Molecular screening of lactic acid bacteria enzymes and their regulation under oenological conditions

by

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DECLARATION

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SUMMARY

During winemaking, a number of biochemical changes occur as a result of the metabolic activity of wine lactic acid bacteria (LAB) associated with malolactic fermentation (MLF). The latter process, which occurs mostly after alcoholic fermentation by wine yeasts, involves the conversion of L-malate to L-lactate and CO_2, thus resulting to wine acidity reduction, microbiological stabilization and alterations of wine organoleptic quality.

Although *Oenococcus oeni* is predominantly the most preferred species suitable for carrying out MLF in wine owing to its desirable oenological properties, *Lactobacillus plantarum* has also been considered as a potential candidate for MLF induction. Other species in the genera of *Lactobacillus* and *Pediococcus* are often associated with wine spoilage. These microorganisms induce wine spoilage by producing off-flavours derived from their metabolic activity. It is therefore of paramount importance to understand the mechanism by which wine microbiota cause spoilage.

The purpose of this study was to investigate the presence of genes encoding enzymes of oenological relevance in wine-associated LAB strains. In order to achieve this, different sets of specific primers were designed and employed for a wide-scale genetic screening of wine LAB isolates for the presence of genes encoding enzymes involved in various metabolic pathways, such as citrate metabolism, amino acid metabolism, hydrolysis of glycosides, degradation of phenolic acids as well as proteolysis and peptidolysis. PCR detection results showed that the majority of the tested strains possessed most of the genes tested for. It was also noted that, among the *O. oeni* strains tested for the presence of the *pad* gene encoding a phenolic acid decarboxylase, only two strains possessed this gene. None of the *O. oeni* strains has previously been shown to possess the *pad* gene, and this study was the first to report on the presence of this gene in *O. oeni* strains. In an attempt to genetically characterize this putative gene, DNA fragments from the two positive *O. oeni* strains were sequenced. The newly determined sequences were compared to other closely related species. Surprisingly, no match was found when these sequences were compared to the published genomes of three *O. oeni* strains (PSU-1, ATCC BAA-1163 and AWRI B429). This reinforced a speculation that the *pad* gene in these two strains might have been acquired via the horizontal gene transfer. In addition, it remains to be further determined if the presence of this gene translates to volatile phenol production in wine.

In this study, a novel strain isolated from South African grape and wine samples was also identified and characterized. The identification of this strain was performed through the 16S rDNA sequence analysis, which indicated that this strain belongs to *Lactobacillus florum* (99.9% sequence identity). A novel PCR assay using a species-specific primer for the rapid detection and identification of *Lb. florum* strains was also established. For further characterization, this strain was also investigated for the presence of genes encoding enzymes of oenological relevance. PCR detection results indicated that the *Lb. florum* strain also possess some of the genes tested for.

In addition to genetic screening of wine LAB isolates for the presence of different genes, this study was also aimed at evaluating the regulation of the *mleA* gene encoding malate decarboxylase in three oenological strains of *O. oeni*. The regulation of this gene was tested in a synthetic wine medium under various conditions of pH and ethanol. From the expression analysis, it was observed that the *mleA* gene expression was negatively affected by high ethanol content in the medium. On the other hand, low pH of the medium seemed to favour

the expression of this gene as the *mleA* gene expression was more pronounced at pH 3.2 than at pH 3.8.

The findings from this study have shed more light on the distribution of a wide array of enzyme-encoding genes in LAB strains associated with winemaking. However, it remains unknown if the enzymes encoded by these genes are functional under oenological conditions, given that wine is such a hostile environment encompassing a multitude of unfavourable conditions for the enzymes to work on. Evaluating the expression of these genes will also help give more insights on the regulation of the genes under winemaking conditions.

OPSOMMING

Gedurende wynmaak, sal 'n aantal biochemiese veranderinge plaasvind as gevolg van die metaboliese aktiwiteit van wyn melksuurbakterieë (MSB) wat betrokke is by appelmelksuurgisting (AMG). Die laasgenoemde proses, wat meestal na alkoholiese fermentasie deur wyngiste plaasvind, behels die omskepping van L-malaat na L-laktaat en CO_2, om sodoende die wyn se suur te verminder, mikrobiologiese stabiliteit en verandering van wyn organoleptiese kwaliteit.

