
BGL-2	TCAAAACCCATTCCGTTCCCCA - rev		
MLE-1	GCGATGACAAAAACTGCAAGTGA - fwd	Malolactic enzyme gene	This work
MLE-2	CTATTTGCTGATGGCCCGGTA - rev		
MLE-int-1	GAAGCAACTTTGAAGAATGC - fwd	Internal primers for malolactic enzyme gene	This work
MLE-int-2	CGTGTCGTCAAATAGTAAACCTTGC - rev		
Prt-1	GCATGGCTAATAAATCATTAATCAAAG - fwd	Serine protease HtrA gene	This work
Prt-2	GCTTAGTTACTTTGTTTAGTTAACGTTTTG - rev		
Est-1	GCTAATTTGTAACCGTATCCGCC - fwd	Putative esterase gene	This work
Est-2	CGCGCATGTTAACTTTTAGTAGAAC - rev		
Gluc-1	CGCATGAAGAGACTAAAATTAGTGCC - fwd	Putative glucanase gene	This work
Gluc-2	GCGCTACATTTTAGCAGCATCTAAA - rev		
Lip-1	CGCGCATGAAACTTACAGATAAAATT - fwd	Putative lipase gene	This work
Lip-2	GCGCGTTATTTACTCATATGTTCTCTG - rev		
Lipdel-1	ATGAAGAAAGTCGTGCTTTTTGGCG - fwd	Putative lipase gene	This work
Lipdel-2	CTATGCCATCTTATTGATTTGGTCAG - rev		

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Table 3.2 PCR amplification programs

Primer pair	***Initial denaturation temp (°C), time (min)***	**Main cycling conditions**				***Final extension temp (°C), time (min)***	***Reference***
		Number of cycles	***Denaturing temp (°C), time***	***Annealing temp (°C), time***	***Extension temp (°C), time (min)***		
BGL-1/BGL-2	94, 5	30	94, 1 min	50, 40 s	72, 1.2	72, 10	Spano *et al.*, 2005
MLE-1/MLE-2	94, 5	30	94, 1 min	55, 30 s	72, 1	72, 10	This work
Prt-1/Prt-2	94, 5	30	94, 1 min	55, 30 s	72, 1	72, 10	This work
Est-1/Est-2	94, 5	30	94, 1 min	53, 30 s	72, 1	72, 10	This work

Table 3.3 Determination of enzymes on the plates[a,b]

Isolate no.	Species name	Species code	Enzymes: BGL[c] (arbutin)	Enzymes: Glucanase (CMC)[d]	Enzymes: Glucanase (lichenan)
2.1	*Lb. plantarum*	Lplant 2.1	●	●	●
2.1	Non-identified	Non-id 2.1	●	●*	●*
2.1	Non-identified	Non-id 2.1	●	●	●*
3	*Lb. hilgardii*	Lhil 3	●	●	●
3.2	*Leuc. mesenter.*	Leuc 3.2	--	●*	●*
3.3	*Lb. brevis*	Lbrev 3.3	--	ns[e]	ns
4	*Leuc. mesenter.*	Leuc 4	--	--	--
5	*Leuc. mesenter.*	Leuc 5	--	--	--
5.1	*Leuc. mesenter.*	Leuc 5.1	--	●	●*
6.1	*Lb. plantarum*	Lplant 6.1	--	●*	●*
9.1	Non-identified	Non-id 9.1	ns	ns	ns
13.1	Non-identified	Non-id 13.1	--	ns	ns
14	*Lb. plantarum*	Lplant 14	●	ns	ns
14.1	*Lb. plantarum*	Lplant 14.1	●	●	●*
16.1	*Lb. hilgardii*	Lhil 16.1	--	ns	ns
17	*Lb. hilgardii*	Lhil 17	--	ns	ns
19.4	Non-identified	Non-id 19.4	--	ns	ns
21.1	*Lb. plantarum*	Lplant 21.1	●	ns	ns
21.1	Non-identified	Non-id 21.1	ns	●**	--
21.2	Non-identified	Non-id 21.2	ns	ns	ns
21.3	Non-identified	Non-id 21.3	--	ns	ns
21.8	Non-identified	Non-id 21.8	●	ns	ns
23.1	Non-identified	Non-id 23.1	●	ns	ns
29	Non-identified	Non-id 29	●	ns	ns
29.1	*Lb. paracasei*	Lpar 29.1	--	--	--
29.2	*Lb. paracasei*	Lpar 29.2	--	--	--
30	*Lb. paracasei*	Lpar 30	--	--	--
30.1	Non-identified	Non-id 30.1	--	ns	ns
31	*Lb. plantarum*	Lplant 31	--	--	--
39	*Lb. paracasei*	Lpar 39	--	ns	ns
39.3	Non-identified	Non-id 39.3	--	ns	ns
40.3	*Lb. plantarum*	Lplant 40.3	●	●*	●*
41.1	*Lb. plantarum*	Lplant 41.1	ns	ns	ns
42	*Lb. pentosus*	Lpent 42	--	●	●
42.1	*Lb. pentosus*	Lpent 42.1	--	●	●
42.2	*Lb. pentosus*	Lpent 42.2	--	●	●**
43	*Lb. plantarum*	Lplant 43	--	●	●
43.1	*Lb. plantarum*	Lplant 43.1	--	●	●
44	*Lb. paracasei*	Lpar 44	--	●	●*
45	*Lb. plantarum*	Lplant 45	--	●	●
46	*Lb. pentosus*	Lpent 46	--	●	--
50	*Lb. plantarum*	Lplant 50	--	●	●
51.2	*Lb. hilgardii*	Lhil 51.2	--	ns	ns
52	*Lb. pentosus*	Lpent 52	--	●*	●*
52.1	*Lb. pentosus*	Lpent 52.1	--	●	●*

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Table 3.3 (contd)

53	*Lb. pentosus*	Lpent 53	--	●	--
53.1	*Lb. pentosus*	Lpent 53.1	--	●	●**
54	*Lb. paracasei*	Lpar 54	--	●	--
55	*Lb. plantarum*	Lplant 55	●	●	--
55.1	*Lb. plantarum*	Lplant 55.1	●	●	●
56	*Lb. plantarum*	Lplant 56	--	●	●
56.1	Non-identified	Non-id 56.1	--	●	●
57	Non-identified	Non-id 57	--	●	●*
65	*Lb. plantarum*	Lplant 65	●	●	●
65.1	*Lb. pentosus* *Lb. plantarum*	Lpentplan 65.1	--	●	●
66	*Lb. plantarum*	Lplant 66	●	●	●
66.1	*Lb. plantarum*	Lplant 66.1	--	●**	●**
68	*Lb. plantarum*	Lplant 68	●	●	●
69	*Lb. plantarum*	Lplant 69	●	●	●
69.1	*Lb. plantarum*	Lplant 69.1	●	●	●
70	*Lb. plantarum*	Lplant 70	●	●	●
71	*Lb. plantarum*	Lplant 71	●	●	●
71.1	*Lb. plantarum*	Lplant 71.1	●	●	●
73.1	*Lb. plantarum*	Lplant 73.1	●	●	●*
73.2	*Lb. plantarum*	Lplant 73.2	ns	●	●
75	*Lb. plantarum*	Lplant 75	--	●	●
76.1	*O. oeni*	Oenos 76.1	--	ns	ns
76.2	*Lb. plantarum*	Lplant 76.2	●	●	●
77	*Lb. paracasei*	Lpar 77	●	●*	●*
77.1	*Lb. plantarum*	Lplant 77.1	●	●*	--
77.1	*Lb. plantarum*	Lplant 77.1	●	●	●*
78	*Lb. plantarum*	Lplant 78	●	●*	●*
78.1	*Lb. plantarum*	Lplant 78.1	●	●	●
79	*Lb. paracasei*	Lpar 79	●	●	●*
79.1	*Lb. plantarum*	Lplant 79.1	●	●	--
79.1	*Lb. plantarum*	Lplant 79.1	●	●	●
79.2	*Lb. pentosus*	Lpent 79.2	●	●*	--
79.3	*Lb. plantarum*	Lplant 79.3	--	--	--
80	*Lb. plantarum*	Lplant 80	●	●	●
80.1	Non-identified	Non-id 80.1	--	●	--
80.2	*Lb. plantarum*	Lplant 80.2	●	●	●*
81.1	*Lb. brevis*	Lbrev 81.1	●	●	●*
81.2	*Lb. plantarum*	Lplant 81.2	●	●	●*
81.2	*Lb. plantarum*	Lplant 81.2	●	●	●*
82	*Lb. plantarum*	Lplant 82	●	●	●
82.2	*Lb. plantarum*	Lplant 82.2	--	●*	●*
83	*Lb. plantarum*	Lplant 83	●	●	●*
83.1	*Lb. paracasei*	Lpar 83.1	●	●	●
84	*Lb. paracasei*	Lpar 84	--	●	●
84.1	Non-identified	Non-id 84.1	--	●	●
85	*Lb. plantarum*	Lplant 85	●	●*	●*
85.1	*Lb. plantarum*	Lplant 85.1	●	●	--
85.2	*Lb. plantarum*	Lplant 85.2	●	●	●**
86	*Lb. plantarum*	Lplant 86	--	●	●

Pectora roborant cultus recti

Table 3.3 (contd)

87.1	*Lb. hilgardii*	Lhil 87.1	●	●	●
89	*Lb. brevis*	Lbrev 89	--	--	--
89.1	Non-identified	Non-id 89.1	--	ns	ns
89.2	Non-identified	Non-id 89.2	ns	●*	●*
92.1	Non-identified	Non-id 92.1	--	●	●*
94.1	*Lb. plantarum*	Lplant 94.1	--	ns	ns
94.1	*Lb. plantarum*	Lplant 94.1	--	ns	ns
98	Non-identified	Non-id 98	--	●**	●**
105	*Pediococcus*	Peds 105	--	ns	ns
105.1	Non-identified	Non-id 105.1	--	ns	ns
105.2	Non-identified	Non-id 105.2	--	--	--
105.3	Non-identified	Non-id 105.3	--	ns	ns
105.4	Non-identified	Non-id 105.4	--	ns	ns
105.5	Non-identified	Non-id 105.5	--	ns	ns
105.6	Non-identified	Non-id 105.6	--	ns	ns
105.7	Non-identified	Non-id 105.7	--	ns	ns
105.8	*Lb. paracasei*	Lpar 105.8	--	--	--
106	Non-identified	Non-id 106	--	ns	ns
106.1	*Lb. plantarum*	Lplant 106.1	--	ns	ns
106.4	*Lb. plantarum*	Lplant	--	ns	ns
106.5	*Lb. plantarum*	Lplant 106.5	--	ns	ns
106.6	*Lb. plantarum*	Lplant 106.6	--	ns	ns
106.7	Non-identified	Non-id 106.7	--	ns	ns
106.8	*Lb. plantarum*	Lplant 106.8	--	ns	ns
106.9	Non-identified	Non-id 106.9	--	ns	ns
107	*Lb. plantarum*	Lplant 107	--	●	--
107.1	*Lactobacillus sp.*	Lact sp.	●	●	--
107.2	*Lb. plantarum*	Lplant 107.2	--	--	--
107.4	*Lb. plantarum*	Lplant 107.4	--	--	--
107.5	*Lb. plantarum*	Lplant 107.5	●	●	●
108	Non-identified	Non-id 108	●	ns	ns
108.2	*Lb. brevis*	Lbrev 108.2	--	ns	ns
108.3	Non-identified	Non-id 108.3	--	ns	ns
108.4	Non-identified	Non-id 108.4	●	ns	ns
108.5	*Lb. paraplantarum*	Lparplant 108.5	●	ns	ns
109	*Lb. plantarum*	Lplant 109	--	ns	ns
109.1	Non-identified	Non-id 109.1	●	ns	ns
109.2	*Lb. plantarum*	Lplant 109.2	●	ns	ns
109.3	*Lb. plantarum*	Lplant 109.3	--	ns	ns
111	*Lb. brevis*	Lbrev 111	--	●*	●*
111.1	Non-identified	Non-id 111.1	--	●*	●*
112	Non-identified	Non-id 112	--	ns	ns
112.1	Non-identified	Non-id 112.1	--	ns	ns
113	*Lb. pentosus*	Lpent 113	●	●	●*
113.1	*Lb. plantarum*	Lplant 113.1	●	●	●
113.2	Non-identified	Non-id 113.2	--	--	--
113.3	Non-identified	Non-id 113.3	●	●	●
113.4	*Lb. plantarum*	Lplant 113.4	●	●	●
113.5	Non-identified	Non-id 113.5	--	--	--
115	Non-identified	Non-id 115	--	--	--

Table 3.3 (contd)

115.3	Non-identified	Non-id 115.3	●	--	--
116	*Lb. brevis*	Lbrev 116	--	ns	ns
116.1	Non-identified	Non-id 116.1	●	ns	ns
116.2	*Lb. brevis*	Lbrev 116.2	--	ns	ns
116.3	*Lb. brevis*	Lbrev 116.3	●	ns	ns
116.4	*Lb. plantarum*	Lplant 116.4	--	ns	ns
116.5	Non-identified	Non-id 116.5	●	ns	ns
117	*Lb. brevis*	Lbrev 117	●	●*	--
117.1	*Lb. brevis*	Lbrev 117.1	●	●	●*
117.2	*Lb. brevis*	Lbrev 117.2	●	●	●
118	*P. acidilactici*	Pedaci 118	●	ns	ns
118.2	*P. acidilactici*	Pedaci 118.2	--	ns	ns
119	*Lb. plantarum*	Lplant 119	●	●	●
120	*Lb. plantarum*	Lplant 120	●	ns	ns
120.1	*Lb. plantarum*	Lplant 120.1	●	ns	ns
120.3	*Lb. pentosus* *Lb. plantarum*	Lpentplan 120.3	●	ns	ns
120.4	Non-identified	Non-id 120.4	--	--	--
121	*Pediococcus*	Peds 121	--	ns	ns
121.1	*Pediococcus*	Peds 121.1	--	ns	ns
121.2	*Pediococcus*	Peds 121.2	--	ns	ns
122	Non-identified	Non-id 122	--	ns	ns
122.1	Non-identified	Non-id 122.1	--	ns	ns
122.2	Non-identified	Non-id 122.2	--	ns	ns
122.5	Non-identified	Non-id 122.5	--	--	--
122.6	Non-identified	Non-id 122.6	--	--	--
122.7	*Lb. plantarum*	Lplant 122.7	●	--	--
122.9	Non-identified	Non-id 122.9	--	--	--
122.10	Non-identified	Non-id 122.10	--	--	--
124.1	*Lb. paracasei*	Lpar 124.1	--	●*	●*
124.2	*Lb. paracasei* *Lb. plantarum*	Lparaplan 124.2	--	●	●
127	Non-identified	Non-id 127	--	●*	●**
130	*Lb. plantarum*	Lplant 130	--	●*	--
130.1	*Lb. plantarum*	Lplant 130.1	●	●*	●*
130.2	*Lb. brevis* *Lb. plantarum*	Lbrevplan 130.2	--	--	--
130.3	*Lb. brevis* *Lb. plantarum*	Lbrevplan 130.3	●	●**	--
130.4	*Lb. brevis* *Lb. plantarum*	Lbrevplan 130.4	●	●	--
130.6	*Lb. brevis* *Lb. plantarum*	Lbrevplan 130.6	--	●*	--
131	*Lb. plantarum*	Lplant 131	--	ns	ns
131.1	*Lb. plantarum*	Lplant 131.1	--	ns	ns
131.2	*Lb. plantarum*	Lplant 131.2	●	ns	ns
131.3	*Lb. plantarum*	Lplant 131.3	●	ns	ns
151	*O. oeni*	Oenos 151	--	ns	ns
152	*O. oeni*	Oenos 152	--	ns	ns
152.1	*O. oeni*	Oenos 152.1	--	ns	ns

Table 3.3 (contd)

152.2	*O. oeni*	Oenos 152.2	●	ns	ns
21.7.2	Non-identified	Non-id 21.7.2	●	●	--
21.7.2	Non-identified	Non-id 21.7.2	--	ns	ns

[a] (●) denotes the presence of enzyme activity; (--) denotes the absence of activity.
[b] (*) denotes weak activity; (**) denotes very weak activity.
[c] BGL, β-glucosidase.
[d] CMC, carboxymethylcellulose.
[e] ns, not tested.

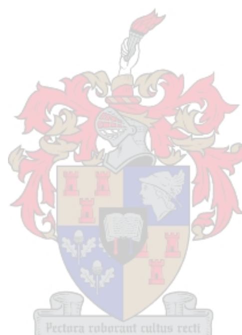

Table 3.4 Determination of enzymes using colony PCR[a]

Isolate no.	Species name	Species code	Enzymes			
			BGL[b]	Protease	MLE[c]	Esterase
2.1	*Lb. plantarum*	Lplant 2.1	●	●	●	●
2.1	Non-identified	Non-id 2.1	●	●	●	●
2.1	Non-identified	Non-id 2.1	●	●	●	●
3	*Lb. hilgardii*	Lhil 3	●	●	●	●
3.2	*Leuc. mesenter.*	Leuc 3.2	ns[d]	--	--	--
3.3	*Lb. brevis*	Lbrev 3.3	ns	--	●	--
4	*Leuc. mesenter.*	Leuc 4	ns	--	--	--
5	*Leuc. mesenter.*	Leuc 5	ns	--	--	--
5.1	*Leuc. mesenter.*	Leuc 5.1	ns	--	--	--
6.1	*Lb. plantarum*	Lplant 6.1	ns	--	--	--
9.1	Non-identified	Non-id 9.1	ns	--	--	--
13.1	Non-identified	Non-id 13.1	ns	--	--	--
14	*Lb. plantarum*	Lplant 14	●	●	●	●
14.1	*Lb. plantarum*	Lplant 14.1	●	●	●	●
16.1	*Lb. hilgardii*	Lhil 16.1	ns	--	--	--
17	*Lb. hilgardii*	Lhil 17	ns	--	--	--
19.4	Non-identified	Non-id 19.4	ns	--	--	--
21.1	*Lb. plantarum*	Lplant 21.1	●	●	--	●
21.1	Non-identified	Non-id 21.1	ns	--	--	--
21.2	Non-identified	Non-id 21.2	ns	--	--	--
21.3	Non-identified	Non-id 21.3	ns	--	--	--
21.8	Non-identified	Non-id 21.8	--	ns	ns	ns
23.1	Non-identified	Non-id 23.1	ns	--	--	--
29	Non-identified	Non-id 29	--	--	--	--
29.1	*Lb. paracasei*	Lpar 29.1	ns	--	--	--
29.2	*Lb. paracasei*	Lpar 29.2	--	--	--	--
30	*Lb. paracasei*	Lpar 30	ns	--	--	--
30.1	Non-identified	Non-id 30.1	ns	--	--	--
31	*Lb. plantarum*	Lplant 31	ns	--	--	--
39	*Lb. paracasei*	Lpar 39	ns	--	--	--
39.3	Non-identified	Non-id 39.3	ns	--	--	--
40.3	*Lb. plantarum*	Lplant 40.3	●	●	●	●
41.1	*Lb. plantarum*	Lplant 41.1	ns	--	--	--
42	*Lb. pentosus*	Lpent 42	ns	--	--	--
42.1	*Lb. pentosus*	Lpent 42.1	ns	--	--	--
42.2	*Lb. pentosus*	Lpent 42.2	ns	--	●	--
43	*Lb. plantarum*	Lplant 43	ns	--	--	--
43.1	*Lb. plantarum*	Lplant 43.1	ns	--	--	--
44	*Lb. paracasei*	Lpar 44	--	--	--	--
45	*Lb. plantarum*	Lplant 45	ns	--	--	--
46	*Lb. pentosus*	Lpent 46	ns	--	●	--
50	*Lb. plantarum*	Lplant 50	ns	●	●	●
51.2	*Lb. hilgardii*	Lhil 51.2	ns	--	--	--
52	*Lb. pentosus*	Lpent 52	ns	--	--	--
52.1	*Lb. pentosus*	Lpent 52.1	ns	--	--	--
53	*Lb. pentosus*	Lpent 53	ns	--	--	--
53.1	*Lb. pentosus*	Lpent 53.1	ns	--	--	--