Alhoewel *Oenococcus oeni* hoofsaaklik die mees gewenste spesies is wat geskik is vir die uitvoering van AMG in wyn weens sy geskikte wynkundige eienskappe, *Lactobacillus plantarum* word ook beskou as 'n potensiële kandidaat vir AMG induksie. Ander spesies in die genera *Lactobacillus* en *Pediococcus* word dikwels geassosieer met wynbederf. Hierdie mikro-organismes veroorsaak wynbederf deur die produksie van wangeure as gevolg van hul metaboliese aktiwiteite. Dit is dus van kardinale belang dat die meganisme van die wynbederf verstaan word.

Die doel van hierdie studie was om die teenwoordigheid van koderend ensieme gene van wynkundige belang in wynverwante MSB stamme te ondersoek. Ten einde dit te bereik, was verskillende stelle van spesifieke peilers ontwerp en toegepas vir 'n groot skaal se genetiese toetsing van wyn MSB isolate vir die teenwoordigheid van ensiemkoderende gene betrokke by verskeie metaboliese paaie, soos sitraat metabolisme, aminosuur metabolisme, hidrolise van glikosiede, agteruitgang van fenoliese sure sowel as proteolise en peptidolise. PKR opsporings resultate het getoon dat die meerderheid van die stamme getoets, die meeste van die gene getoets voor besit. Dit is ook opgemerk dat, onder die *O. oeni* stamme getoets vir die teenwoordigheid van die *pad* geen, slegs twee stamme hierdie geen besit. Geen *O. oeni* stamme het voorheen gewys dat hul die *pad* geen besit, en hierdie studie was die eerste bewys oor die teenwoordigheid van hierdie geen in *O. oeni* stamme. In 'n poging om die geen geneties te karakteriseer, is DNA-fragmente van die twee positiewe *O. oeni* stamme se sekwens volgorde bepaal. Die DNA volgorde is vergelyk met ander nouverwante spesies. Verrassend, was geen passende DNA volgorde gevind met die gepubliseerde genome van drie *O. oeni* stamme (PSU-1, ATCC BAA-1163 en AWRI B429) nie. Dit versterk die spekulasie dat die *pad* geen in hierdie twee stamme via die horisontale geen-oordrag verkry is. Verder moet dit nog bepaal word of die teenwoordigheid van hierdie geen lei na vlugtige fenol produksie in wyn.

In hierdie studie, is ongeïdentifiseerde stam geïsoleerd van Suid-Afrikaanse druiwe en wyn monsters ook geïdentifiseer en karakteriseer. Die identifisering van hierdie stam is uitgevoer deur middel van die 16S rDNA volgorde analise, wat aangedui het dat hierdie stam behoort aan *Lactobacillus florum* (99.9% volgorde identiteit). PKR toetse met behulp van die spesie-spesifieke peiler vir die vinnige opsporing en identifikasie van *Lb. florum* stamme is ook ontwikkel. Vir verdere karakterisering, was hierdie stam ook ondersoek vir die teenwoordigheid van koderende ensiem gene van wynkundige belang. PKR opsporings resultate het aangedui dat die *Lb. florum* stam ook oor 'n paar van die gene getoets voor besit.

Bykomend tot genetiese toetsing van wyn MSB isolate vir die teenwoordigheid van verskillende gene, het die studie ook die evaluering van die regulering van die *mleA* geen, kodering malaatdekarboksilase in drie wyn stamme van *O. oeni*. Die regulering van hierdie geen was getoets in die sintetiese wynmedium onder verskillende pH en etanol kondisies. Van die uitdrukkingsresultate, is daar waargeneem dat die *mleA* geenuitdrukking is negatief geraak deur hoë etanol-inhoud in die medium. Aan die ander kant, in die lae pH medium was die uitdrukking van hierdie geen bevoordeel by pH 3.2 as by pH 3.8.

Die bevindinge van hierdie studie het meer lig gewerp op die verspreiding van die wye verskeidenheid van ensiem-koderende gene in MSB stamme wat verband hou met wynmaak. Dit bly egter steeds onbekend of die ensieme gekodeer deur hierdie gene funksioneel is onder wynkondisies, gegewe dat wyn so 'n vyandige omgewing is menigte ongunstige toestande vir die werking van ensieme. Evaluering van die uitdrukking van hierdie gene sal ook help om meer insigte gee oor die regulering van die gene onder wynmaak toestande.

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 and obtained the degree in 2007. In the same year, he enrolled for PhD in Wine Biotechnology at the same institution.

DEDICATION

This dissertation is dedicated to my mother Khethiwe kaButhelezi-Mtshali

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Hierdie proefskrif is opgedra aan my moeder Khethiwe kaButhelezi-Mtshali

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Lolu cwaningo-mbhalo lubhekiswe kuMama wami uKhethiwe kaButhelezi-Mtshali

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PREFACE

This dissertation is presented as a compilation of 10 chapters. Each chapter is introduced separately and is written according to the style of the journal to which it will be submitted for publication.

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Chapter 2 **Literature Review**
Wine lactic acid bacteria: using '-omics' technologies to unravel the future

Chapter 3 **Research Results I**
Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines

Chapter 4 **Research Results II**
A survey of genes encoding enzymes of oenological relevance in *Oenococcus oeni* strains of South African wine origin

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

CHAPTER 1

1.1 INTRODUCTION

During vinification, two fermentation processes take place. The primary or alcoholic fermentation is carried out by yeasts, typically *Saccharomyces cerevisiae* strains, in which grape sugars are converted to ethanol and CO_2. Malolactic fermentation (MLF) occurs mostly after alcoholic fermentation and consists of the conversion of a dicarboxylic acid L-malate into a monocarboxylic acid L-lactate and CO_2 by lactic acid bacteria (LAB) of the genera *Lactobacillus* and *Oenococcus*. This process is catalyzed by the malolactic enzyme in the presence of two co-factors: Mn^{2+} and NAD^+ (Wibowo *et al.* 1985).

Given that wine is such a hostile environment encompassing a variety of stressful conditions, very few species are able to survive in wine during MLF. Among the wine-associated LAB species associated with spontaneous MLF, *Oenococcus oeni* remains predominantly the most suitable candidate well adapted to conduct MLF due to its tolerance to survive harsh oenological conditions of nutrient depletion, high ethanol, low pH, low temperatures and the presence of ethanol (Davis *et al.* 1988; Drici-Cachon *et al.* 1996; Lerm *et al.* 2010). *Lactobacillus plantarum* has also been considered as a potential candidate to be used as the starter culture to induce MLF, particularly for wines with higher pH (Bou and Krieger 2004; Du Toit *et al.* 2010). The first culture of *Lact. plantarum* for MLF was released in 2010 by Lallemand (Toulouse, France). This bacterium has also been assessed in wine to conduct MLF in a co-culture with *O. oeni* (Lerm 2010).

The reduction in wine acidity is not the only known effect of MLF. There are also other desirable attributes that this process imparts in wine. The conversion of L-malate to L-lactate confers increased microbial stability to wine due to the depletion of L-malate as the energy source for spoilage microorganisms. In addition, MLF influences desirable aroma and flavour formation in wine by modifying fruit-derived aromas and by producing aroma-active compounds (Bartowsky 2005). The possible pathways by which the malolactic bacteria modulate wine aroma were described recently by Swiegers *et al.* (2005). Amongst them, the most important metabolic pathways with potential to alter the wine organoleptic quality include citrate metabolism, amino acid metabolism, metabolism of polysaccharides, metabolism of polyols, catabolism of aldehydes, hydrolysis of glycosides, synthesis and hydrolysis of esters, proteolysis and peptidolysis, and the degradation of phenolic acids (Liu 2002; Matthews *et al.* 2004).