Pectora roborant cultus recti

Table 3.4 (contd)

54	*Lb. paracasei*	Lpar 54	ns	--	--	--
55	*Lb. plantarum*	Lplant 55	●	●	--	●
55.1	*Lb. plantarum*	Lplant 55.1	●	--	--	●
56	*Lb. plantarum*	Lplant 56	ns	●	●	●
56.1	Non-identified	Non-id 56.1	--	●	--	●
57	Non-identified	Non-id 57	ns	●	●	●
65	*Lb. plantarum*	Lplant 65	--	●	●	●
65.1	*Lb. pentosus* *Lb. plantarum*	Lpentplan 65.1	ns	--	--	●
66	*Lb. plantarum*	Lplant 66	--	--	●	●
66.1	*Lb. plantarum*	Lplant 66.1	--	●	●	●
68	*Lb. plantarum*	Lplant 68	●	●	●	●
69	*Lb. plantarum*	Lplant 69	●	●	●	●
69.1	*Lb. plantarum*	Lplant 69.1	●	●	--	●
70	*Lb. plantarum*	Lplant 70	●	●	●	●
71	*Lb. plantarum*	Lplant 71	--	●	●	●
71.1	*Lb. plantarum*	Lplant 71.1	--	--	●	●
73.1	*Lb. plantarum*	Lplant 73.1	--	●	●	●
75	*Lb. plantarum*	Lplant 75	ns	●	●	●
76.2	*Lb. plantarum*	Lplant 76.2	●	●	●	●
77	*Lb. paracasei*	Lpar 77	●	--	--	●
77.1	*Lb. plantarum*	Lplant 77.1	●	●	●	●
77.1	*Lb. plantarum*	Lplant 77.1	●	●	●	●
78	*Lb. plantarum*	Lplant 78	ns	--	●	●
78.1	*Lb. plantarum*	Lplant 78.1	--	●	●	●
79	*Lb. paracasei*	Lpar 79	--	●	●	●
79.1	*Lb. plantarum*	Lplant 79.1	●	●	--	●
79.1	*Lb. plantarum*	Lplant 79.1	●	●	--	●
79.2	*Lb. pentosus*	Lpent 79.2	●	●	●	●
79.3	*Lb. plantarum*	Lplant 79.3	--	--	●	●
80	*Lb. plantarum*	Lplant 80	--	--	--	--
80.1	Non-identified	Non-id 80.1	ns	●	●	●
80.2	*Lb. plantarum*	Lplant 80.2	●	●	●	●
81.1	*Lb. brevis*	Lbrev 81.1	●	●	●	●
81.2	*Lb. plantarum*	Lplant 81.2	●	●	●	●
81.2	*Lb. plantarum*	Lplant 81.2	●	●	●	●
82	*Lb. plantarum*	Lplant 82	--	●	●	●
82.2	*Lb. plantarum*	Lplant 82.2	ns	●	--	●
83	*Lb. plantarum*	Lplant 83	--	--	--	●
83.1	*Lb. paracasei*	Lpar 83.1	--	●	●	●
84	*Lb. paracasei*	Lpar 84	ns	●	●	●
84.1	Non-identified	Non-id 84.1	ns	●	●	●
85	*Lb. plantarum*	Lplant 85	●	●	●	●
85.1	*Lb. plantarum*	Lplant 85.1	●	--	--	●
85.2	*Lb. plantarum*	Lplant 85.2	●	●	●	●
86	*Lb. plantarum*	Lplant 86	ns	●	●	●
87.1	*Lb. hilgardii*	Lhil 87.1	●	●	●	●
89	*Lb. brevis*	Lbrev 89	ns	--	--	--
89.1	Non-identified	Non-id 89.1	--	--	--	--

Table 3.4 (contd)

92.1	Non-identified	Non-id 92.1	--	--	--	--
94.1	*Lb. plantarum*	Lplant 94.1	--	--	--	--
94.1	*Lb. plantarum*	Lplant 94.1	--	--	--	--
98	Non-identified	Non-id 98	--	--	--	--
105	*Pediococcus*	Peds 105	--	--	--	--
105.1	Non-identified	Non-id 105.1	--	--	--	--
105.2	Non-identified	Non-id 105.2	--	--	--	--
105.3	Non-identified	Non-id 105.3	--	--	--	--
105.4	Non-identified	Non-id 105.4	--	--	--	--
105.5	Non-identified	Non-id 105.5	--	--	--	--
105.6	Non-identified	Non-id 105.6	--	--	--	--
105.7	Non-identified	Non-id 105.7	--	--	--	--
105.8	*Lb. paracasei*	Lpar 105.8	--	--	--	--
106	Non-identified	Non-id 106	ns	--	ns	--
106.1	*Lb. plantarum*	Lplant 106.1	ns	--	ns	--
106.4	*Lb. plantarum*	Lplant	ns	--	ns	--
106.5	*Lb. plantarum*	Lplant 106.5	ns	--	ns	--
106.6	*Lb. plantarum*	Lplant 106.6	ns	--	ns	--
106.7	Non-identified	Non-id 106.7	ns	--	--	ns
106.8	*Lb. plantarum*	Lplant 106.8	ns	--	--	ns
106.9	Non-identified	Non-id 106.9	ns	--	--	ns
107	*Lb. plantarum*	Lplant 107	--	●	●	●
107.1	*Lactobacillus sp.*	Lact sp.	●	--	--	--
107.2	*Lb. plantarum*	Lplant 107.2	ns	--	--	--
107.4	*Lb. plantarum*	Lplant 107.4	--	--	--	--
107.5	*Lb. plantarum*	Lplant 107.5	--	●	--	●
108	Non-identified	Non-id 108	ns	--	--	ns
108.2	*Lb. brevis*	Lbrev 108.2	ns	--	--	ns
108.3	Non-identified	Non-id 108.3	ns	--	--	ns
108.4	Non-identified	Non-id 108.4	--	--	--	ns
108.5	*Lb. paraplantarum*	Lparplant 108.5	ns	--	●	ns
109	*Lb. plantarum*	Lplant 109	ns	●	●	ns
109.1	Non-identified	Non-id 109.1	--	●	●	ns
109.2	*Lb. plantarum*	Lplant 109.2	--	●	●	ns
109.3	*Lb. plantarum*	Lplant 109.3	ns	●	●	ns
111	*Lb. brevis*	Lbrev 111	ns	--	--	--
111.1	Non-identified	Non-id 111.1	ns	--	--	--
112	Non-identified	Non-id 112	ns	--	--	ns
112.1	Non-identified	Non-id 112.1	ns	--	ns	ns
113	*Lb. pentosus*	Lpent 113	●	--	--	--
113.1	*Lb. plantarum*	Lplant 113.1	●	--	--	--
113.2	Non-identified	Non-id 113.2	--	--	--	--
113.3	Non-identified	Non-id 113.3	--	--	--	--
113.4	*Lb. plantarum*	Lplant 113.4	--	--	--	--
113.5	Non-identified	Non-id 113.5	--	●	--	●
115	Non-identified	Non-id 115	ns	●	●	●
115.3	Non-identified	Non-id 115.3	ns	●	●	●
116	*Lb. brevis*	Lbrev 116	ns	--	ns	--
116.1	Non-identified	Non-id 116.1	--	--	ns	--
116.2	*Lb. brevis*	Lbrev 116.2	ns	--	ns	--

Table 3.4 (contd)

116.3	*Lb. brevis*	Lbrev 116.3	●	●	●	●
116.4	*Lb. plantarum*	Lplant 116.4	ns	--	ns	--
116.5	Non-identified	Non-id 116.5	ns	--	ns	--
117	*Lb. brevis*	Lbrev 117	--	--	--	--
117.1	*Lb. brevis*	Lbrev 117.1	--	--	--	--
117.2	*Lb. brevis*	Lbrev 117.2	●	●	●	●
118	*P. acidilactici*	Pedaci 118	--	--	ns	--
118.2	*P. acidilactici*	Pedaci 118.2	ns	--	ns	--
119	*Lb. plantarum*	Lplant 119	●	●	●	●
120	*Lb. plantarum*	Lplant 120	--	●	ns	●
120.1	*Lb. plantarum*	Lplant 120.1	●	●	ns	●
120.3	*Lb. pentosus* *Lb. plantarum*	Lpentplan 120.3	--	--	ns	--
120.4	Non-identified	Non-id 120.4	--	--	--	--
121	*Pediococcus*	Peds 121	--	--	--	--
121.1	*Pediococcus*	Peds 121.1	--	--	--	--
121.2	*Pediococcus*	Peds 121.2	--	--	--	--
122	Non-identified	Non-id 122	ns	--	--	--
122.1	Non-identified	Non-id 122.1	ns	--	--	--
122.2	Non-identified	Non-id 122.5	ns	--	--	--
122.5	Non-identified	Non-id 122.5	ns	--	--	--
122.6	Non-identified	Non-id 122.6	ns	--	--	--
122.7	*Lb. plantarum*	Lplant 122.7	●	●	--	●
122.9	Non-identified	Non-id 122.9	ns	--	--	--
122.10	Non-identified	Non-id 122.10	ns	--	--	--
124.1	*Lb. paracasei*	Lpar 124.1	ns	--	--	--
124.2	*Lb. paracasei* *Lb. plantarum*	Lparaplan 124.2	ns	●	●	●
127	Non-identified	Non-id 127	ns	--	--	--
130	*Lb. plantarum*	Lplant 130	ns	--	--	●
130.1	*Lb. plantarum*	Lplant 130.1	ns	--	--	--
130.2	*L. brevis* *L. plantarum*	Lbrevplan 130.2	ns	--	ns	--
130.3	*L. brevis* *L. plantarum*	Lbrevplan 130.3	--	●	ns	●
130.4	*L. brevis* *L. plantarum*	Lbrevplan 130.4	●	●	ns	●
130.6	*L. brevis* *L. plantarum*	Lbrevplan 130.6	ns	--	ns	--
131	*L. plantarum*	Lplant 131	ns	●	ns	●
131.1	*L. plantarum*	Lplant 131.1	ns	●	ns	●
131.2	*L. plantarum*	Lplant 131.2	--	--	ns	--
131.3	*L. plantarum*	Lplant 131.3	--	--	ns	--
21.7.2 *(a)*	Non-identified	Non-id 21.7.2	--	●	●	●
21.7.2 *(b)*	Non-identified	Non-id 21.7.2	--	--	--	--

[a] (●) denotes the presence of enzyme activity; (--) denotes the absence of activity.
[b] BGL, β-glucosidase.
[c] MLE, malolactic enzyme.
[d] ns, not tested.

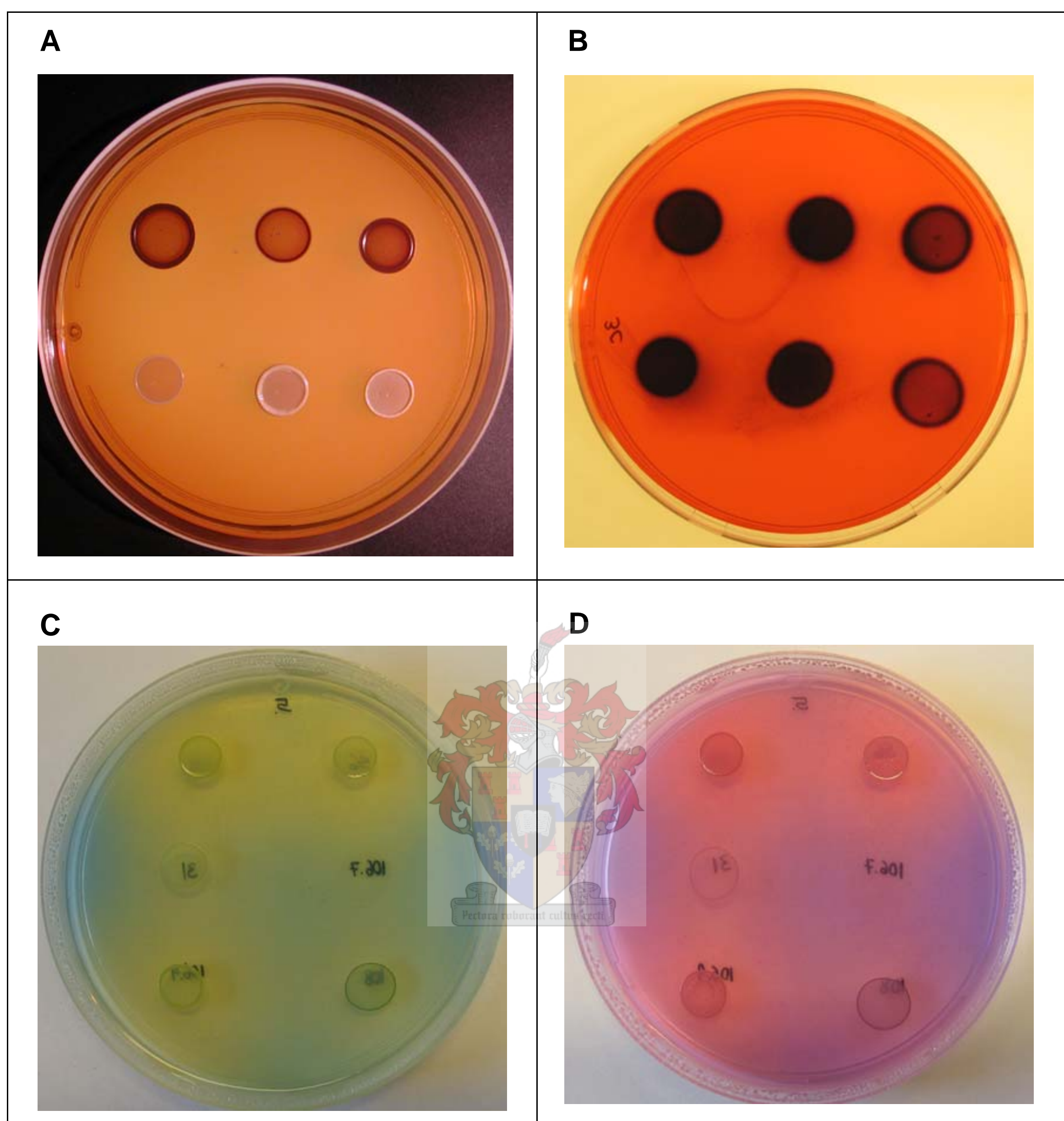

Figure 3.1 Detection of enzymes on the plates. (A) Identification of β-glucosidase activity on MRS agar with arbutin. Isolates with activity contain dark halo (top row) while those without activity remain white (bottom row). (B) Identification of glucanase activity on PHB agar medium with CMC. (C) Detection of lipolytic activity on tributyrin agar supplemented with tributyrin. (D) Detection of lipolytic activity on tributyrin agar supplemented with tributyrin and rhodamine B dye.