In order for malolactic bacteria, more specifically *O. oeni*, to impart desirable sensory attributes in wine, they produce a variety of compounds arising from their metabolic

activity. Glycosides present in the grape are an important source of wine aroma. These compounds are chemically bound to sugar molecules; in this form, they are odourless and therefore do not contribute directly to the varietal aroma of wine. The presence of glycosidases has been shown to be the key in liberating aroma fractions from glycosylated precursor components (Sarry and Gunata 2004). This enzyme family breaks down the bond between the sugar component and the aglycones, thereby releasing aroma compounds. The presence of glycosidase activities in *O. oeni* and other wine LAB has been documented (D'Incecco *et al*. 2004; Grimaldi *et al*. 2000, 2005a, b; Ugliano and Moio 2006), albeit these enzymes are prone to inhibition by winemaking parameters (Spano *et al*. 2005).

Another important contributor to wine aroma is diacetyl, which confers a desirable "buttery" attribute to wine (Bartowsky and Henschke, 2004). This compound is an intermediate metabolite of citric acid metabolism in LAB (Ramos *et al*. 1995). However, the presence of diacetyl at excessive concentrations (i.e. those exceeding 4 mg/L) is undesirable as this compound may become a spoilage character (Martineau *et al*. 1995). There are a number of factors that influence diacetyl synthesis, and these include citrate concentration, malolactic bacterial strain used, sulphur dioxide content, fermentation temperature, oxygen exposure and duration of MLF (Bartowsky and Henschke 2004).

Apart from flavour-active compounds conferring desirable traits in wine, winemakers are faced with the challenge of fighting off wine spoilage microbes which are responsible for the production of off-flavours in wine. Recently, Bartowsky (2009) listed some of the pathways by which the malolactic bacteria induce wine spoilage. Amongst the wine substrates metabolized by LAB, amino acids represent the most important source of nitrogen, carbon and sulphur for sulphur-containing amino acids (Swiegers *et al*. 2005). It has been demonstrated that *O. oeni* and wine-associated *Lactobacillus* species are able to catabolize methionine to produce diverse volatile sulphur compounds (VCSs) (Pripis-Nicolau *et al*. 2004). Although the VSCs can impact positively to the bouquet of wine (Mestres *et al*. 2000; Landaud *et al*. 2008), certain other VSCs are classified as detrimental to wine organoleptic quality, depending on their concentration (Knoll *et al*. 2011).

In relation to the metabolism of arginine, one of the major amino acids present in grape juice and wine, some arginine-degrading wine LAB can catabolize this compound via the arginine deiminase pathway (Liu *et al*. 1996). The physiological importance of arginine metabolism relates to the formation of energy in the form of ATP, and this reaction is accompanied by an increase in pH due to ammonium production (Tonon and Lonvaud-Funel 2000). However, the citrulline formed as an intermediate in the degradation of arginine by wine LAB can serve as the precursor for ethyl carbamate formation (Liu 2002).

In addition, arginine metabolism can also be linked to amine production. Ornithine, another intermediate in arginine catabolism, can result to the formation of putrescine. Together with other biologically active amines (histamine, tyramine), the latter compound is implicated in food poisoning incidents associated with the consumption of fermented foods including wine (Silla 1996; Smit *et al.* 2008).

1.2 PROJECT AIMS

The overriding goal of this project was to genetically test LAB isolates of South African wine origin for the presence of genes encoding enzymes of relevance in winemaking and to evaluate gene regulation under oenological conditions.

The specific objectives were as follows:

(a) Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines.

(b) A survey of genes encoding enzymes of oenological relevance in *Oenococcus oeni* strains of South African wine origin.

(c) PCR detection of enzyme-encoding genes in *Leuconostoc mesenteroides* strains of wine origin.

(d) Identification and characterization of *Lactobacillus florum* strains isolated from South African grape and wine samples.

(e) Expression analysis of *Oenococcus oeni* malolactic enzyme gene under oenological conditions.

(f) Molecular detection of *Lactobacillus* and *Pediococcus* genes related to peptide and amino acid utilization in wine.

1.3 REFERENCES

Bartowsky, E.J. (2009) Bacterial spoilage of wine and approaches to minimize it. *Lett Appl Microbiol* **48**, 149–156.

Bartowsky, E.J. (2005) *Oenococcus oeni* and malolactic fermentation – moving into the molecular arena. *Aust J Grape Wine Res* **11**, 174–187.

Bartowsky, E.J. and Henschke, P.A. (2004) The ‘buttery’ attribute of wine – diacetyl – desirability, spoilage and beyond. *Int J Food Microbiol* **96**, 235–252.

Bou, M. and Krieger, S. (2004) Alcohol-tolerant malolactic strains for the maturation of wines with average or high pH. PCT Pub. No.: WO/2004/111179.

D'Incecco, N., Bartowsky, E., Kassara, S., Lante, A., Spettoli, P. and Henschke, P. (2004) Release of glycosidically bound flavour compounds of Chardonnay by *Oenococcus oeni* during malolactic fermentation. *Food Microbiol* **21**, 257–265.

Davis, C.R., Wibowo, D., Fleet, G.H. and Lee, T.H. (1988) Properties of wine lactic acid bacteria: their potential enological significance. *Am J Enol Vitic* **39**, 137–142.

Drici-Cachon, Z., Guzzo, J., Cavin, F. and Diviès, C. (1996) Acid tolerance in *Leuconostoc oenos*. Isolation and characterisation of an acid-resistant mutant. *Appl Microbiol Biotechnol* **44**, 785–789.

Du Toit, M., Engelbrecht, L., Lerm, E. and Krieger-Weber, S. (2010) *Lactobacillus*: the next generation of malolactic fermentation starter cultures–an overview. *Food Bioprocess Technol*. doi: 10.1007/s11947-010-0448-8 (In press).

Grimaldi, A., Bartowsky, E. and Jiranek, V. (2005a) Screening of *Lactobacillus* spp. and *Pediococcus* spp. for glycosidase activities that are important in oenology. *J Appl Microbiol* **99**, 1061–1069.

Grimaldi, A., Bartowsky, E. and Jiranek, V. (2005b) A survey of glycosidase activities of commercial wine strains of *Oenococcus oeni*. *Int J Food Microbiol* **105**, 233–244.

Grimaldi, A., McLean, H. and Jiranek, V. (2000) Identification and partial characterization of glycosidic activity of commercial strains of the lactic acid bacterium *Oenococcus oeni*. *Am J Enol Vitic* **51**, 362–369.

Knoll, C., du Toit, M., Schnell, S., Rauhut, D. and Irmler, S. (2011) Cloning and characterisation of a cystathionine β/γ-lyase from two *Oenococcus oeni* oenological strains. *Appl Microbiol Biotechnol* 89, 1051–1060.

Landaud, S., Helinck, S. and Bonnarme, P. (2008) Formation of volatile sulfur compounds and metabolism of methionine and other sulfur compounds in fermented food. *Appl Microbiol Biotechnol* **77**, 1191–1205.

Lerm, E. (2010) The selection and characterisation of lactic acid bacteria to be used as a mixed starter culture for malolactic fermentation. Masters Thesis. Institute for Wine Biotechnology, Stellenbosch University, South Africa.

Lerm, E., Engelbrecht, L. and Du Toit, M. (2010) Malolactic Fermentation: The ABC's of MLF. *S Afr J Enol Vitic* **31**, 186–212.

Liu, S.-Q. (2002) Malolactic fermentation in wine – beyond deacidification. *J Appl Microbiol* **92**, 589–601.

Liu, S.Q., Pritchard, G.G., Hardman, M.J. and Pilone, G.J. (1996) Arginine catabolism in wine lactic acid bacteria: is it via the arginine deiminase pathway or the arginase-urease pathway? *J Appl Bacteriol* **81**, 486–492.

Martineau, B., Acree, T.E. and Henick-Kling, T. (1995) Effect of wine type on the detection threshold for diacetyl. *Food Res Int* **28**, 139–143.

Matthews, A., Grimaldi, A., Walker, M., Bartowsky, E., Grbin, P. and Jiranek, V. (2004) Lactic acid bacteria as a potential source of enzymes for use in vinification. *Appl Environ Microbiol* **70**, 5715–5731.

Mestres, M., Busto, O. and Guasch, J. (2000) Analysis of organic sulfur compounds in wine aroma. *J Chromatogr A* **881**, 569–581.