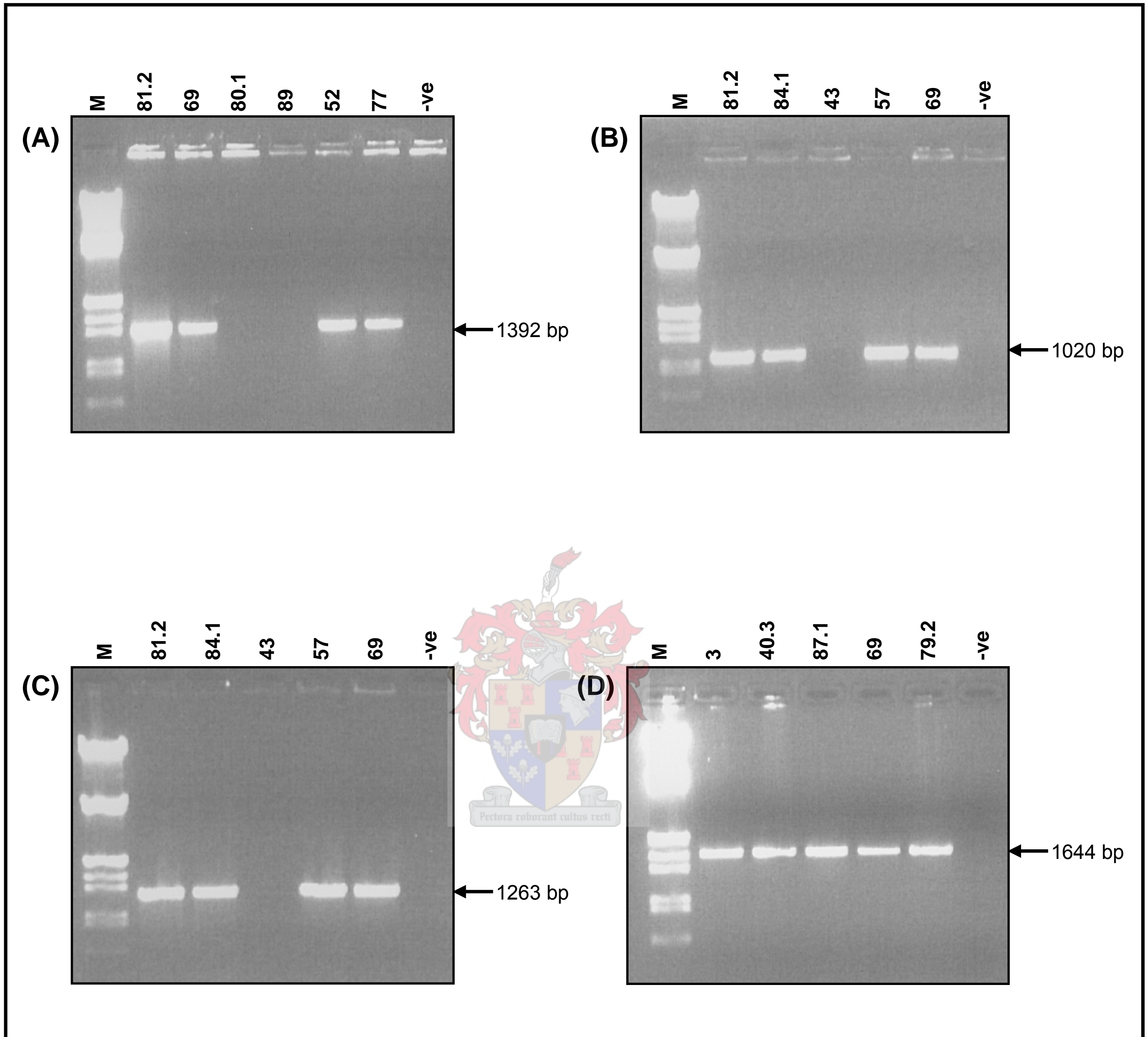

Figure 3.2 PCR amplifications showing the presence of β-glucosidase genes (A), esterase genes (B), serine protease HtrA genes (C) and malolactic enzyme genes (D). **M** is the standard molecular weight marker. Species codes: 81.2 - *Lb. plantarum*; 69 - *Lb. plantarum*; 80.1 - Non-identified sp.; 89 - *Lb. brevis*; 52 - *Lb. pentosus*; 77 - *Lb. paracasei*; 84.1 - Non-identified sp.; 43 - *Lb. plantarum*; 57 - Non-identified sp.; 3 - *Lb. hilgardii*; 40.3 - *Lb. plantarum*; 87.1 - *Lb. hilgardii*; 79.2 - *Lb. pentosus*.

```
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Lbrev 116.3    ATGACAAAAACTGCAAGTGAAATTTTAAATAACCCATTCTTAAATAAAGGCACTGCCTTTACCAAAGAGGAACGTCAAGCACTTGGTTTGACGGGGACGT
Lbrev 117.2    ATGACAAAAACTGCAAGTGAAATTTTAAATAACCCATTCTTAAATAAAGGCACTGCCTTTACCAAAGAGGAACGTCAAGCACTTGGCTTGACGGGGACGT
Lplant 40.3    ATGACAAAAACTGCAAGTGAAATTTTAAATAACCCATTCTTAAATAAAGGCACTGCCTTTACCAAAGAGGAACGTCAAGCACTTGGCTTGACGGGGACGT
Lplant 69      ATGACAAAAACTGCAAGTGAAATTTTAAATAACCCATTCTTAAATAAAGGCACTGCCTTTACCAAAGAGGAACGTCAAGCACTTGGCTTGACGGGGACGT
Lhil 87.1      ATGACAAAAACTGCAAGTGAAATTTTAAATAACCCATTCTTAAATAAAGGCACTGCCTTTACCAAAGAGGAACGTCAAGCACTTGGCTTGACGGGGACGT
Lhil 3         ATGACAAAAACTGCAAGTGAAATTTTAAATAACCCATTCTTAAATAAAGGCACTGCCTTTACCAAAGAGGAACGTCAAGCACTTGGCTTGACGGGGACGT
Lbrev 81.1     ATGACAAAAACTGCAAGTGAAATTTTAAATAACCCATTCTTAAATAAAGGCACTGCCTTTACCAAAGAGGAACGTCAAGCACTTGGCTTGACGGGGACGT

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Lplant WCFS1*  TACCAAGCAAGGTTCAAACAATTGATGAACAGGCGACGCAAGCTTATGCCCAATTCAAGAGCAAGCCTAGTCGTTTGGAACAACGGATTTTCTTAATGAA
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Lplant 69      TACCGAGCAAGGTTCAAACAATTGATGAACAGGCGACGCAAGCTTATGCCCAATTCAAGAGCAAGCCTAGTCGTTTGGAACAACGGATTTTCTTAATGAA
Lhil 87.1      TACCGAGCAAGGTTCAAACAATTGATGAACAGGCGACGCAAGCTTATGCCCAATTCAAGAGCAAGCCTAGTCGTTTGGAACAACGGATTTTCTTAATGAA
Lhil 3         TACCAAGCAAGGTTCAAACAATTGATGAACAGGCGACGCAAGCTTATGCCCAATTCAAGAGCAAGCCTAGTCGTTTGGAACAACGGATTTTCTTAATGAA
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Lbrev 117.2    TCTCTTTAACGAAAACCGGACGTTGTTCTTCCATCTGATGGATGAACACGTGGTTGAATTTATGCCAATCGTTTATGATCCGGTCGTTGCTGATTCCATC
Lplant 40.3    TCTCTTTAACGAAAACCGGACGTTGTTCTTCCATCTGATGGATGAACACGTGGTTGAATTTATGCCAATCGTTTATGATCCGGTCGTTGCTGATTCCATC
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Lhil 3         TCTCTTTAACGAAAACCGGACGTTGTTCTTCCATCTGATGGATGAACACGTGGTTGAATTTATGCCAATCGTTTATGATCCGGTCGTTGCTGATTCCATC
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Lbrev 117.2    GAACAGTATAATGAATTGTTCTTGGATCCACAGAATGCGGCCTTCGTTTCCGTCGATGCACCTGAAGATATTGAAGCAACTTTGAAGAATGCCGCGGACG
Lplant 40.3    GAACAGTATAATGAATTGTTCTTGGATCCACAGAATGCGGCCTTCGTTTCCGTCGATGCACCTGAAGATATTGAAGCAACTTTGAAGAATGCCGCGGACG
Lplant 69      GAACAGTATAATGAATTGTTCTTGGATCCACAGAATGCGGCCTTCGTTTCCGTCGATGCACCTGAAGATATTGAAGCAACTTTGAAGAATGCCGCGGACG
Lhil 87.1      GAACAGTATAATGAATTGTTCTTGGATCCACAGAATGCGGCCTTCGTTTCCGTCGATGCACCTGAAGATATTGAAGCAACTTTGAAGAATGCCGCGGACG
Lhil 3         GAACAGTATAATGAATTGTTCTTGGATCCACAGAATGCGGCCTTCGTTTCCGTCGATGCACCTGAAGATATTGAAGCAACTTTGAAGAATGCCGCGGACG
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Lplant WCFS1*  GCCGTGATATTCGGTTAGTCGTTGTTACGGATGCTGAAGGTATTCTTGGTATGGGTGACTGGGGTGTTAACGGTGTTGATATTGCCATTGGTAAATTAAT
Lpar 83.1      GCCGTGATATTCGGTTAGTCGTTGTTACGGATGCTGAAGGTATTCTTGGTATGGGTGACTGGGGTGTTAACGGTGTTGATATTGCCATTGGTAAATTAAT
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Lbrev 117.2    GCCGTGATATTCGCTTAGTCGTTGTGACGGATGCTGAAGGGATTCTTGGTATGGGTGACTGGGGTGTCAACGGTGTTGATATCGCCATTGGTAAATTAAT
Lplant 40.3    GCCGTGATATTCGCTTAGTCGTTGTGACGGATGCTGAAGGGATTCTTGGTATGGGTGACTGGGGTGTCAACGGTGTTGATATCGCCATTGGTAAATTAAT
Lplant 69      GCCGTGATATTCGCTTAGCCGTTGTGACGGATGCTGAAGGGATTCTTGGTATGGGTGACTGGGGTGTCAACGGTGTTGATATCGCCATTGGTAAATTAAT
Lhil 87.1      GCCGTGATATTCGCTTAGTCGTTGTGACGGATGCTGAAGGGATTCTTGGTATGGGTGACTGGGGTGTCAACGGTGTTGATATCGCCATTGGTAAATTAAT
Lhil 3         GCCGTGATATTCGCTTAGTCGTTGTGACGGATGCTGAAGGGATTCTTGGTATGGGTGACTGGGGTGTCAACGGTGTTGATATCGCCATTGGTAAATTAAT
Lbrev 81.1     GCCGTGATATTCGCTTAGTCGTTGTGACGGATGCTGAAGGGATTCTTGGTATGGGTGACTGGGGTGTCAACGGTGTTGATATCGCCATTGGTAAATTAAT

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Lbrev 117.2    GGTTTACACCGCCGCCGCTGGTATTGACCCATCACAAGTACTGCCAGTTAGCATCGATGCTGGGACGAACAACCAGAAGTTATTAGATGATCCATTGTAT
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Lplant 69      GGTTTACACCGCCGCCGCTGGTATTGACCCATCACAAGTACTGCCAGTTAGCATCGATGCTGGGACGAACAACCAGAAGTTATTAGATGATCCATTGTAT
Lhil 87.1      GGTTTACACCGCCGCCGCTGGTATTGACCCATCACAAGTACTGCCAGTTAGCATCGATGCTGGGACGAACAACCAGAAGTTATTAGATGATCCATTGTAT
Lhil 3         GGTTTACACCGCCGCCGCTGGTATTGACCCATCACAAGTACTGCCAGTTAGCATCGATGCTGGGACGAACAACCAGAAGTTATTAGATGATCCATTGTAT
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Lbrev 116.3    CTTGGTAACCGGCACAAGCGTGTTTCCGGCGAACAATACTATGATGTGATCGACAAGTTCGTTGCTGCTGAACAACAATTATTCCCAGATTCATTGTTAC
Lbrev 117.2    CTTGGTAACCGTCACAAGCGTGTGTCCGGCGAACAATACTATGACGTGATCGACAAGTTCGTTGCTGCTGAACAACAATTATTCCCAGATTCATTGTTAC
Lplant 40.3    CTTGGTAACCGTCACAAGCGTGTGTCCGGCGAACAATACTATGACGTGATCGACAAGTTCGTTGCTGCTGAACAACAATTATTCCCAGATTCATTGTTAC
Lplant 69      CTTGGTAACCGTCACAAGCGTGTGTCCGGCGAACAATACTATGACGTGATCGACAAGTTCGTTGCTGCTGAACAACAATTATTCCCAGATTCATTGTTAC
Lhil 87.1      CTTGGTAACCGTCACAAGCGTGTGTCCGGCGAACAATACTATGACGTGATCGACAAGTTCGTTGCTGCTGAACAACAATTATTCCCAGATTCATTGTTAC
Lhil 3         CTTGGTAACCGTCACAAGCGTGTGTCCGGCGAACAATACTATGACGTGATCGACAAGTTCGTTGCTGCTGAACAACAATTATTCCCAGATTCATTGTTAC
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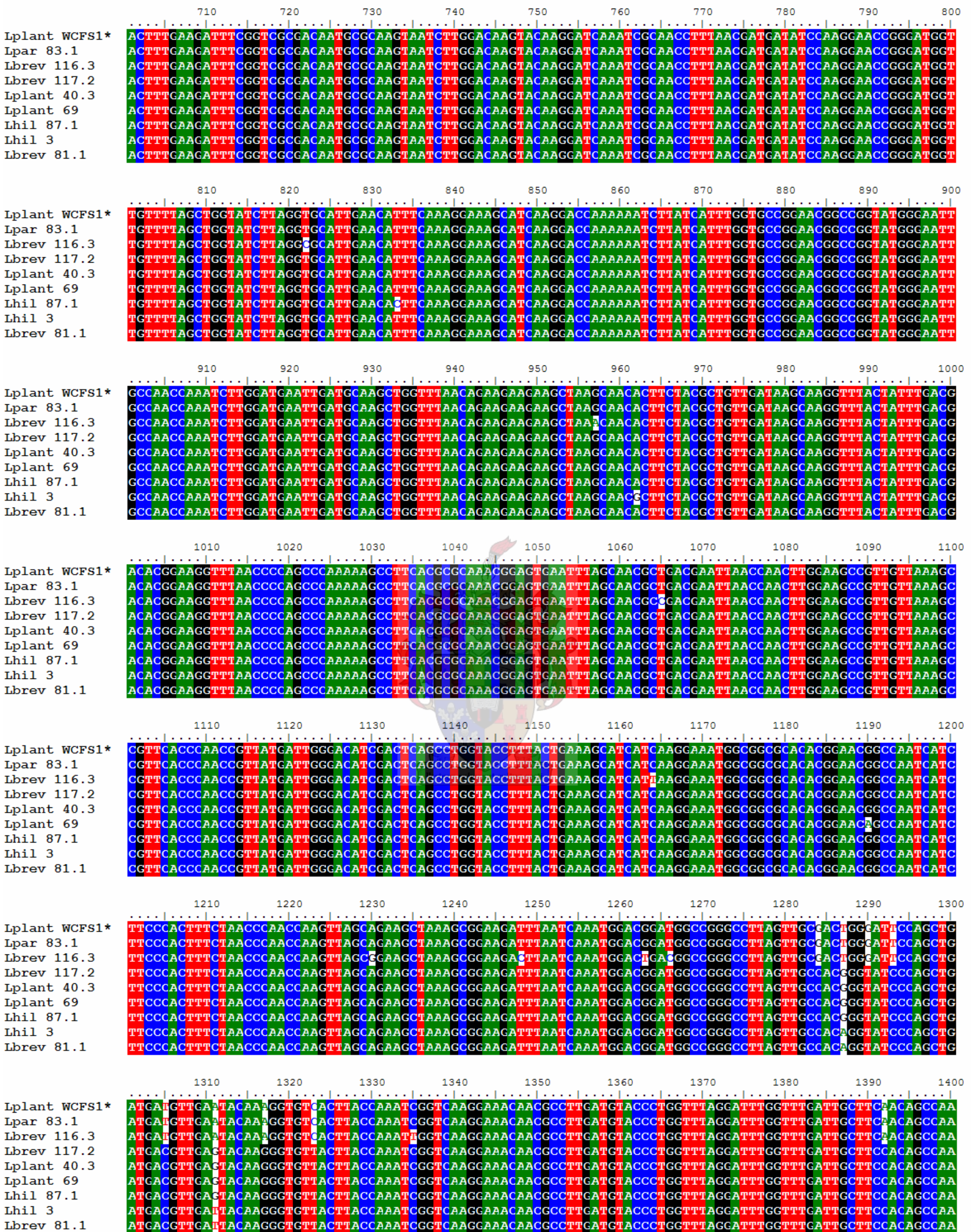
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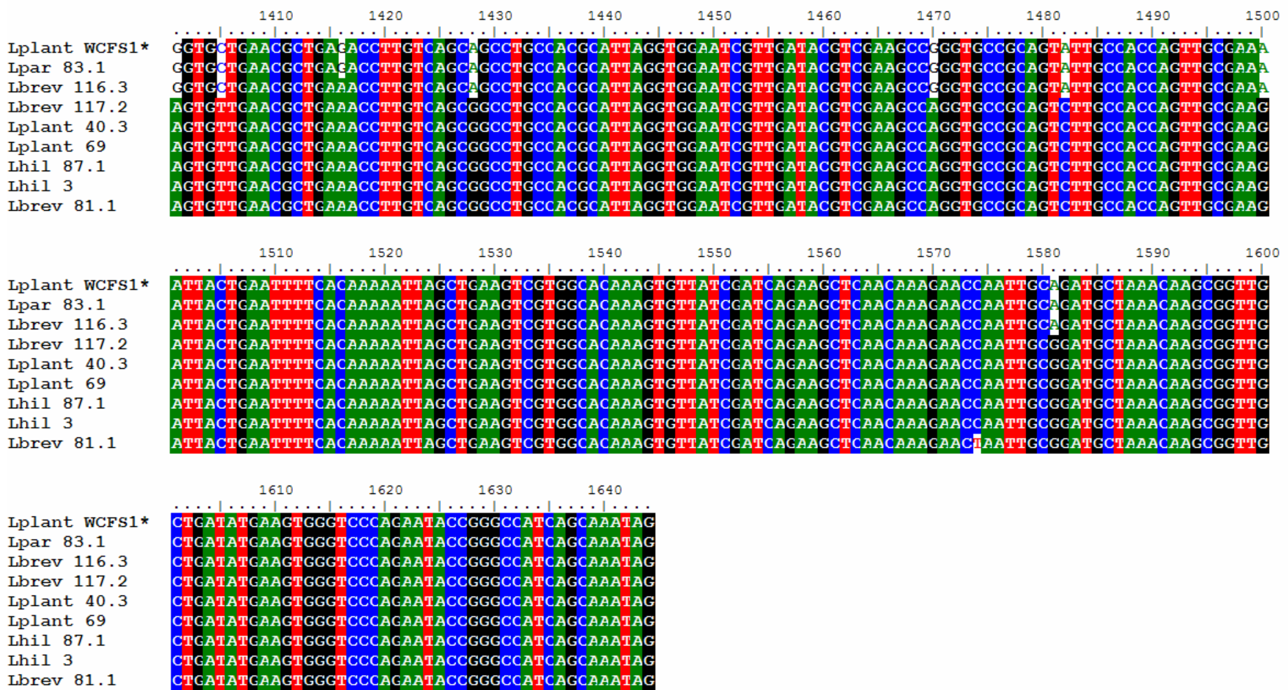
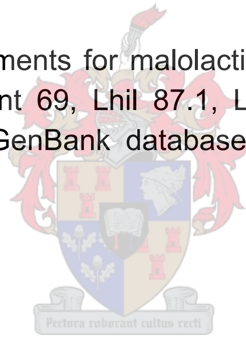


Figure 3.3 Nucleotide sequence alignments for malolactic enzyme genes from Lpar 83.1, Lbrev 116.3, Lbrev 117.2, Lplant 40.3, Lplant 69, Lhil 87.1, Lhil 3 and Lbrev 81.1. Gene sequence indicated by (*) was extracted from GenBank database. Highlighted residues indicate regions which are highly similar or conserved.



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O. oeni*        GTGACTATGGTAGAGTTTCCGGAAGGCTTTGTCTGGGGGGCGGCAACGTCGGGGCCCCAGACAGAAGGTAACTTTCATAAACAGCATCAGAATGTTTTCG
Lpent 79.2      GTGACTATGGTAGAGTTTCCGGAAGGCTTTGTCTGGGGGGCGGCAACGTCGGGCCCCCAGACAGAAGGTAACTTTCATAAACAGCATCAGAATGTTTTCG
Lhil 3          GTGACTATGGTAGAGTTTCCGGAAGGCTTTGTCTGGGGGGCGGCAACGTCGGGCCCCCAGACAGAAGGTAACTTTCATAAACAGCATCAGAATGTTTTCG
Lhil 87.1       GTGACTATGGTAGAGTTTCCGGAAGGCTTTGTCTGGGGGGTGGCAACGTCGGGCCCCCAGACAGAAGGTAACTTTCATAAACAGCATCAGAATGTTTTCG
Lplant 69       GTGACTATGGTAGAGTTTCCGGAAGGCTTTGTCTGGGGGGTGGCAACGTCGGGCCCCCAGACAGAAGGTAACTTTCATAAACAGCATCAGAATGTTTTCG
Lplant 40.3     GTGACTATGGTAGGGTTTCCGGAAGGCTTTGTCTGGGGGGTGGCAACGTCGGGCCCCCAGACAGAAGGTAACTTTCATAAACAGCATCAGAATGTTTTCG
Lbrev 81.1      GTGACTATGGTAGAGTTTCCGGAAGGCTTTGTCTGGGGGGCGGCAACGTCGGGCCCCCAGACAGAAGGTAACTTTCATAAACAGCATCAGAATGTTTTCG
Lplant 113.1    GTGACTATGGTAGAGTTTCCGGAAAGCTTTGTCTGGGGGGCGGCAACGTCGGGCCCCCAGACAGAAGGTAACTTTCATAAACAGCATCAGAATGTTTTCG
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O. oeni*        ATTATTGGTTCGCAACGGAGCCGGAACAATTCGATGCCGGTGTTGGACCAGATACGGCGTCGAACTTTTATAATGATTATGATCATGACCTTGCACTTAT
Lpent 79.2      ATTATTGGTTCGCAACGGAGCCGGAACAATTCGATGCCGGTGTTGGACCAGATACGGCGTCGAACTTTTATAATGATTATGATCATGACCTTGCACTTAT
Lhil 3          ATTATTGGTTCGCAACGGAGCCGGAACAATTCGATGCCGGTGTTGGACCAGATACGGCGTCGAACTTTTATAATGATTATGATCATGACCTTGCACTTAT
Lhil 87.1       ATTATTGGTTCACAACGGAGCCGGAACAATTCGATGCCGGTGTTGGACCAGATACGGCGTCGAACTTTTATAATGATTATGATCATGACCTTGCACTTAT
Lplant 69       ATTATTGGTTCACAACGGAGCCGGAACAATTCGATGCCGGTGTTGGACCAGATACGGCGTCGAACTTTTATAATGATTATGATCATGACCTTGCACTTAT
Lplant 40.3     ATTATTGGTTCACAACGGAGCCGGAACAATTCGATGCCGGTGTTGGACCAGATACGGCGTCGAACTTTTATAATGATTATGATCATGACCTTGCACTTAT
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O. oeni*        GGCGCAGGCGGGGGTTCAAGGTTTGCGGACATCAATCCAATGGACACGGCTAATTGATGACTTTGAAACGGCAAGCTTAAATGCTGATGGTGTCGCCTTC
Lpent 79.2      GGCGCAGGCGGGGGTTCAAGGTTTGCGGACATCAATCCAATGGACACGGCTAATTGATGACTTTGAAACGGCAAGCTTAAATGCTGATGGTGTCGCCTTC
Lhil 3          GGCGCAGGCGGGGGTTCACGGTTTGCGGACATCAATCCAATGGACACGGCTAATTGATGACTTTGAAACGGCAAGCTTAAATGCTGATGGTGTCGCCTTC
Lhil 87.1       GGCGCAGGCGGGGGTTCAAGGTTTGCGGACATCAATCCAATGGACACGGCTAATTGATGACTTTGAAACGGCAAGCTTAAATGCTGATGGTGTCGCCTTC
Lplant 69       GGCGCAGGCGGGGGTTCAAGGTTTGCGGACATCAATCCAATGGACACGGCTAATTGATGACTTTGAAACGGCAAGCTTAAATGCTGATGGTGTCGCCTTC
Lplant 40.3     GGCGCAGGCGGGGGTTCAAGGTTTGCGGACATTAATCCAATGGACACGGCTAATTGATGACTTTGAAACGGCAAGCTTAAATGCTGATGGTGTCGCCTTC
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Lplant 113.1    GGCGCAGGCGGGGGTTCAAGGTTTGCGGACATCAATCCAATGGACACGGCTAATTGATGACTTTGAAACGGCAAGCTTAAATGCTGATGGTGTCGCCTTC
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Lhil 3          TATAATCACGTGATTGATTCGATGTTGGCTCACCACATTACACCGTATATTAATTTGCACCACTTTGACT~ACCGGTGGCACTCTATGATAAATATCATG
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Lplant 40.3     TATAATCACGTGATTGATTCGATGTTGGCTCACCACATTACACCGTATATTAATTTGCACCACTTTGACTTACCGGTGGCACTCTATGATAAATATCATG
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Lplant 113.1    ACCTAAAGTCGTTGTTGATGGTCAATATTTGTATGGCTGGCATTATCCACAGGTGATTAATGGCCCTAAAGCAGTACAGGTCGCCTATAACATGAATTTG
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                         610       620       630       640       650       660       670       680       690       700
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O. oeni*        GCATCAGCTAAAACGGTTGCTCGGTTTCATGAACTTTGTGTGCGACCGGAACAACAGATTGGGATTATTTTGAATTTGACGCCAGCGTATGCCGCGTCAG
Lpent 79.2      GCATCCGCTAAAACGGTTGCTCGGTTTCATGAACTTTGCGTGCGACCGGAACAACAGATTGGGATTATTTTGAATTTGACGCCAGCGTATGCCGCGTCAG
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Lhil 87.1       GCATCCGCTAAAACGGTTGCTCGGTTTCATGAACTTTGCGTGCGACCGGAACAACAGATTGGGATTATTTTGAATTTGACGCCAGCGTATGCCGCGTCAG
Lplant 69       GCATCCGCTAAAACGGTTGCTCGGTTTCATGAACTTTGCGTGCGACCGGAACAACAGATTGGGATTATTTTGAATTTGACGCCAGCGTATGCCGCGTCAG
Lplant 40.3     GCATCCGCTAAAACGGTTGCTCGGTTTCATGAACTTTGCGTGCGACCGGAACAACAGATTGGGATTATTTTGAATTTGACGCCAGCGTATGCCGCGTCAG
Lbrev 81.1      GCATCCGCTAAAACGGTTGCTCGGTTTCATGAACTTTGCGTGCGACCGGAACAACAGATTGGGATTATTTTGAATTTGACGCCAGCGTATGCCGCGTCAG
Lplant 113.1    GCATCCGCTAAAACGGTTGCTCGGTTTCATGAACTTTGCGTGCGACCGGAACAACAGATTGGGATTATTTTGAATTTGACGCCAGCGTATGCCGCGTCAG
Lbrev 116.3     GCATCCGCTAAAACGGTTGCTCGGTTTCATGAACTTTGCGTGCGACCGGAACAACAGATTGGGATTATTTTGAATTTGACGCCAGCGTATGCCGCGTCAG

                         710       720       730       740       750       760       770       780       790       800
Lplant WCFS1*   ATGATCCAGCTGATTTGGCGGCTGCGGAATTTGCTGAGTTGTGGTCCAATAACCTATTCTTAGATCCAGCGGTGTTAGGACATTTTCCAGAAAAGTTGGT
O. oeni*        ATGATCCAGCTGATTTGGCGGCTGCGGAATTTGCTGAGTTGTGGTCCAATAACCTATTCTTAGATCCAGCGGTGTTAGGACATTTTCCAGAAAAGTTGGT
Lpent 79.2      ATGATCCAGCTGATTTGGCGGCTGCGGAATTTGCTGAGTTGTGGTCCAATAACCTCTTCTTAGATCCAGCGGTGTTAGGACATTTTCCAGAAAAGTTGGT
Lhil 3          ATGATCCAGCTGATTTGGCGGCTGCGGAATTTGCTGAGTTGTGGTCCAATAACCTCTTCTTAGATCCAGCGGTGTTAGGACATTTTCCAGAAAAGTTGGT
Lhil 87.1       ATGATCCAGCTGATTTGGCGGCTGCGGAATTTGCTGAGTTGTGGTCCAATAACCTCTTCTTAGATCCAGCGGTGTTAGGACATTTTCCAGAAAAGTTGGT
Lplant 69       ATGATCCAGCTGATTTGGCGGCTGCGGAATTTGCTGAGTTGTGGTCCAATA~CCTCTTCTTAGATCCAGCGGTGTTAGGACATTTTCCAGAAAAGTTGGT
Lplant 40.3     ATGATCCAGCTGATTTGGCGGCTGCGGAATTTGCTGAGTTGTGGTCCAATAACCTCTTCTTAGATCCAGCGGTGTTAGGACATTTTCCAGAAAAGCTGGT
Lbrev 81.1      ATGATCCAGCTGATTTGGCGGCTGCGGAATTTGCTGAGTTGTGGTCCAATAACCTCTTCTTAGATCCAGCGGTGTTAGGACATTTTCCAGAAAAGTTGGT
Lplant 113.1    ATGATCCAGCTGATTTAGCGGCTGCGGAATTTGCTGAGTTGTGGTCCAATAACCTCTTCTTAGATCCAGCGGTGTTAGGACATTTTCCAGAAAAGTTGGT
Lbrev 116.3     ATGATCCAGCTGATTTAGCGGCTGCGGAATTTGCTGAGTTGTGGTCCAATAACCTCTTCTTAGATCCAGCGGTGTTAGGACATTTTCCAGAAAAGTTGGT

                         810       820       830       840       850       860       870       880       890       900
Lplant WCFS1*   CGAGCGACTGACAATGGATGGGGTACTATGGGATGCGACCCCAACTGAATTGGCAATTATCGCTGCTAATCCCGTTGACTGTTTGGGTGTTAATTACTAT
O. oeni*        CGAGCGACTGACAATGGATGGGGTACTATGGGATGCGACCCCAACTGAATTGGCAATTATCGCTGCTAATCCCGTTGACTGTTTGGGTGTTAATTACTAT
Lpent 79.2      CGAGCGACTGACAATGGATGGGGTACTATGGGATGCGACCCCAACTGAATTGGCAATTATCGCTGCTAATCCCGATGACTGTTTGGGTGTAAATTACTAT
Lhil 3          CGAGCGACTGACAATGGATGGGGTACTATGGGATGCGACCCCAACTGAATTGGCAATTATCGCTGCTAATCCCGTTGACTGTTTGGGTGTTAATTACTAT
Lhil 87.1       CGAGCGACTGACAATGGATGGGGTACTATGGGATGCGACCCCAACTGAATTGGCAATTATCGCTGCTAATCCCGTTGACTGTTTGGGTGTTAATTACTAT
Lplant 69       CGAGCGACTGACAATGGATGGGGTACTATGGGATGCGACCCCAACTGAATTGGCAATTATCGCTGCTAATCCCGTTGACTGTTTGGGTGTTAATTACTAT
Lplant 40.3     CGAGCGACTGACAATGGATGGGGTACTATGGGATGCGACCCCAACTGAATTGGCAATTATCGCTGCTAATCCCGTTGACTGTTTGGGTGTTAATTACTAT
Lbrev 81.1      CGAGCGACTGACAATGGATGGGGTACTATGGGATGCGACCCCAACTGAATTGGCAATTATCGCTGCTAATCCCGTTGACTGTTTGGGTGTTAATTACTAT
Lplant 113.1    CGAGCGACTGACAATGGATGGGGTACTATGGGATGCGACCCCAACTGAATTGGCAATTATCGCTGCTAATCCCGTTGACTGTTTGGGTGTTAATTACTAT
Lbrev 116.3     CGAGCGACTGACAATGGATGGGGTACTATGGGATGCGACCCCAACTGAATTGGCAATTATCGCTGCTAATCCCGTTGACTGTTTGGGTGTTAATTACTAT

                         910       920       930       940       950       960       970       980       990      1000
Lplant WCFS1*   CACCCCTTCCGTGTTCAGCGACCAGATATCAGCCCAAAGAGTTTGCAACCGTGGATGCCTGATATTTACTTCAAGGAATACGATATGCCAGGTCGAATGA
O. oeni*        CACCCCTTCCGTGTTCAGCGACCAGATATCAGCCCAAAGAGTTTGCAACCGTGGATGCCTGATATTTACTTCAAGGAATACGATATGCCAGGTCGAATGA
Lpent 79.2      CACCCCTTCCGTGTTCAGCGACCAGATATCAGCCCAAAGAGTTTGCAACCGTGGATGCCTGATATTTACTTCAAGGAATCCGATATGCCAGGTCGAATGA
Lhil 3          CACCCCTTCCGTGTTCAGCGACCAGATATCAGCCCAAAGAGTTTGCAACCGTGGATGCCTGATATTTACTTCAAGGAATACGATATGCCAGGTCGAATGA
Lhil 87.1       CACCCCTTCCGTGTTCAGCGACCAGATATCAGCCCAAAGAGTTTGCAACCGTGGATGCCTGATATTTACTTCAAGGAATACGATATACCAGGTCGAATGA
Lplant 69       CACCCCTTCCGTGTTCAGCGACCAGATATCAGCCCAAAGAGTTTGCAACCGTGGATGCCTGATATTTACTTCAAGGAATACGATATGCCAGGTCGAATGA
Lplant 40.3     CACCCCTTCCGTGTTCAGCGACCAGATATCAGCCCAAAGAGTTTGCAACCGTGGATGCCTGATATTTACTTCAAGGAATACGATATGCCAGGTCGAATGA
Lbrev 81.1      CACCCCTTCCGTGTTCAGCGACCAGATATCAGCCCAAAGAGTTTGCAACCGTGGATGCCTGATATTTACTTCAAGGAATACGATATGCCAGGTCGAATGA
Lplant 113.1    CACCCCTTCCGTGTTCAGCGACCAGATATCAGCCCAAAGAGTTTGCAACCGTGGATGCCTGATATT~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Lbrev 116.3     CACCCCTTCCGTGTTCAGCGACCAGATATCAGCCCAAAGAGTTTGCAACCGTGGATGCCTGATATT~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                        1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
Lplant WCFS1*   TGAACGTTGATCGGGGCTGGGAAATTTACCCACAGGCGATGACTGATATTGCACGCAATATTCAAAAAAATTATGGTAACATTCCGTGGATGATCTCTGA
O. oeni*        TGAACGTTGATCGGGGCTGGGAAATTTACCCACAGGCGATGACTGATATTGCACGCAATATTCAAAAAAATTATGGTAACATTCCGTGGATGATCTCTGA
Lpent 79.2      TGAACGTTGATCGGGGCTGGGAAATTTACCCACAGGCGATGAATGATATTGCACGCAATATTCAAAAAAATTATGGTAACATTCCGTGGATGATCTCTGA
Lhil 3          TGAACGTTGATCGGGGCTGGGAAATTTACCCACAGGCGATGACTGATATTGCACGCAATATTCAAAAAAATTATGGTAACATTCCGTGGCTGATCTCTGA
Lhil 87.1       TGAACGTTGATCGGGGCTGGGAAATTTACCCACAGGCGATGACTGATATTGCACGCAATATTCAAAAAAATTATGGTAACATTCCGTGGATGATCTCTGA
Lplant 69       TGAACGTTGATCGGGGCTGGGAAATTTACCCACAGGCGATGACTGATATTGCACGCAATATTCAAAAAAATTATGGTAACATTCCGTGGATGATCTCTGA
Lplant 40.3     TGAACGTTGATCGGGGCTGGGAAATTTACCCACAGGCGATGACTGATATTGCACGCAATATTCAAAAAAATTATGGTAACATTCCGTGGATGATCTCTGA
Lbrev 81.1      TGAACGTTGATCGGGGCTGGGAAATTTACCCACAGGCGATGACTGATATTGCACGCAATATTCAAAAAAATTATGGTAACATTCCGTGGATGATCTCTGA
Lplant 113.1    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GCACGCAATATTCAAAAAAATTATGGTAACATTCCGTGGATGATCTCTGA
Lbrev 116.3     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GCACGCAATATTCAAAAAAATTATGGTAACATTCCGTGGATGATCTCTGA

                        1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
Lplant WCFS1*   AAACGGTATGGCAGTTGCTGGTGAAGAACGGTTTCTGGACAAACAAGGCGTCGTTCAAGATGACTACCGCATCGATTTTATGAAGGAACACCTAACTGCC
O. oeni*        AAACGGTATGGCAGTTGCTGGTGAAGAACGGTTTCTGGACAAACAAGGCGTCGTTCAAGATGACTACCGCATCGATTTTATGAAGGAACACCTAACTGCC
Lpent 79.2      AAACGGTATGGCAGTTGCTGGTGAAGAACGATTTCTGGACAAACAAGGCGTCGTTCAAGATGACTACCGCATCGATTTTATGAAGGAACACCTAACTGCC
Lhil 3          AAACGGTATGGCAGTTGCTGGTGAAGAACGATTTCTGGACAAACAAGGCGTCGTTCAAGATGACTACCGCATCGATTTTATGAAGGAACACCTAACTGCC
Lhil 87.1       AAACGGTATGGCAGTTGCTGGTGAAGAACGGTTTCTGGACAAACAAGGCGTCGTTCAAGATGACTACCGCATCGATTTTATGAAGGAACACCTAACTGCC
Lplant 69       AAACGGTATGGCAGTTGCTGGTGAAGAACGGTTTCTGGACAAACAAGGCGTCGTTCAAGATGACTACCGCATCGATTTTATGAAGGAACACCTAACTGCC
Lplant 40.3     AAACGGTATGGCAGTTGCTGGTGAAGAACGGTTTCCGGACAAACAAGGCGTCGTTCAAGATGACTACCGCATCGATTTTATGAAGGAACACCTAACTGCC
Lbrev 81.1      AAACGGTATGGCAGTTGCTGGTGAAGAACGATTTCTGGACAAACAAGGCGTCGTTCAAGATGACTACCGCATCGATTTTATGAAGGAACACCTAACTGCC
Lplant 113.1    AAACGGTATGGCAGTTGCTGGTGAAGAACGGTTTCTGGACAAACAAGGCGTCGTTCAAGATGACTACCGCATCGATTTTATGAAGGAACACCTAACTGCC
Lbrev 116.3     AAACGGTATGGCAGTTGCTGGTGAAGAACGGTTTCTGGACAAACAAGGCGTCGTTCAAGATGACTACCGCATCGATTTTATGAAGGAACACCTAACTGCC
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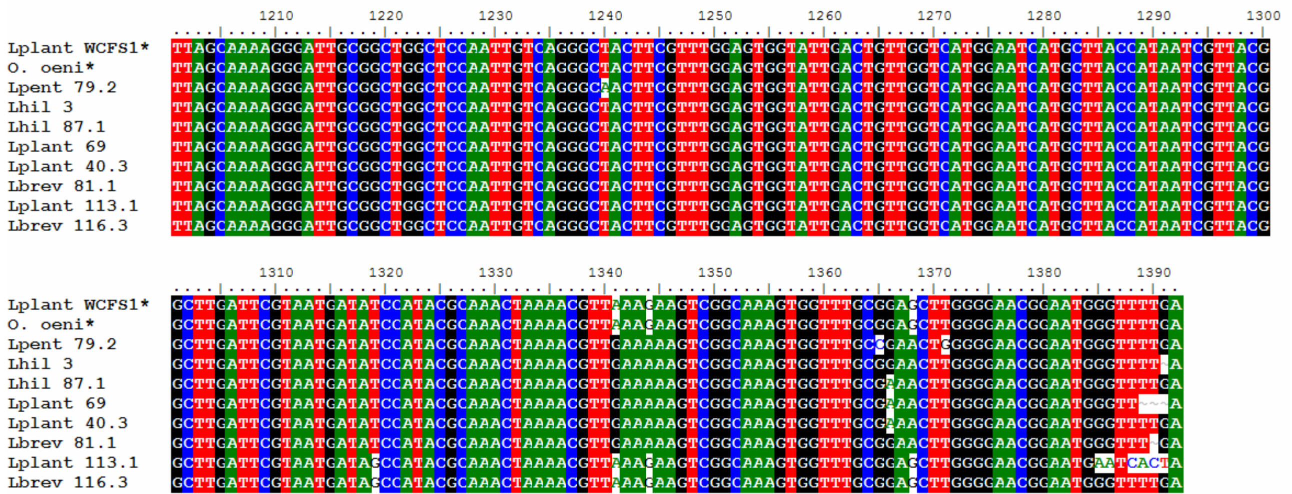

Figure 3.4 Nucleotide sequence alignments for β-glucosidase genes from Lpent 79.2, Lhil 3, Lhil 87.1, Lplant 69, Lplant 40.3, Lbrev 81.1, Lplant 113.1 and Lbrev 116.3. Gene sequences indicated by (*) were extracted from GenBank database. Highlighted residues indicate regions which are highly similar or conserved.

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               10        20        30        40        50        60        70        80        90       100
Lplant WCFS1*  CTAATTTGTAACCGTATCCGCCAACCAAGCCGCAATTAACCGGCGACGTGCTGGAAGGAATACCGAATTATTTGCAACTGGATGCACCCGGTTGGATAAG
Lhil 3         CTAATTTGTAACCGTATCCGCCAACCAAGCCGCAATTAACCGGCGACGTGCTGGAAGGAATACCGAATTATTTGCAACTGGATGCACCCGGTTGGATAAG
Lpar 79        ~TAATTTGTAACCGTATCCGCCAACCAAGCCGCAATTAACCGGCGACGTGCTGGAAGGAATACCGAATTATTTGCAACTGGATGCACCCGGTTGGATAAG
Lplant 69      CTAATTTGTAACCGTATCCGCCAACCAAGCCGCAATTAACCGGCGACGTGCTGGAAGGAATACCGAATTATTTGCAACTGGATGCACCCGGTTGGATAAG
Lhil 87.1      CTAATTTGTAACCGTATCCGCCAACCAAGCCGCAATTAACCGGCGACGTGCTGGAAGGAATACCGAATTATTTGCAACTGGATGCACCCGGTTGGATAAG
Lplant 40.3    CTAATTTGTAACCGTATCCGCCAACCAAGCCGCAATTAACCGGCGACGTGCTGGAAGGAATACCGAATTATTTGCAACTGGATGCACCCGGTTGGATAAG
Lbrev 117.2    CTAATTTGTAACCGTATCCGCCAACCAAGCCGCAATTAACCGGCGACGTGCTGGAAGGAATACCGAATTATTTGCAACTGGATGCACCCGGTTGGATAAG

              110       120       130       140       150       160       170       180       190       200
Lplant WCFS1*  AAAACAAGGCCCCGTTGCCGTTGACGGTCAAGCATCAGGAGCGTACCAGTATAACCAGTATGAAAAATCACCGGCAGTGTCTCATCAGAACGGTGCCAGA
Lhil 3         AAAACAAGGCCCCGTTGCCGTTGACAGTCAAGCATCAGGAGCGTACCAGTATAACCAGTATGAAAAATCACCGGCAGTGTCTCATCAGAACGGTGCCAGA
Lpar 79        AAAACAAGGCCCCGTTGCCGTTGACGGTCAAGCATCAGGAGCGTGCCAGTATAACCAGTATGAAAAATCACCGGCAGCGTCTCATTAGAACGGTGCCAGA
Lplant 69      AAAACAAGGCCTCGTTGCCGTTGACGGTCAAGCATCAGGAGCGTACCAGTATAACCAGTATGAAAAATCACCGGCAGTGTCTCATCAGAACGGTGCCGGA
Lhil 87.1      AAAACAAGGCCTCGTTGCCGTTGACGGTCAAGCATCAGGAGCGTACCAGTATAACCAGTATGAAAAATCACCGGCAGTGTCTCATCAGAACGGTGCCAGA
Lplant 40.3    AAAACAAGGCCTCGTTGCCGTTGACGGTCAAGCATCAGGAGCGTACCAGTATAACCAGTATGAAAAATCACCGGCAGTGTCTCATCAGAACGGTGCCAGA
Lbrev 117.2    AAAACAAGGCCTCGTTGCCGTTGACGGTCAAGCATCAGGAGCGTACCAGTATAACCAGTATGAAAAATCACCGGCAGTGTCTCATCAGAACGGTGCCAGA

              210       220       230       240       250       260       270       280       290       300
Lplant WCFS1*  GGTTCCAACCAAAGCTCCGGCGTAAGTGTTGATTGGGCGTCCAATCTTGATACAAGCTTGCAATCGTAGCCTGTGATAGCACAGCTGGTACGTCGATTTG
Lhil 3         GGTTCCAACCAAAGCTCCGGCGTAAGTGTTGATTGGGCGTCCAATCTTGATACAAGCTTGCAATCGTAGCCTGTGATAGCACAGCTGGTACGTCGATTTG
Lpar 79        GGTTCCAACCAAAGCTCCGGCGTAAGTGTTGATTGGGCGTCCAATCTTGATACAAGCTTGCAATCGTGGCCTGTGATAGCACAGCTGGTACGTCGATTTG
Lplant 69      GGTTCCAACCAAAGCTCCGGCGTAAGTGTTGATGGGGCGTCCAATCTTGATACAAGCTTGCAATCGTAGCCTGTGATAGCACAGCTGGTACGTCGATTTG
Lhil 87.1      GGTTCCAACCAAAGCTCCGGCGTAAGTGTTGATTGGGCGTCCAATCTTGATACAAGCTTGCAATCGTAGCCTGTGATAGCACAGCTGGTACGTCGATTTG
Lplant 40.3    GGTTCCAACCAAAGCTCCGGCGTAAGTGTTGATTGGGCGTCCAATCTTGATACAAGCTTGCAATCGTAGCCTGTGATAGCACAGCTGGTACGTCGATTTG
Lbrev 117.2    GGTTCCAACCAAAGCTCCGGCGTAAGTGTTGATTGGGCGTCCAATCTTGATACAAGCTTGCAATCGTAGCCTGTGATAGCACAGCTGGTACGTCGATTTG

              310       320       330       340       350       360       370       380       390       400
Lplant WCFS1*  GCCGAGCATCAGTTGTGAAAACCGAACAAGGTCTGTCACACTGGCAAATAAACCGGCGGAACCACAGTGTTCACCTAAGACTCTGGCCTTAGGATCATGA
Lhil 3         GCCGAGCATCAGTTGTGAAAACCGAACAAGGTCTGTCACACTGGCAAATAAACCGGCGGAACCACAGTGTTCACCTAAGACTCTGGCCTTAGGATCATGA
Lpar 79        GCCGAGCATCAATTGTGAAAACCGAACAAGGTCTGTCACACTGGCAAATAAACCGGCGGAACCACAGTGTTCACCTAAGACTCTGGCCTTAGGATCATGA
Lplant 69      GCCGAGCATCAGTTGTGAAAACCGAACAAGGTCTGTCACACTGGCAAATAAACCGGCGGAACCACAGTGTTCACCTAAGACTCTGGCCTTAGGATCATGA
Lhil 87.1      GCCGAGCATCAGTTGTGAAAACCGAACAAGGTCTGTCACACTGGCAAATAAACCGGCGGAACCACAGTGTTCACCTAAGACTCTGGCCTTAGGATCATGA
Lplant 40.3    GCCGAGCATCAGTTGTGAAAACCGAACAAGGTCTGTCACACTGGCAAATAAACCGGCGGAACCACAGTGTTCACCTAAGACTCTGGCCTTAGGATCATGA
Lbrev 117.2    GCCGAGCATCAGTTGTGAAAACCGAACAAGGTCTGTCACACTGGCAAATAAACCGGCGGAACCACAGTGTTCACCTAAGACTCTGGCCTTAGGATCATGA

              410       420       430       440       450       460       470       480       490       500
Lplant WCFS1*  ACGATCCCCCGTAACAACCCAGTTTTCATGGAATACGTCGTCGGGACGGTTTGCGCTAGTTGAGGTTGAAAACTCGTGTGTGCTAACCCTAATGGCTGCA
Lhil 3         ACGATCCCCCGTAACAACCCAGTTTTCATGGAATACGTCGGCGGAACGGTTTGCGCTAGTTGAGGTTGAAAACTCGTGTGTGCCAACCCTAATGGCTGCA
Lpar 79        ACGATCCCCCGTAACAACCCAGTTTTCATGGAATACGTCGTCGGAACGGTTTGCGCTAGTTGAGGTTGGAAACTCGTGTGTACTAACCCTAATGGCTGCA
Lplant 69      ACGATCCCCCGTAACAACCCAGTTTTCATGGAATACGTCGTCGGAACGGTTTGCGCTAGTCGAGGTTGAAAACTCGTGTGTGCTAACCCTAATGGCTGCA
Lhil 87.1      ACGATCCCCCGTAACAACCCAGTTTTCATGGAATACGTCGTCGGAACGGTTTGCGCTAGTCGAGGTTGAAAACTCGTGTGTGCTAACCCTAATGGCTGCA
Lplant 40.3    ACGATCCCCCGTAACAACCCAGTTTTCATGGAATACGTCGTCGGAACGGTTTGCGCTAGTCGAGGTTGAAAACTCGTGTGTGCTAACCCTAATGGCTGCA
Lbrev 117.2    ACGATCCCCCGTAACAACCCAGTTTTCATGGAATACGTCGTCGGAACGGTTTGCGCTAGTCGAGGTTGAAAACTCGTGTGTGCTAACCCTAATGGCTGCA

              510       520       530       540       550       560       570       580       590       600
Lplant WCFS1*  AGACGCGCTCAGTGATAATTGGCTGAATGGCCGCTCCAAGAATTCGTTCAATGACTTGCCCCATGATGATCAAGCCTAAATCCGTATAAACCACCCGATG
Lhil 3         AGACGCGCTCAGTGATAATTGGCTGAATGGCCGCTCCAAGAATTCGTTCAATGACTTGCCCCATGATGATCAAGCCTAAATCCGTATAAACCACCCGATG
Lpar 79        AAACGCGCTCAGTGATAATTGGCTGAATGGCTGCTCCAAGAATTCGTTCAATGACTTGCCCCATGATGATCAAGCCTAAATCCGTATAAACCACCCGATG
Lplant 69      AGACGCGCTCAGTGATAATTGGCTGAATGGCCGCTCCAAGAATTCGTTCAATGACTTGCCCCATGATGATCAAGCCTAAATCCGTATAAACCACCCGATG
Lhil 87.1      AGACGCGCTCAGTGATAATTGGCTGAATGGCCGCTCCAAGAATTCGTTCAATGACTTGCCCCATGATGATCAAGCCTAAATCCGTATAAACCACCCGATG
Lplant 40.3    AGACGCGCTCAGTGATAATTGGCTGAATGGCCGCTCCAAGAATTCGTTCAATGACTTGCCCCATGATGATCAAGCCTAAATCCGTATAAACCACCCGATG
Lbrev 117.2    AGACGCGCTCAGTGATAATTGGCTGAATGGCCGCTCCAAGAATTCGTTCAATGACTTGCCCCATGATGATCAAGCCTAAATCCGTATAAACCACCCGATG

              610       620       630       640       650       660       670       680       690       700
Lplant WCFS1*  ATTGCGGTTACGCTCACTGATTGGTAGCTGTTGAATCGCGGCTAATAACGCATCCGCTGACAACGCATTCCGATTTGGAATATAACCACTCAGCCCACTT
Lhil 3         ATTGCGGTTACGCTCACTGATTGGTAGCTGTTGAATCGCGGCTAATAACGCATCCGCTGACAACGCATTCCGATTTGGAATATAACCACTCGGCCCACTT
Lpar 79        ATTGCGGTTACGCTCACTGATTGGTAGCTGTTGAATCGCGGCTAATAACGCATCTGCAGACAACGCATTCCGATTTGGAATATAACCACTCAGCCCACTT
Lplant 69      ATTGCGGTTACGCTCACTGATTGGTAGCTGTTGAATCGCGGCTAATAACGCATCCGCTGACAACGCATTCCGATTTGGAATATAACCACTCAGCCCACTT
Lhil 87.1      ATTGCGGTTACGCTCACTGATTGGTAGCTGTTGAATCGCGGCTAATAACGCATCCGCTGACAACGCATTCCGATTTGGAATATAACCACTCAGCCCACTT
Lplant 40.3    ATTGCGGTTACGCTCACTGATTGGTAGCTGTTGAATCGCGGCTAATAACGCATCCGCTGACAACGCATTCCGATTTGGAATATAACCACTCAGCCCACTT
Lbrev 117.2    ATTGCGGTTACGCTCACTGATTGGTAGCTGTTGAATCGCGGCTAATAACGCATCCGCTGACAACGCATTCCGATTTGGAATATAACCACTCAGCCCACTT

              710       720       730       740       750       760       770       780       790       800
Lplant WCFS1*  GTATGGGTCATTAAATTACGAACCGTGATCGTTGTGGACTGTAAAGTCGGTAGGTAGTCATGCACGGGGCGATCAATTTCAAGTTGCCCATTTTGCATCA
Lhil 3         GTATGGGTCATTAAATTACGAACCGTGATCGTTGTGGACTGTAAAGTCGGTAGGTAGTCATGCACGGGGCGATCAATTTCAAGTTGCCCATTTTGCATCA
Lpar 79        GTATGGGTCATCAAATTACGACCCGTAATCGTTGTGGACTGTAAAGCCGGTAGGTAATCATGCACGGGGCGGTCAATTTCAAGTTGCCCATTTTGCATCA
Lplant 69      GTATGGGTCATTAAATTACGAACCGTGATCGTTGTGGACTGTAAAGTCGGTAGGTAGTCATGCACGGGGCGATCAATTTCAAGTTGCCCATTTTGCATCA
Lhil 87.1      GTATGGGTCATTAAATTACGAACCGTGATCGTTGTGGACTGTAAAGTCGGTAGGTAGTCATGCACGGGGCGATCAATTTCAAGTTGCCCATTTTGCATCA
Lplant 40.3    GTATGGGTCATTAAATTACGAACCGTGATCGTTGTGGACTGTAAAGTCGGTAGGTAGTCATGCACGGGGCGATCAATTTCAAGTTGCCCATTTTGCATCA
Lbrev 117.2    GTATGGGTCATTAAATTACGAACCGTGATCGTTGTGGACTGTAAAGTCGGTAGGTAGTCATGCACGGGGCGATCAATTTCAAGTTGCCCATTTTGCATCA

              810       820       830       840       850       860       870       880       890       900
Lplant WCFS1*  ACTGCATCGCCACGGTTGTCGTTCCCACGACCTTAGTAACCGAGGCCAAATCATACATCGCATCCGGCCATAATGGTTTGATCGTTGGTTTTATTTGAGC
Lhil 3         ACTGCATCGCCACGGTTGTCGTTCCCACGACCTTAGTAACCGAGGCCAAATCATACATCGCATCCGGCCATAATGGTTTGATCGTTGGTTTTATTTGAGC
Lpar 79        ACTGCATCGCCACGGTTGTTGTTCCCACGACCTTAGTAACCGAGGCTAAATCATACATCGCATCCGGCCATAATGGTTTGATCGTTGGTTTTATTTGAGC
Lplant 69      ACTGCATCGCCACGGTTGTCGTTCCCACGACCTTAGTAACCGAGGCCAAATCATACATCGCATCCGGCCATAATGGTTTGATCGTTGGTTTTATTTGAGC
Lhil 87.1      ACTGCATCGCCACGGTTGTCGTTCCCACGACCTTAGTAACCGAGGCCAAATCATACATCGCATCCGGCCATAATGGTTTGATCGTTGGTTTTATTTGAGC
Lplant 40.3    ACTGCATCGCCACGGTTGTCGTTCCCACGACCTTAGTAACCGAGGCCAAATCATACATCGCATCCGGCCATAATGGTTTGATCGTTGGTTTTATTTGAGC
Lbrev 117.2    ACTGCATCGCCACGGTTGTCGTTCCCACGACCTTAGTAACCGAGGCCAAATCATACATCGCATCCGGCCATAATGGTTTGATCGTTGGTTTTATTTGAGC
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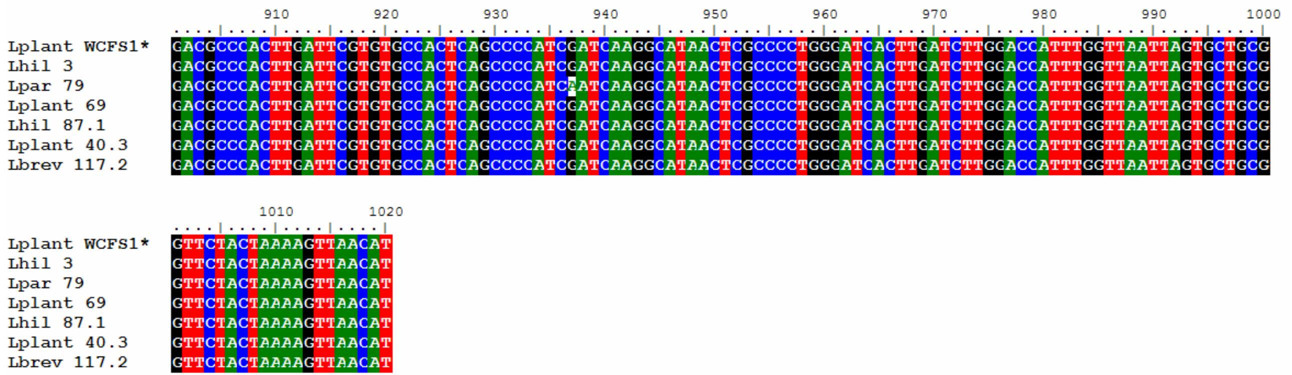

Figure 3.5 Nucleotide sequence alignments for putative esterase genes from Lplant 69, Lhil 87.1, Lhil 3, Lplant 40.3 and Lbrev 117.2. Gene sequence indicated by (*) was extracted from GenBank database. Highlighted residues indicate regions which are highly similar or conserved.