Pripis-Nicolau, L., Revel, G., Bertrand, A. and Lonvaud-Funel, A. (2004) Methionine catabolism and production of volatile sulphur compounds by *Oenococcus oeni*. *J Appl Microbiol* **96**, 1176–1184.

Ramos, A., Lolkema, J.S., Konings, W.N. and Santos, H. (1995) Enzyme basis for pH regulation of citrate and pyruvate metabolism by *Leuconostoc oenos*. *Appl Environ Microbiol* **61**, 1303–1310.

Sarry, J.E. and Gunata, Z. (2004) Plant and microbial glycoside hydrolases: volatile release from glycosidic aroma precursors. *Food Chem* **87**, 509–521.

Silla, M.H. (1996) Biogenic amines: their importance in foods. *Int J Food Microbiol* **29**, 213–231.

Smit, A.Y., du Toit, W.J. and Du Toit, M. (2008) Biogenic amines in wine: understanding the headache. *S Afr J Enol Vitic* **29**, 109–127.

Spano, G., Rinaldi, A., Ugliano, M., Moio, L., Beneduce, L. and Massa, S. (2005) A β-glucosidase gene isolated from wine *Lactobacillus plantarum* is regulated by abiotic stresses. *J Appl Microbiol* **98**, 855–861.

Swiegers, J.H., Bartowsky, E., Henschke, P. and Pretorius, I.S. (2005) Yeast and bacteria modulation of wine aroma and flavour. *Aust J Grape Wine Res* **11**, 139–173.

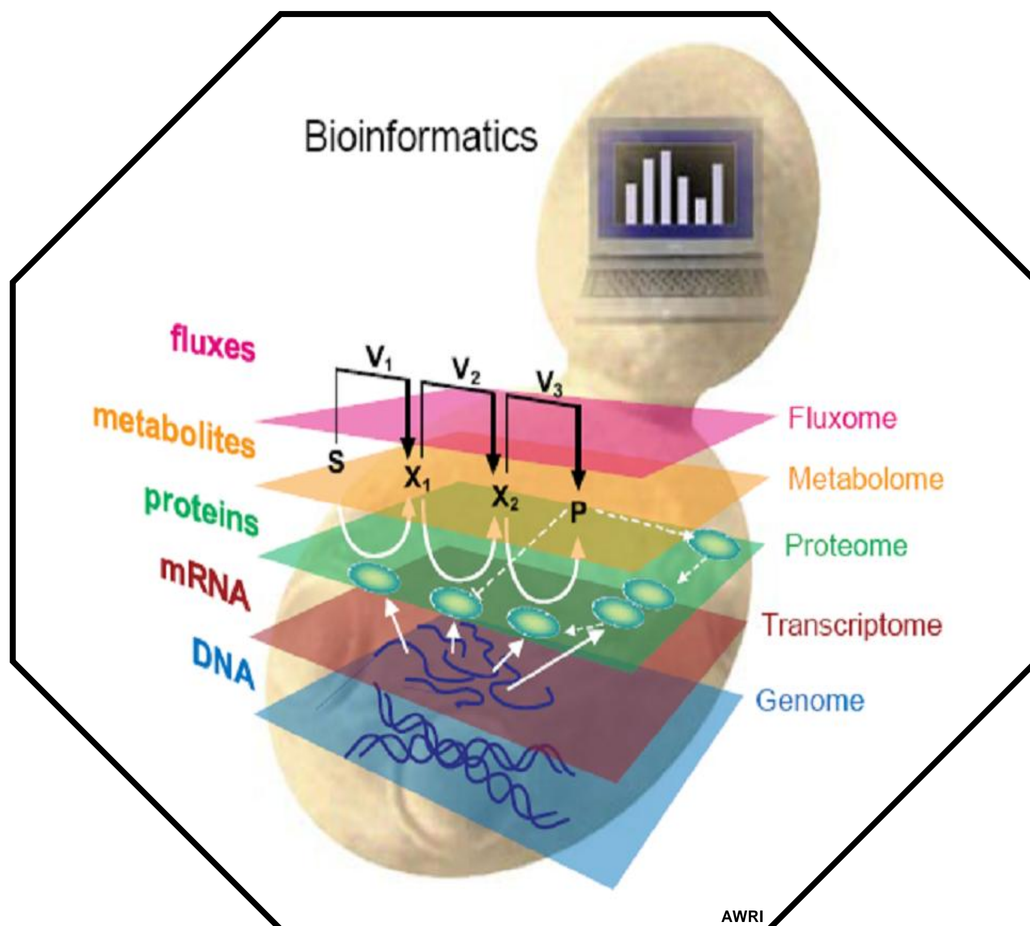
Tonon, T. and Lonvaud-Funel, A. (2000) Metabolism of arginine and its positive effect on growth and revival of *Oenococcus oeni*. *J Appl Microbiol* **89**, 526–531.