```
                       10        20        30        40        50        60        70        80        90       100
Lplant WCFS1*  ATGGCTAATAAATCATTAATCAAAGTCGCAGTAACCGCACTAGTAGCTGGTTTAATCGGTGGTGGTGTTGCTTACGGCGGTATTAATTATTTCCAAAACA
Lhil 87.1      ATGGCTAATAAATCATTAATCAAAGTCGCAGTAACCGCACTAGTAGCTGGTTTAATCGGTGGTGGTGTTGCTTACGGCGGTATTAATTATTTCCAAAACA
Lbrev 117.2    ATGGCTAATAAATCATTAATCAAAGTCGCAGTAACCGCACTAGTAGCTGGTTTAATCGGTGGTGGTGTTGCTTACGGCGGTATTAATTATTTCCAAAACA
Lpar 79        ATGGCTAATAAATCATTAATCAAAGTCGCAGTAACCGCACTAGTAGCTGGTTTAATCGGTGGTGGTGTTGCTTACGGCGGTATTAATTATTTCCAAAACA
Lpar 83.1      ATGGCTAATAAATCATTAATCAAAGTCGCAGTAACCGCACTAGTAGCTGGTTTAATCGGTGGTGGTGTTGCTTACGGCGGTATTAATTATTTCCAAAACA
Lplant 40.3    ATGGCTAATAAATCATTAATCAAAGTCGCAGTAACCGCACTAGTAGCTGGTTTAATCGGTGGTGGTGTTGCTTACGGCGGTATTAATTATTTCCAAAACA
Lplant 69      ATGGCTAATAAATCATTAATCAAAGTCGCAGTAACCGCACTAGTAGCTGGTTTAATCGGTGGTGGTGTTGCTTACGGCGGTATTAATTATTTCCAAAACA
Lbrev 81.1     ATGGCTAATAAATCATTAATCAAAGTCGCAGTAACCGCACTAGTAGCTGGTTTAATCGGTGGTGGTGTTGCTTACGGCGGTATTAATTATTTCCAAAACA
Lhil 3         ATGGCTAATAAATCATTAATCAAAGTCGCAGTAACCGCACTAGTAGCTGGTTTAATCGGTAGTGGTGTTGCTTACGGCGGTATTAATTATTTCCAAAACA
Lpent 79.2     ATGGCTAATAAATCATTAATCAAAGTCGCAGTAACCGCACTAGTAGCTGGTTTAATCGGTGGTGGTGTTGCTTACGGCGGTATTAATTATTTCCAAAACA

                      110       120       130       140       150       160       170       180       190       200
Lplant WCFS1*  ATAACATCGCAACGTCATCGACCAGTGTACCAACTGGTTCTAATAAATCGGGGTCAACGTCAACGACGAACGTTAAGGTCAATGTCAGTTCACAGGCGAC
Lhil 87.1      ATAACATCGCAACGTCATCGACCAGTGTACCAACTGGTTCTAATAAATCGGGGTCAACGTCAACGACGAACGTTAAGGTCAATGTCAGTTCACAGGCGAC
Lbrev 117.2    ATAACATCGCAACGTCATCGACCAGTGTACCAACTGGTTCTAATAAATCGGGGTCAACGTCAACGACGAACGTTAAGGTCAATGTCAGTTCACAGGCGAC
Lpar 79        ATAACATCGCAACGTCATCGACCAGTGTACCAACTGGTTCTAATAAATCGGGGTCAACGTCAACGACGAACGTTAAGGTCAATGTCAGTTCACAGGCGAC
Lpar 83.1      ATAACATCGCAACGTCATCGACCAGTGTACCAACTGGTTCTAATAAATCGGGGTCAACGTCAACGACGAACGTTAAGGTCAATGTCAGTTCACAGGCGAC
Lplant 40.3    ATAACATCGCAACGTCATCGACCAGTGTACCAACTGGTTCTAATAAATCGGGGTCAACGTCAACGACGAACGTTAAGGTCAATGTCAGTTCACAGGCGAC
Lplant 69      ATAACATCGCAACGTCATCGACCAGTGTACCAACTGGTTCTAATAAATCGGGGTCAACGTCAACGACGAACGTTAAGGTCAATGTCAGTTCACAGGCGAC
Lbrev 81.1     ATAACATCGCAACGTCATCGACCAGTGTACCAACTGGTTCTAATAAATCGGGGTCAACGGCAACGACGAACGTTAAGGTCAATGTCAGTTCACAGGCGAC
Lhil 3         ATAACATCGCAACGTCATCGACCAGTGTACCAACTGGTTCTAATAAATCGGGGTCAACGGCAACGACGAACGTTAAGGTCAATGTCAGTTCACAGGCGAC
Lpent 79.2     ATAACATCGCAACGTCATCGACCAGTGTACCAACTGGTTCTAATAAATCGGGGTCAACGGCAACGACGAACGTTAAGGTCAATGTCAGTTCACAGGCGAC

                      210       220       230       240       250       260       270       280       290       300
Lplant WCFS1*  CAAGGTGTTTGAAAATAACAAGGCGGCCGTTGTATCGGTCATTAATCTCCAAAAGAAGAGCTCTTCAAGCAGTTGGAGTGGTATTTTGGGTGGCGATGAC
Lhil 87.1      CAAGGTGTTTGAAAATAACAAGGCGGCCGTTGTATCGGTCATTAATCTCCAAAAGAAGAGCCCTTCAAGCAGTTGGAGTGGTATTTTGGGTGGCGATGAC
Lbrev 117.2    CAAGGTGTTTGAAAATAACAAGGCGGCCGTTGTATCGGTCATTAATCTCCAAAAGAAGAGCTCTTCAAGCAGTTGGAGTGGTATTTTGGGTGGCGATGAC
Lpar 79        CAAGGTGTTTGAAAATAACAAGGCGGCCGTTGTATCGGCCATTAATCTCCAAAAGAAGAGCTCTTCAAGCAGTTGGAGTGGTATTTTGGGTGGCGATGAC
Lpar 83.1      CAAGGTGTTTGAAAATAACAAGGCGGCCGTTGTATCGGTCATTAATCTCCAAAAGAAGAGCTCTTCAAGCAGTTGGAGTGGTATTTTGGGTGGCGATGAC
Lplant 40.3    CAAGGTGTTTGAAAATAACAAGGCGGCCGTTGTATCGGTCATTAATCTCCAAAAGAAGAGCTCTTCAAGCAGTTGGAGTGGTATTTTGGGTGGCGATGAC
Lplant 69      CAAGGGGTTTGAAAATAACAAGGCGGCCGTTGTATCGGTCATTAATCTCCAAAAGAAGAGCTCTTCAAGCAGTTGGAGTGGTATTTTGGGTGGCGATGAC
Lbrev 81.1     CAAAGGGGTTGAAAATAACAAGGCGGCCGTTGTATCGGTCATTAATCTCCAAAAGAAGAGCTCTTCAAGCAGTTGGAGTGGTATTTTGGGTGGCGATGAC
Lhil 3         CAAGGTGTTTGAAAATAACAAGGCGGCCGTTGTATCGGTCATTAATCTCCAAAAGAAGAGCTCTTCAAGCAGTTGGAGTGGTATTTTGGGTGGCGATGAC
Lpent 79.2     CAAGGTGTTTGAAAATAACAAGGCGGCCGTTGTATCGGTCATTAATCTCCAAAAGAAGAGCTCTTCAAGCAGTTGGAGTGGTATTTTGGGTGGCGATGAC

                      310       320       330       340       350       360       370       380       390       400
Lplant WCFS1*  TCGTCTGGCAGTGATAGTTCATCCAGTTCAGATTCTAGCTCTAGTAAGCTGGAAGAGTACAGTGAAGGTTCTGGGTTGATCTATAAGAAGAGCGGTGACG
Lhil 87.1      TCGTCTGGCAGTGATAGTTCATCCAGTTCAGATTCTAGCTCTAGTAAGCTGGAAGAGTACAGTGAAGGTTCTGGGTTGATCTATAAGAAGAGCGGTGACG
Lbrev 117.2    TCGTCTGGCAGTGATAGTTCATCCAGTTCAGATTCTAGCTCTAGTAAGCTGGAAGAGTACAGTGAAGGTTCTGGGTTGATCTATAAGAAGAGCGGTGACG
Lpar 79        TCGTCTGGCAGTGATAGTTCATCCTGTTCAGATTCTAGCTCTAGTAAGCTGGAAGAGCACAGTGAAGGTTCTGGGTTGATCTATAATAAGAGCGGTGACG
Lpar 83.1      TCGTCTGGCAGTGATAGTTCATCCAGTTCAGATTCTAGCTCTAGTAAGCTGGAAGAGTACAGTGAAGGTTCTGGGTTGATCTATAAGAAGAGCGGTGACG
Lplant 40.3    TCGTCTGGCAGTGATAGTTCATCCAGTTCAGATTCTAGCTCTAGTAAGCTGGAAGAGTACAGTGAAGGTTCTGGGTTGATCTATAAGAAGAGCGGTGACG
Lplant 69      TCGTCTGGCAGTGATAGTTCATCCAGTTCAGATTCTAGCTCTAGTAAGCTGGAAGAGTACAGTGAAGGTTCTGGGTTGATCTATAAGAAGAGCGGTGACG
Lbrev 81.1     TCGTCTGGCAGTGATAGTTCATCCAGTTCAGATTCTAGCTCTAGTAAGCTGGAAGAGTACAGTGAAGGTTCTGGGTTGATCTATAAGAAGAGCGGTGACG
Lhil 3         TCGTCTGGCAGTGATAGTTCATCCAGTTCAGATTCTAGCTCTAGTAAGCTGGAAGAGTACAGTGAAGGTTCTGGGTTGATCTATAAGAAGAGCGGTGACG
Lpent 79.2     TCGTCTGGCAGTGATAGTTCATCCAGTTCAGATTCTAGCTCTAGTAAGCTGGAAGAGTACAGTGAAGGTTCTGGGTTGATCTATAAGAAGAGCGGTGACG

                      410       420       430       440       450       460       470       480       490       500
Lplant WCFS1*  CGGCTTATATTGTAACGAATAATCACGTGGTGAGTGGTTCAAGTGCCATTCGAGTGATTATGAGTGATGGGACTAAGTTGTCAGCTAAAATTGTCGGAAC
Lhil 87.1      CGGCTTATATTGTAACGAATAATCACGTGGTGAGTGGTTCAAGTGCCATTCGAGTGATTATGAGTGATGGGACTAAGTTGTCAGCTAAAATTGTCGGAAC
Lbrev 117.2    CGGCTTATATTGTAACGAATAATCACGTGGTGAGTGGTTCAAGTGCCATTCGAGTGATTATGAGTGATGGGACTAAGTTGTCAGCTAAAATTGTCGGAAC
Lpar 79        CGGCTTATATTGTAACGAATAATCTCGTGGTGAGTGGTTCAAGTGCCATTCGAGTGATTATGAGTGATGGGACTAAGTTGTCAGCTAAAATTGCCGGAAC
Lpar 83.1      CGGCTTATATTGTAACGAATAATCACGTGGTGAGTGGTTCAAGTGCCATTCGAGTGATTATGAGTGATGGGACTAAGTTGTCAGCTAAAATTGTCGGAAC
Lplant 40.3    CGGCTTATATTGTAACGAATAATCACGTGGTGAGTGGTTCAAGTGCCATTCGAGTGATTATGAGTGATGGGACTAAGTTGTCAGCTAAAATTGTCGGAAC
Lplant 69      CGGCTTATATTGTAACGAATAATCACGTGGTGAGTGGTTCAAGTGCCATTCGAGTGATTATGAGTGATGGGACTAAGTTGTCAGCTAAAATTGTCGGAAC
Lbrev 81.1     CGGCTTATATTGTAACGAATAATCACGTGGTGAGTGGTTCAAGTGCCATTCGAGTGATTATGAGTGATGGGACTAAGTTGTCAGCTAAAATTGTCGGAAC
Lhil 3         CGGCTTATATTGTAACGAATAATCACGTGGTGAGTGGTTCAAGTGCCATTCGAGTGATTATGAGTGATGGGACTAAGTTGTCAGCTAAAATTGTCGGAAC
Lpent 79.2     CGGCTTATATTGTAACGAATAATCACGTGGTGAGTGGTTCAAGTGCCATTCGAGTGATTATGAGTGATGGGACTAAGTTGTCAGCTAAAATTGTCGGAAC

                      510       520       530       540       550       560       570       580       590       600
Lplant WCFS1*  CGATTCTGTGACTGACTTGGCCGTTCTGAAAATCAATTCTTCTAAAGTAACGAAGACGGCTAGCTTTGGTAACTCAGATAATATCAAGGTTGGTGAAACG
Lhil 87.1      CGATTCTGTGACTGACTTGGCCGTTCTGAAAATCAATTCTTCTAAAGTAACGAAGACGGCTAGCTTTGGTAACTCAGATAATATCAAGGTTGGTGAAACG
Lbrev 117.2    CGATTCTGTGACTGACTTGGCCGTTCTGAAAATCAATTCTTCTAAAGTAACGAAGACGGCTAGCTTTGGTAACTCAGATAATATCAAGGTTGGTGAAACG
Lpar 79        CGATTCTGTGACTGACTTGGCCGATCTGAAAATCAATTCTTCTAAAGTAACGAAGACGGCTAGCTTTGGTAACTCAGATAATATCAAGGTTGGTGAAACC
Lpar 83.1      CGATTCTGTGACTGACTTGGCCGTTCTGAAAATCAATTCTTCTAAAGTAACGAAGACGGCTAGCTTTGGTAACTCAGATAATATCAAGGTTGGTGAAACG
Lplant 40.3    CGATTCTGTGACTGACTTGGCCGTTCTGAAAATCAATTCTTCTAAAGTAACGAAGACGGCTAGCTTTGGTAACTCAGATAATATCAAGGTTGGTGAAACG
Lplant 69      CGATTCTGTGACTGACTTGGCCGTTCTGAAAATCAATTCTTCTAAAGTAACGAAGACGGCTAGCTTTGGTAACTCAGATAATATCAAGGTTGGTGAAACG
Lbrev 81.1     CGATTCTGTGACTGACTTGGCCGTTCTGAAAATCAATTCTTCTAAAGTAACGAAGACGGCTAGCTTTGGTAACTCAGATAATATCAAGGTTGGTGAAACG
Lhil 3         CGATTCTGTGACTGACTTGGCCGTTCTGAAAATCAATTCTTCTAAAGTAACGAAGACGGCTAGCTTTGGTAACTCAGATAATACCAAGGTTGGTGAAACG
Lpent 79.2     CGATTCTGTGACTGACTTGGCCGTTCTGAAAATCAATTCTTCTAAAGTAACGAAGACGGCTAGCTTTGGTAACTCAGATAATATCAAGGTTGGTGAAACG

                      610       620       630       640       650       660       670       680       690       700
Lplant WCFS1*  GCCTTAGCCATTGGGTCACCGATGGGCTCTAATTACGCAACGACCTTGACGCAAGGAATCATTTCAGCGAAGAAGCGGACCGTGGCGACGACGAATACAT
Lhil 87.1      GCCTTAGCCATTGGGTCACCGATGGGCTCTAATTACGCAACGACCTTGACGCAAGGAATCATTTCAGCGAAGAAGCGGACCGTGGCGACGACGAATACAT
Lbrev 117.2    GCCTTAGCCATTGGGTCACCGATGGGCTCTAATTACGCAACGACCTTGACGCAAGGAATCATTTCAGCGAAGAAGCGGACCGTGGCGACGACGAATACAT
Lpar 79        GCCTTAGCCATTGGGTCACCGATGGGCTCTAATTACGCAACGACCTTGACGCAAGGAATCATTTCAGCGAAGAAGCGGACCGTGGCGACGACGAATACAT
Lpar 83.1      GCCTTAGCCATTGGGTCACCGATGGGCTCTAATTACGCAACGACCTTGACGCAAGGAATCATTTCAGCGAAGAAGCGGACCGTGGCGACGACGAATACAT
Lplant 40.3    GCCTTAGCCATTGGGTCACCGATGGGCTCTAATTACGCAACGACCTTGACGCAAGGAATCATTTCAGCGAAGAAGCGAACCGTGGCGACGACGAATACAT
Lplant 69      GCCTTAGCCATTGGGTCACCGATGGGCTCTAATTACGCAACGACCTTGACGCAAGGAATCATTTCAGCGAAGAAGCGGACCGTGGCGACGACGAATACAT
Lbrev 81.1     GCCTTAGCCATTGGATCACCGATGGGCTCTAATTACGCAACGACCTTGACGCAAGGAATCATTTCAGCGAAGAAGCGGACCGTGGCGACGACGAATACAT
Lhil 3         GCCTTAGCCATTGGATCACCGATGGGCTCTAATTACGCAACGACCTTGACGCAAGGAATCATTTCAGCGAAGAAGCGGACCGTGGCGACGACGAATACAT
Lpent 79.2     GCCTTAGCCATTGGATCACCGATGGGCTCTAATTACGCAACGACCTTGACGCAAGGAATCATTTCAGCGAAGAAGCGGACCGTGGCGACGACGAATACAT
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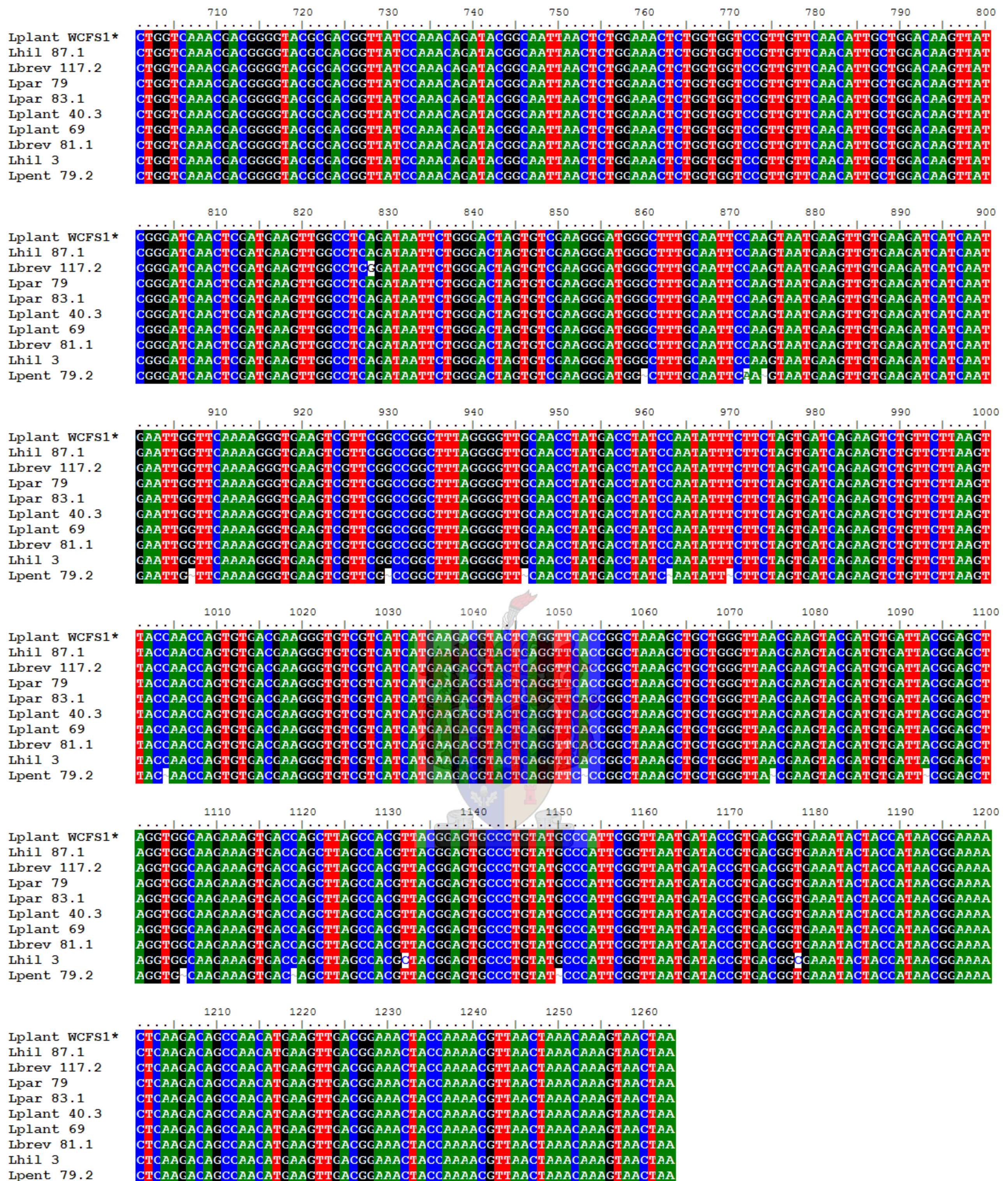
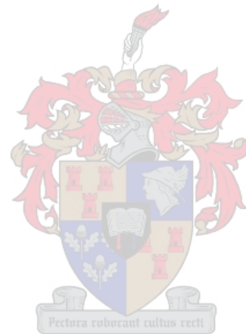


Figure 3.6 Nucleotide sequence alignments for serine protease HtrA genes from Lhil 87.1, Lbrev 117.2, Lpar 79, Lpar 83.1, Lplant 40.3, Lplant 69, Lbrev 81.1, Lhil 3 and Lpent 79.2. Gene sequence indicated by (*) was extracted from GenBank database. Highlighted residues indicate regions which are highly similar or conserved.

Chapter 4



GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 4

4.1 CONCLUDING REMARKS AND OTHER PERSPECTIVES

Among the factors contributing to wine quality and complexity, wine aroma is one of the most prominent factors. A large number of volatile aromatic components contributing to wine aroma have been identified in wine. With respect to their origin, these aroma compounds are divided into four categories: (i) the primary aroma components originating from the grapes; (ii) the aromatic compounds produced or changed due to the modifications caused by specific technological steps such as grape crushing and must treatment; (iii) the secondary aroma components produced by microorganisms during fermentation; and (iv) the tertiary aroma compounds formed as a result of enzymatic or physicochemical actions during ageing (Schreier, 1979).

Apart from its origin from the grapes, fungi and yeasts, wine aroma can also originate from the metabolic activity of wine LAB. These microorganisms are usually associated with MLF that normally occurs after alcoholic fermentation. MLF is beneficial to wine due to its contribution to deacidification, microbiological stabilisation and wine aroma formation (Wibowo *et al*., 1985). MLF is conducted by LAB of the genera *Lactobacillus, Oenococcus, Pediococcus* and *Leuconostoc*. Not all these genera are desirable for MLF. *O. oeni* is the most beneficial species which predominantly occurs in wines with pH values below 3.5 (Henick-Kling, 1993). Species which are associated with wine spoilage are generally members of lactobacilli and pediococci (Du Toit and Pretorius, 2000; Jackson, 1994).