Ugliano, M. and Moio, L. (2006) The influence of malolactic fermentation and *Oenococcus oeni* strain on glycosidic aroma precursors and related volatile compounds of red wine. *J Sci Food Agric* **86**, 2468–2476.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H. and Lee, T.H. (1985) Occurrence and growth of lactic acid bacteria in wine: a review. *Am J Enol Vitic* **36**, 302–313.

CHAPTER 2

LITERATURE REVIEW



Wine lactic acid bacteria: using '-omics' technologies to unravel the future

CHAPTER 2

2.1 MALOLACTIC FERMENTATION

In winemaking, two fermentation processes occur. The primary (alcoholic) fermentation process, conducted mainly by *Saccharomyces cerevisiae* wine yeast, involves the conversion of grape sugars to form ethanol as the main product. Malolactic fermentation (MLF), which usually occurs after alcoholic fermentation, is carried out by the lactic acid bacteria (LAB) of the genera *Lactobacillus* and *Oenococcus* (Wibowo *et al.*, 1985). This process is catalysed by the malolactic enzyme which decarboxylates L-malic acid into a monocarboxylic L-lactic acid and CO_2 in the presence of Mn^{2+} and NAD^+ as co-factors, thus bringing about the reduction in wine acidity (Kunkee, 1991). Besides wine deacidification as the well-known effect of MLF, this process also offers increased microbial stability and causes changes in the sensory properties of wine (Liu, 2002; Swiegers *et al.*, 2005; Du Toit *et al.*, 2010; Lerm *et al.*, 2010). Changes in the chemical profile of wine are usually associated with the microbiological metabolism of precursor components present in wine during fermentation. The summary of biochemical changes that could occur in wine during MLF is depicted in Figure 2.1 below.