Besides wine deacidification as the most well-known outcome of the metabolic activity of LAB, MLF can also alter the organoleptic quality of the wine through the production of aromatic compounds. The production of these volatile components contributing towards the formation of wine aroma can be achieved through the hydrolytic action of enzymes such as β-glucosidase, protease, esterase, lipase and glucanase. Enzymes can hydrolyse the problematic high molecular weight substances such as β-glucans, thereby improving clarification and filtration. In addition, enzymes can also allow for enhanced development of flavour by hydrolysing compounds contributing to wine aroma (www.biocatalysts.com).

Many studies on LAB enzymes are primarily based on dairy products (Andersen *et al*., 1995; Magboul *et al*., 1997). Our study therefore forms the basis for the survey of enzymes in wine LAB. To our knowledge this is the first study to investigate the presence of different enzymes in wine LAB isolates under the South African winemaking conditions. We screened bacterial isolates for different enzymes using both classical and molecular methods. Isolates were first screened on agar media supplemented with appropriate substrate analogues. From the results obtained, it was noteworthy that the isolates possessed different combinations of the enzymes investigated in this study. But due to the fact that enzyme analyses on the plates were conducted on different agar media, a major

challenge would therefore be to develop a cost-effective plate assay that would allow screening of all enzymes in one medium. However, there are some limitations associated with the success in developing this plate assay. Firstly, the incubation periods of the indicator plates differ with respect to the requirements of the enzyme tested. Some plate assays do not require prolonged incubation of the plates before detecting enzyme activity whereas some require longer incubation periods. A second constraint is based on the differences in the composition of the media. Moreover, some enzymes function properly at certain pH levels and the media should therefore be adjusted to a pH level that is suitable for enzyme activity. For example, β-glucosidase activity can be detected on agar medium adjusted to pH 5.5 whereas lipase activity can be observed on a different agar medium adjusted to pH 7.0. Some enzymes can also function in the presence of certain cofactors that would stimulate their activity. The addition of these cofactors may, in turn, have a negative effect on the activity of other enzymes being tested.

In testing the isolates for the presence of lipase activity, nutrient agar medium supplemented with tributyrin was employed (Lee and Rhee, 1993). Isolates exhibiting enzyme activity were identified by yellow colouration in the medium around the bacterial colonies. However, tributyrin is not a suitable substrate for the detection of true lipase activity because it can be hydrolysed by both lipase and esterase. This could be overcome by using a lipase-specific dye method that involves the inclusion of a fluorescent dye, such as rhodamine B, in the medium. True lipase activity would therefore be recognisable by the formation of orange fluorescence around the bacterial colonies, and that would be visible upon irradiating the plates under UV light at 350 nm. From the few isolates that were tested, none exhibited orange fluorescence. Since LAB are acknowledged for being weakly lipolytic in comparison to other groups of bacteria (Kalogridou-Vassiliadou, 1984), one possibility may be that, from the few isolates that we tested, none were positive. On the other hand, failure to observe orange fluorescence may be attributed to the fact that we did not have UV light at a wavelength of 350 nm but, instead, we exposed the plates under UV light at 365 nm.

Glucanase activity was tested on PHB agar plates supplemented with lichenan or CMC (Heng *et al.*, 1997). From the two substrates tested, enzyme activity was more pronounced on CMC than on lichenan. The principle behind using Congo red system for the assay of β-glucanase activity lies on a previous demonstration that Congo red shows a strong interaction with polysaccharides such as cellulose (Teather and Wood, 1982). The potential advantage of using this assay system is that it allows for the development of intense colour of the dye-glucan complex. This system also allows for a corresponding decrease in time required to detect lower levels of enzyme activity. In general, polysaccharides can affect wine processing due to the increased viscosity. These macromolecules reduce juice extraction and are primarily responsible for fouling of filters during clarification steps. Polysaccharides may also affect sensory properties of wine through changes in clarity. The effect of viscosity may influence mouthfeel and body.

Excessive levels of polysaccharides in wine are undesirable in terms of inducing ropiness. However, moderate levels may add complexity to wine (Liu, 2002). Further studies are required to elucidate the potential of wine LAB to hydrolyse polysaccharides.

Although major advances have been made with regard to the development of efficient and rapid techniques for detecting different enzymes on the plates, it should be noted that most screening systems are based on the laboratory media. The activity of enzymes may change in the actual winemaking. This emanates from the fact that wine is somehow a hostile environment encompassing different compounds that can subsequently affect the activity of these enzymes. In addition, almost all β-glucosidases are subjected to an end product inhibition (Saha and Bothast, 1996), which is an important constraint for industrial exploitation of this enzyme. β-Glucosidases release glucose as the major end product of their hydrolysis. In turn, this compound inhibits the activity of β-glucosidase. Therefore, the availability of β-glucosidase which is not sensitive to glucose inhibition is a major challenge in the world of research.

Besides testing the activity of enzymes on the plates, we also screened isolates with colony PCR using enzyme-specific primers. These primers amplified single PCR products with sizes corresponding to respective genes (i.e. malolactic enzyme, protease, β-glucosidase and esterase). As in the case for plate assays, a similar trend of enzyme secretion was observed. Isolates also possessed different combinations of the enzymes even during PCR detection. *Lb. acidophilus* was used as the basis for designing the primer sets for amplifying lipase and glucanase genes. But none of these primers gave amplification products and this may be attributed to the fact that *Lb. acidophilus* does not occur in wine. This could also suggest the absence of genetic similarity between *Lb. acidophilus* and other wine-associated lactobacilli. A second set of primers for detecting the presence of lipase genes was designed from a putative lipase gene of *Lb. delbrueckii.* This primer pair only produced non-specific bands. Several endeavours of troubleshooting proved unsuccessful.

In an attempt to study sequence homologies between different genes, genomic DNA was extracted from 11 selected isolates belonging to different *Lactobacillus* species and subsequently used as template to amplify the coding sequences of the respective genes. The selected isolates possessed all four enzyme genes (i.e. β-glucosidase, esterase, protease and malolactic enzyme). The same sets of enzyme-specific primers were employed for PCR amplifications. Purified amplicons were cloned into pGEM-T easy vector and sequenced. From the alignment results, gene sequences exhibited a significant similarity with GenBank nucleotide gene sequences (Benson *et al.*, 1999) and these sequences were also highly conserved between the species. During sequence analyses, it was also interesting to note that β-glucosidase genes of Lplant 113.1 and Lbrev 116.3 did not contain a certain portion of the sequence (83 nucleotides missing). This could be due to sequencing error, deletions or evolution within the two genes. But no conclusions could be inferred from these results unless the two genes could be sequenced again.

Furthermore, an analysis of protease genes from the tested isolates revealed that these genes belong to the class of serine proteinase HtrA enzymes. HtrA homologs have been identified in a variety of bacteria including *E. coli* (Skórko-Glonek *et al*., 1997), *Lb. helveticus* (Smeds *et al*., 1998), *Lactococcus lactis* (Poquet *et al*., 2000) and *Streptococcus pyogenes* (Lyon and Caparon, 2004). This group of enzymes is involved in the folding and maturation of secreted proteins, as well as in the degradation of proteins that misfold during secretion (Clausen *et al*., 2002; Pallen and Wren, 1997). Depletion of HtrA has been shown to affect the sensitivity of many organisms to thermal and environmental stresses (Skórko-Glonek *et al*., 1999). During wine fermentation, microbes present in wine are exposed to a variety of stresses, and the presence of HtrA genes in LAB could confer a resistance against harsh physicochemical conditions in wine. This area therefore merits further studies to elucidate the potential of wine LAB to possess serine proteinase HtrA enzyme that would minimise the risk associated with failure to cope with winemaking conditions.

In summary, our study has demonstrated the existence of different enzymes in several LAB isolates associated with wine during MLF. It is therefore apparent that LAB can be the potential source of enzymes for use in vinification. Further analyses of the tested isolates therefore merit further research in quest of "competent" isolates possessing desired characteristics that can subsequently contribute toward the formation of wine aroma. In our study, none of *O. oeni* species were tested molecularly for possessing one of these enzymes. In previous studies, more research on enzymes in wine fermentation were concerned with β-glucosidase produced by *O. oeni*. We have therefore shown by using different approaches that other genera of wine LAB also possess different combinations of enzymes. For future studies, it will also be interesting to test *O. oeni* isolates for possessing all the enzymes investigated in this study. A possible application of biotechnology, such as gene cloning, in some of the isolates that we tested would also be of great interest in taking this research further as a means of advancing our knowledge on wine LAB enzymes.

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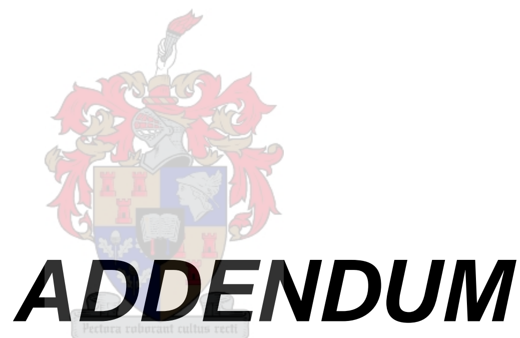
Pectora roborant cultus recti

Chapter 5



ADDENDUM

Partial characterisation of β-glucosidase from certain wine lactic acid bacteria isolated from South African wines

CHAPTER 5

Partial characterisation of β-glucosidase from certain wine lactic acid bacteria isolated from South African wines

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ABSTRACT

Lactic acid bacteria are microorganisms normally associated with wine during malolactic fermentation (MLF). Their metabolic activity results to the modification of wine aroma and flavour through the production of hydrolytic enzymes such as β-glucosidase. Six isolates belonging to *Lactobacillus plantarum*, *Lb. hilgardii, Lb. paracasei, Lb. pentosus* and *Lb. brevis* were tested for β-glucosidase activity against *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as substrate. The activity of β-glucosidase was assayed under various physicochemical conditions simulating those of winemaking. All the isolates exhibited enzymatic activity against this substrate, with the exception of *Lb. paracasei* which was used as negative control. β-Glucosidase activity of all the isolates tested was competitively affected by various concentrations of glucose as well as a temperature of 50°C. There was no significant reduction in enzyme activity at various concentrations of ethanol. Since wine contains glycosides which serve as the potential source of aromatic flavour, the possible use of β-glucosidase for the hydrolysis of sugar-bound components can enhance the sensory properties of the wine.

Keywords**:** Lactic acid bacteria, enzyme, β-glucosidase, *Lactobacillus*, aroma, wine

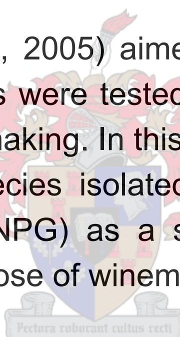
5.1 INTRODUCTION

Many aromatic compounds found in grapes, must and wines occur in two different forms: free and sugar-bound forms. The sugar-bound components are generally non-volatile and therefore do not contribute to wine aroma. The glycosidic precursors which impart an important aroma in wines can be hydrolysed either enzymatically through glucosidases or via acid hydrolysis (Günata *et al*., 1988). Acid hydrolysis has been studied as a method for the release of bound aroma compounds, where samples are adjusted to lower pH levels to break glycosidic bonds (Williams *et al*., 1981). Unlike acidic hydrolysis which can consequently interfere with wine aroma, enzymatic hydrolysis is alternatively preferred for

hydrolysing sugar-conjugated flavour precursors. Under the latter conditions, the changes in the natural monoterpenol distribution are minimal (Günata *et al.*, 1988). Some aromatic aglycones may be released through the sequential hydrolytic action of glycosidases. In general, the mechanism for enzymatic hydrolysis of glycosidic precursors occurs through two successive steps. In the first phase, the glucose is separated from the terminal sugars by a hydrolase group (α-L-arabinofuranosidase) before, in the second phase, β-D-glucosidase breaks the bond between the aglycone and glucose (Günata *et al.*, 1988; Spagna *et al.*, 1998), hence liberating the volatile flavour precursor.

Although glycosidase activities have been investigated from sources other than lactic acid bacteria (LAB), little is known about the potential of different genera of wine LAB to possess glycosidase activities. Preliminary studies done on LAB β-glucosidase have focused on evaluating the activity of this enzyme mainly in *Oenococcus oeni*, which is the main bacterial species preferred for conducting malolactic fermentation (MLF). This is due to its tolerance against the harsh physicochemical conditions of high acidity, nutrient depletion and high alcohol content present in wine after alcoholic fermentation (Wibowo *et al.*, 1985). However, the research is now directed towards evaluating glycosidase activities of other genera of wine LAB.

In a recent study (Grimaldi *et al.*, 2005) aimed at evaluating β-glucosidase activity, *Lactobacillus* and *Pediococcus* strains were tested with all the *p*-nitrophenyl forms of the key glycosides of importance in winemaking. In this study we have evaluated the activity of β-glucosidase from *Lactobacillus* species isolated from the South African wines using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as a substrate. Enzyme activity was tested under various conditions simulating those of winemaking.



5.2 MATERIALS AND METHODS

5.2.1 Bacterial isolates

Bacterial isolates used in this study were from the culture collection of the Institute for Wine Biotechnology and were previously collected from five different commercial wineries situated in the Western Cape region, South Africa. They belonged to *Lactobacillus plantarum* (Lb-113.1), *Lb. hilgardii* (Lb-3), *Lb. paracasei* (Lb-30), *Lb. pentosus* (Lb-79.2) and *Lb. brevis* (Lb-116.3 and Lb-117.2). All these isolates were precultured on MRS agar plates.

5.2.2 Growth curves

In order to study the growth patterns of isolates that were positive for all enzymes, the normal MRS and modified MRS (ModMRS) media were employed for the propagation of these isolates. The two media were tested to see some differences on the growth patterns of isolates when grown in two different media. Growth pattern was monitored by

measuring cell density of cultures using spectrophotometer at 600 nm. ModMRS was filter-sterilised at 0.2 μm rather than autoclaving in order to avoid darkening caused by heating. The composition of ModMRS was similar to that described by Grimaldi *et al.* (2000). According to the latter authors, normal MRS proved unsuitable for the direct determination of glycosidase activities in culture supernatants because of the deep yellow/brown colour of the medium. ModMRS medium with reduced amounts of sugars and yeast extract was therefore adopted as the suitable medium.

The cells were prepared by inoculating a loopful of cells into 10 ml of MRS broth, grown at 30°C for 48 h and subcultured into 50 ml of experimental medium to an optical density of 0.2 at 600 nm. The experimental cultures were incubated at 30°C for 2 days and growth was monitored spectrophotometrically at 600 nm after every three to six hours.

5.2.3 Enzyme activity assay

β-Glucosidase activity was quantified from three selected bacterial isolates using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as the substrate (Grimaldi *et al.*, 2000). One isolate belonging to *Lb. paracasei* was also incorporated as a negative control. Enzyme activity was evaluated under different physicochemical parameters simulating those of winemaking, including pH, temperature, ethanol and glucose.

5.2.3.1 Cell preparation

A loopful of cells was inoculated from MRS agar plate into 10 ml of the liquid medium. After 48 h incubation at 30°C, the inocula were subcultured into 100 ml of experimental medium to an optical density of 0.2 at 600 nm. Experimental cultures were incubated at 30°C for 48 h. At regular intervals, 2 ml samples were taken for monitoring culture growth at an absorbance of 600 nm and separate determination of β-glucosidase activity from the whole cells.

To determine β-glucosidase activity from the whole cells, the cells were harvested from 1 ml of culture (5 000 rpm, 10 min, 4°C), washed with cold 150 mM NaCl and the pellet was resuspended in an appropriate volume of 125 mM citrate-phosphate buffer (pH 3.5) containing *p*NPG and used for enzymatic assay.

5.2.3.2 Enzyme assay

β-Glucosidase activity was determined by measuring the amount of *p*-nitrophenol (*p*NP) released from *p*NPG as chromogenic substrate. Cells were harvested from 1 ml of culture, washed with cold 150 mM NaCl and resuspended in 500 μl of 125 mM citrate-phosphate buffer (pH 3.5) containing 5 mM *p*NPG. The reaction mixture was incubated for 2 h at 25°C and subsequently the reaction was stopped by adding 1 ml of 1 M Na_2CO_3 (pH 10.2). The samples were clarified by centrifugation and the liberated *p*NP was measured at 400 nm in Shimadzu UV-V spectrophotometer (Shimadzu Corporation, Kyoto, Japan). All the assays were performed in duplicate and averaged.

5.2.3.3 Influence of pH, temperature, glucose and ethanol

To study the influence of different physicochemical parameters on β-glucosidase activity, enzyme assay was conducted under conditions of varying temperatures, pH levels as well as different concentrations of ethanol and glucose. To evaluate the influence of different temperatures (25, 30 and 50°C) on β-glucosidase activity, the pH and ethanol were kept at 3.5 and 12% (v/v), respectively. The effect of pH on enzymatic preparations was studied using citrate-phosphate buffer at varying pH levels of 3.5, 3.8 and 5.0 while temperature and ethanol were kept at 25°C and 12%, respectively. An influence of glucose was studied by adding this compound to the reaction mixture in concentrations of 0, 1, 3 and 5% (w/v). Similarly, the influence of 10, 12 and 14% (v/v) ethanol was studied by adding this compound to the reaction mixture at pH 3.5 and 25°C to simulate winemaking conditions. All the reaction mixtures were incubated and analysed as above.

5.3 RESULTS

5.3.1 Growth curves

The growth patterns of different species of *Lactobacillus* are presented in **Figure 5.1**. As shown in the figure, all the bacterial isolates tested exhibited a similar pattern of growth. This information was exploited for determining the stages of growth at which β-glucosidase enzyme assay would be conducted. However, the normal MRS medium seemed to be the preferred medium for exploitation during enzyme assays as it showed a better growth trend of bacterial species in comparison to ModMRS medium. The normal MRS medium was therefore adopted for conducting enzyme assays.

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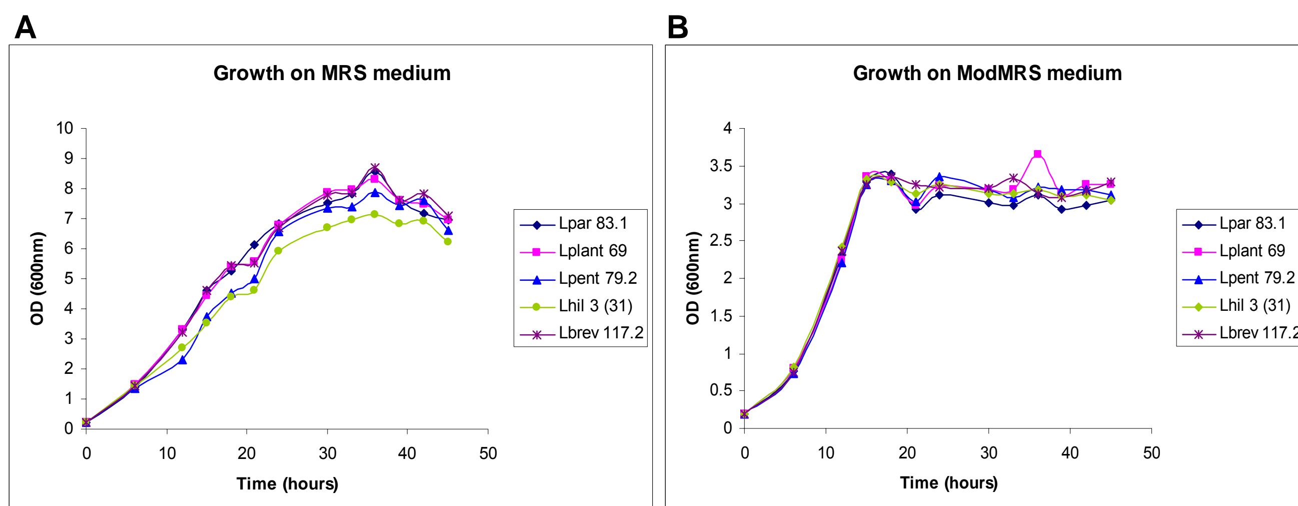

Figure 5.1 The growth patterns of different species of *Lactobacillus* grown in normal MRS (A) and ModMRS (B) media.

5.3.2 Kinetic properties of β-glucosidase

β-Glucosidase was evaluated under different conditions to quantify the amount of enzyme produced by individual isolates. The enzyme was characterised for activity under different physicochemical conditions simulating those of winemaking. In addition to the selected isolates, one isolate known not to posses the enzyme was also incorporated as the negative control during enzyme assay and, indeed, no enzyme activity was observed.

5.3.2 .1 Influence of pH

The influence of pH on enzymatic activity is shown in **Figure 5.2**. The effect of pH on β-glucosidase activity was studied on whole cells using citrate-phosphate buffer at pH 3.5, 3.8 and 5.0. Among these pH levels, enzyme activity was higher at pH 3.8 than at a pH of 3.5 for all the isolates tested. A slight increase in enzyme activity from pH 3.8 to 5.0 was observed in Lb-3 and Lb-79.2 whereas a slight decrease in activity was seen in Lb-117.2, Lb-113.1 and Lb-116.3.

5.3.2.2 Influence of temperature

The influence of temperature on enzymatic activity is presented in **Figure 5.3**. Under the conditions used (12% v/v ethanol, pH 3.5), Lb-3 and Lb-79.2 exhibited a slight increase in enzyme activity when the temperature was increased from 25°C to 30°C while Lb-117.2, Lb-113.1 and Lb-116.3 exhibited a reduction in activity by less than 15%. At a temperature of 50°C, enzyme activity was reduced by 70% for all the isolates tested.

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5.3.2.3 Influence of ethanol

The effect of ethanol on β-glucosidase activity is shown in **Figure 5.4**. The enzyme showed no significant reduction in activity in the presence of various concentrations of ethanol. Relative activity was proportionally reduced with increasing concentration of ethanol from the least inhibitory (10%, v/v) to the most inhibitory (14%, v/v) condition. These findings are similar to those reported on β-glucosidase activity from *O. oeni* strains (Grimaldi *et al*., 2000).

5.3.2.4 Influence of glucose

The influence of glucose on β-glucosidase activity is shown in **Figure 5.5**. The presence of glucose inhibited enzymatic activity in all the wine isolates examined. The inhibition of activity by glucose increased with sugar concentration and relative activity was reduced to less than 50% at a glucose concentration of 5% (w/v). This trend of enzyme inhibition in the presence of sugar is similar to that reported for β-glucosidase from grape (Lecas *et al*., 1991) and from fungal origin (Aryan *et al*., 1987).

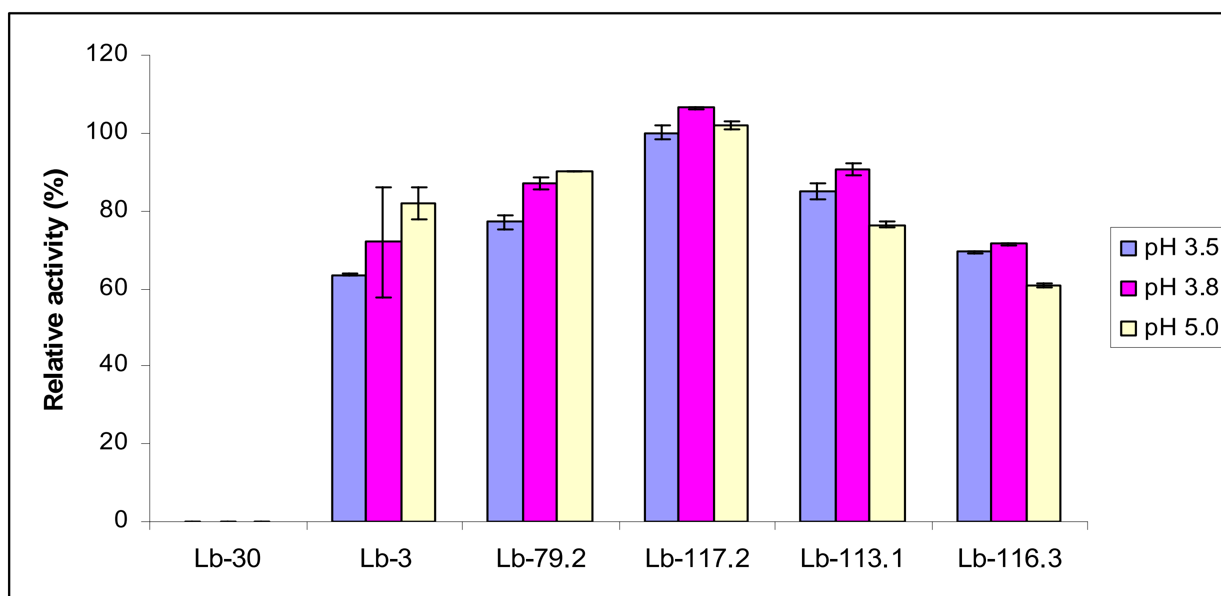

Figure 5.2 The influence of pH on β-glucosidase activity of *Lactobacillus* spp. Experimental conditions of assay mixture: 12% (v/v) ethanol, 25°C. The pH was adjusted by using citrate-phosphate buffer to obtain the desired pH. Values are the mean of two determinations and are expressed relative to the activity of the isolate with highest activity in buffer with pH 3.5. Error bars indicate the standard deviations.

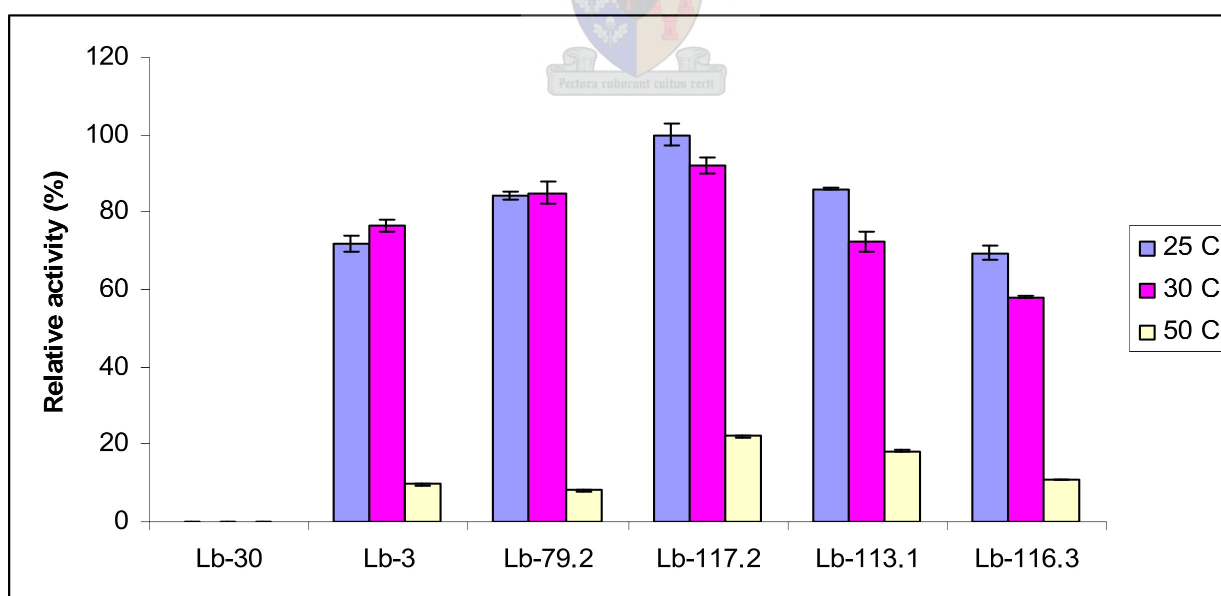

Figure 5.3 The influence of temperature on β-glucosidase activity of *Lactobacillus* spp. Experimental conditions of assay mixture: 12% (v/v) ethanol, pH 3.5. Values are the mean of two determinations and are expressed relative to the activity of the isolate with highest activity at 25°C. Error bars indicate the standard deviations.

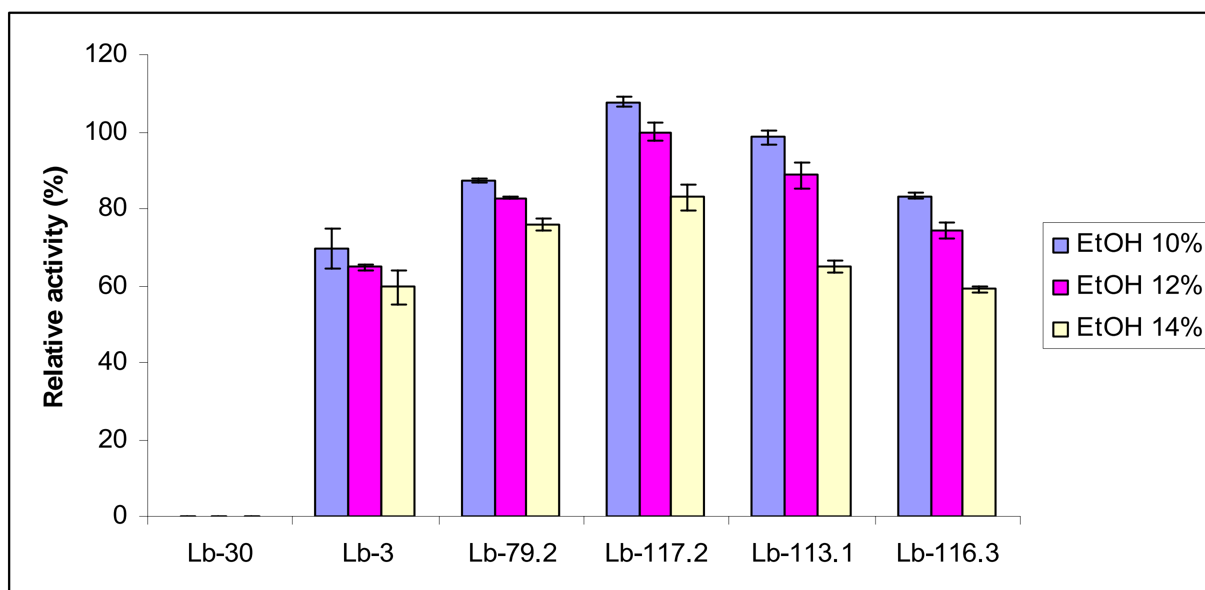

Figure 5.4 The influence of ethanol on β-glucosidase activity of *Lactobacillus* spp. Experimental conditions of assay mixture: pH 3.5, 25°C. Values are the mean of two determinations and are expressed relative to the activity of the isolate with highest activity in buffer with ethanol concentration of 12% (v/v). Error bars indicate the standard deviations.

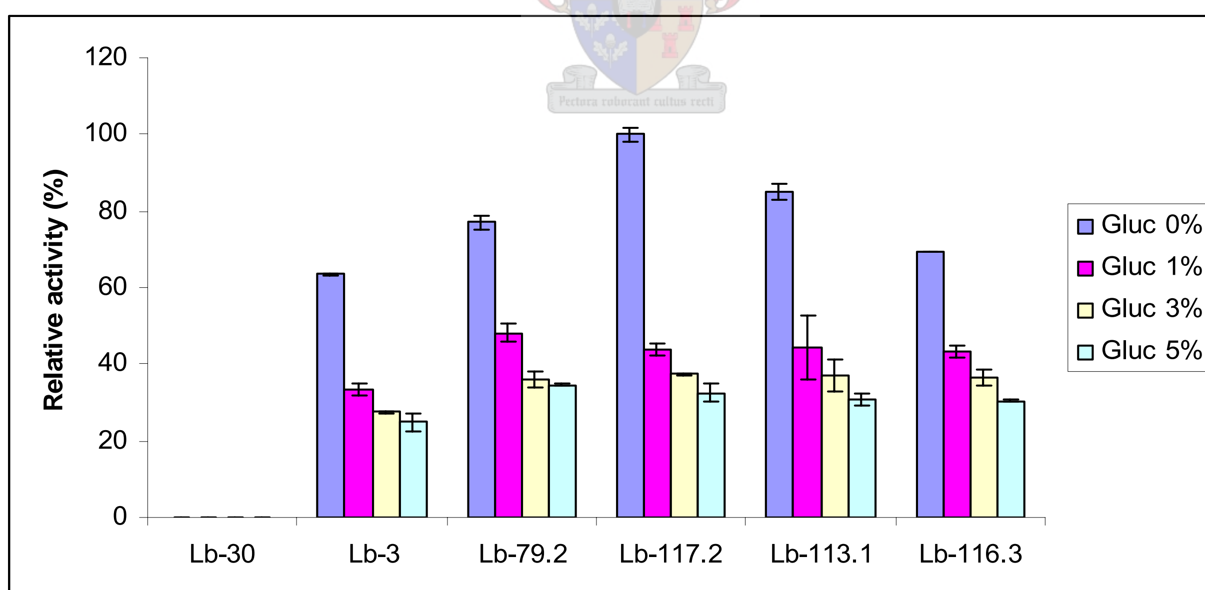

Figure 5.5 The influence of glucose on β-glucosidase activity of *Lactobacillus* spp. Experimental conditions of assay mixture: 12% (v/v) ethanol, pH 3.5, 25°C. Values are the mean of two determinations and are expressed relative to the activity of the isolate with highest activity in buffer lacking glucose. Error bars indicate the standard deviations.

5.4 DISCUSSION

The hydrolysis of volatile compounds contributing to wine aroma is achieved through the hydrolytic action of enzymes such β-glucosidase. This enzyme plays a pivotal role in the liberation of potent aroma components positively influencing the organoleptic quality of wine. The activity of β-glucosidase has been extensively studied in fungi (Spagna *et al*., 1998), grape (Aryan *et al*., 1987), yeasts (Delcroix *et al*., 1994; Mateo and Di Stefano, 1997) and bacteria (mainly *O. oeni*) (Grimaldi *et al*., 2000; McMahon *et al*., 1999). Very few studies have extended their focus on evaluating the activity of this enzyme in other genera of wine LAB. We have investigated the combined effect of wine parameters on the activity of β-glucosidase from the selected species of *Lactobacillus* isolated from the South African wines. In our study, we evaluated the influence of pH, temperature, ethanol and glucose on enzyme activity. From our results, it has been shown that the combined effect of winemaking parameters can inhibit the activity of β-glucosidase in various ways.

In investigating its influence on β-glucosidase activity, ethanol has been shown to have an enhancing effect occurring most often at lower concentrations (e.g. 4%, v/v) (Grimaldi *et al*., 2000, 2005). Such a phenomenon, described by Pemberton *et al*. (1980), is the result of a glycosyl transferase activity. At higher concentrations, however, the glycosidase enzymes are inhibited by ethanol probably because of protein denaturing (Gueguen *et al*., 1995). This is also supported by the findings of Spano *et al*. (2005) who observed a repression of an expression of a β-glucosidase gene from *Lb. plantarum* by 12% (v/v) ethanol. In addition, ethanol partially inhibited glycosidase activities, with complete inhibition being most often seen for α-arabinofuranosidase activity of *O. oeni* (Grimaldi *et al*., 2005).

The natural grape sugars glucose and fructose have been shown to be inhibitory to glycosidase enzymes even at the residual concentrations found in wines (Grimaldi *et al*., 2005). The inclusion of these sugars produced a similar pattern of inhibition for both β-D-glucopyranosidase and α-glucopyranosidase activities in *Lactobacillus* spp. While glucose inhibition increased with an increase in sugar concentration, the strong inhibition was observed even at 0.01% (w/v) glucose. In *Pediococcus* strains, β-D-glucopyranosidase was completely inhibited at all glucose concentrations (Grimaldi *et al*., 2005). These results suggest a limitation of the use of β-glucosidase in winemaking during the presence of sugars.

The results reported on the ability of wine LAB to hydrolyse glyco-conjugates are contradictory. β-Glucosidase activity in wine LAB (mainly *O. oeni*) was discovered about 10 years ago in a synthetic media by Guilloux-Benatier *et al*. (1993). This was further confirmed by Grimaldi *et al*. (2000) who found detectable activity of β-glucosidase in 11 commercial preparation of *O. oeni*. Further studies (Mansfield *et al*., 2002) detected the production of β-glucosidase enzymes in strains of *O. oeni*, although cultures of the same strains failed to hydrolyse native grape glycosides. In contrast, McMahon *et al*. (1999)

observed no enzymatic activity in commercial strains of *O. oeni* against arbutin, an artificial glycosidic substrate.

These findings suggest that even wine LAB have the potential to hydrolyse glycoconjugates consequently affecting wine aroma and colour. However, β-glucosidase enzymes in yeasts and bacteria are usually inhibited by winemaking parameters such as pH, ethanol and sugars (Delcroix *et al*., 1994; McMahon *et al*., 1999; Grimaldi *et al*., 2000). The acidic conditions in wine may result in denaturing and/or inhibition of enzymatic hydrolysis, although strains of *O. oeni* may retain 80% of maximum β-glucosidase activity at pH 3.5 (Grimaldi *et al*., 2000). These findings on enzyme inhibition are also supported by our results on the evaluation of β-glucosidase activity in different *Lactobacillus* species. We investigated the influence of multiple winemaking parameters on the activity of β-glucosidase. The enzyme was competitively inhibited by glucose at various concentrations and enzyme inhibition was proportional to each increase in glucose concentration. In addition, lower temperatures (25°C and 30°C) had a stimulatory effect on β-glucosidase activity and enzyme inhibition to less than 30% was observed at a temperature of 50°C. Ethanol resulted in a 20% reduction of β-glucosidase activity from 12% to 14% (v/v) whereas pH enhanced enzyme activity at pH 3.8 and 5.0. It is therefore crucial to understand if and how β-glucosidase enzymes are regulated by winemaking parameters. This will enable the selection of starter cultures able to positively alter the wine volatile fraction throughout the liberation of glycosidically bound aroma components (Spano *et al*., 2005).

5.5 CONCLUSIONS

In summary, this study forms the basis for the survey performed on wine LAB isolates in our culture collection with intent to search for suitable isolates to be selected as starter cultures for conducting MLF, hence liberating wine aroma components from glycosidically bound compounds. In addition, this study confirms the presence of β-glucosidase activity from various species of *Lactobacillus*. Apart from that, it should also be accounted that most of the assay systems are based on laboratory media. Apparently, the activity of enzymes from the LAB may change in the actual winemaking. This stems from the fact that wine is a complex medium encompassing various compounds, such as phenols, anthocyanins and tannins. These compounds may pose an inhibitory effect on the activity of enzymes. For future studies, it would also be interesting to further explore this area of research by taking into consideration some of the aspects such as the purification of the enzyme before characterisation, determining the specificity of the enzyme by testing it over a large number of substrates with α and β configurations, determining the enzyme optimum pH at various buffers as well as evaluating the influence of various metal ions on the activity of β-glucosidase.

5.6 ACKNOWLEDGEMENTS

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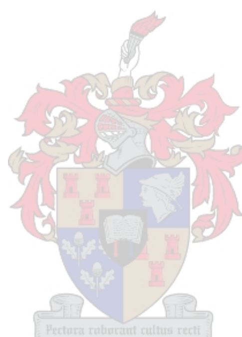

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