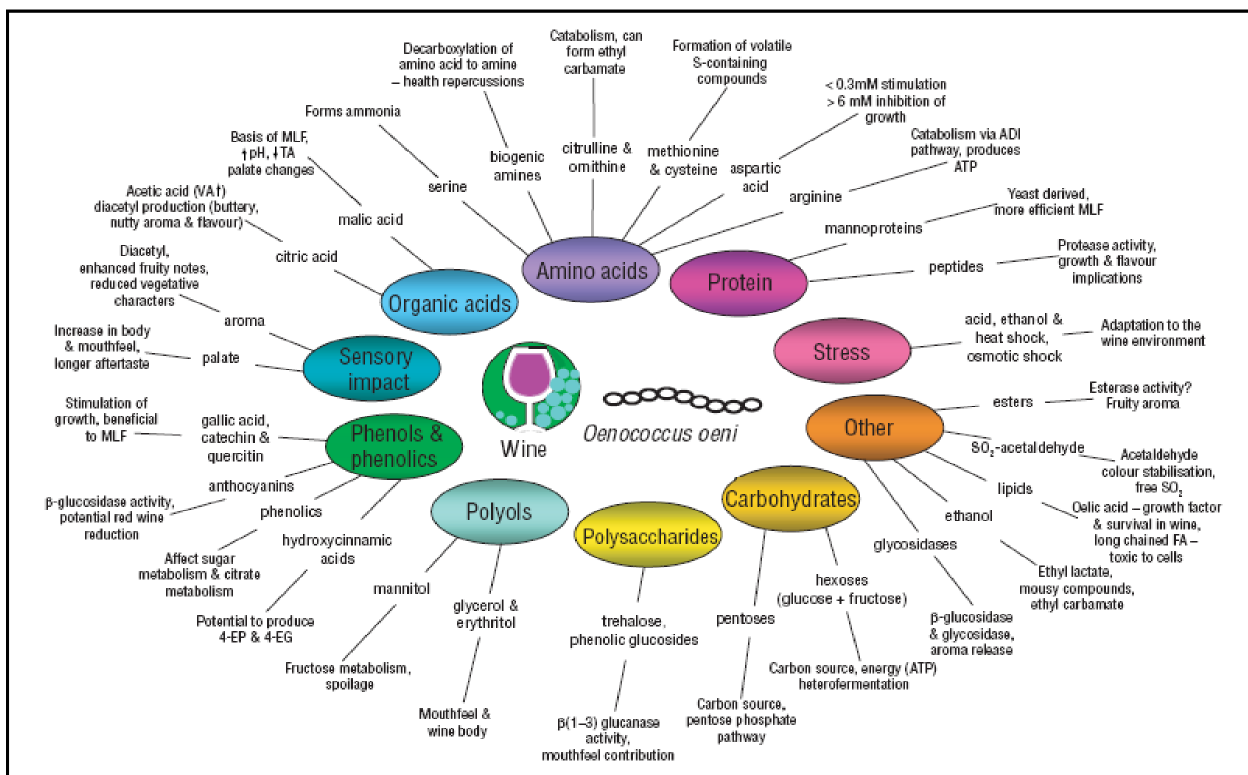


Figure 2.1. An overall summary of the characterised biochemical changes occurring during malolactic fermentation and *Oenococcus oeni* metabolism (Bartowsky, 2005).

The recognition of the presence of bacteria in wine dates back to the mid-1800s when Louis Pasteur described wine bacteria as the spoilage microorganisms of wine (Pasteur, 1866). It was only in the mid 1960s that Garvie (1967) isolated and named *Leuconostoc oenos* as the main bacterial species involved in MLF. However, this bacterium was later reclassified as *Oenococcus oeni* based on the 16S rRNA sequence analysis (Dicks *et al*., 1995). Although there are also a number of other LAB species known to be associated with wine during MLF, *O. oeni* remains main preferred candidate to perform MLF due to its adaptation to tolerate harsh conditions prevalent in wine. Unlike the major spoilage microorganisms of lactobacilli and pediococci, *O. oeni* is also less likely to induce wine spoilage through the production of off-flavours (Lerm *et al*., 2010) apart from the possibility of forming acetate from the degradation of pentose sugars or citrate via the pentose phosphate pathway. Recently, *Lactobacillus plantarum* has again been considered as a commercial candidate for MLF particularly in wines with higher pH (Du Toit *et al*., 2010).

2.2 THE '-OMICS' TECHNOLOGY APPROACHES

The completion of the Human Genome Sequencing Project ushered in a new era of systems biology known as '-omics' technology. During the past few years, there has been a tremendous resurgence of '-omics' technology platforms, including genomics, transcriptomics, proteomics and metabolomics (Fiehn, 2001; Lin & Qian, 2007; Tang *et al*., 2009). These '-omics' technologies, as shown in Figure 2.2, have been applied extensively in various areas of microbial research studies to characterise the complexity of biological systems.

Unlike traditional methods, '-omics' technologies are holistic, data-driven and high-throughput, and they also employ a top-down approach. Another common feature of these high-throughput '-omics' technologies is that they generate large amounts of data and the analysis of these data requires the development in the fields of computational biology and statistics (Zhang *et al*., 2010). Computational biology, or simply bioinformatics, is an integrative and evolving discipline which is used to predict computationally systems of higher complexity, such as the interaction networks in cellular processes and the phenotypes of whole organisms (Bayat, 2002). It has also become clear that any single '-omics' technology may be insufficient in characterising the complexity of biological systems (Gygi *et al*., 1999). The application of integrated multi'-omics' approaches may therefore be a key to decipher complex biological systems (Zhang *et al*., 2010). The sections that follow describe several '-omics' approaches that have been used for LAB species associated with wine.

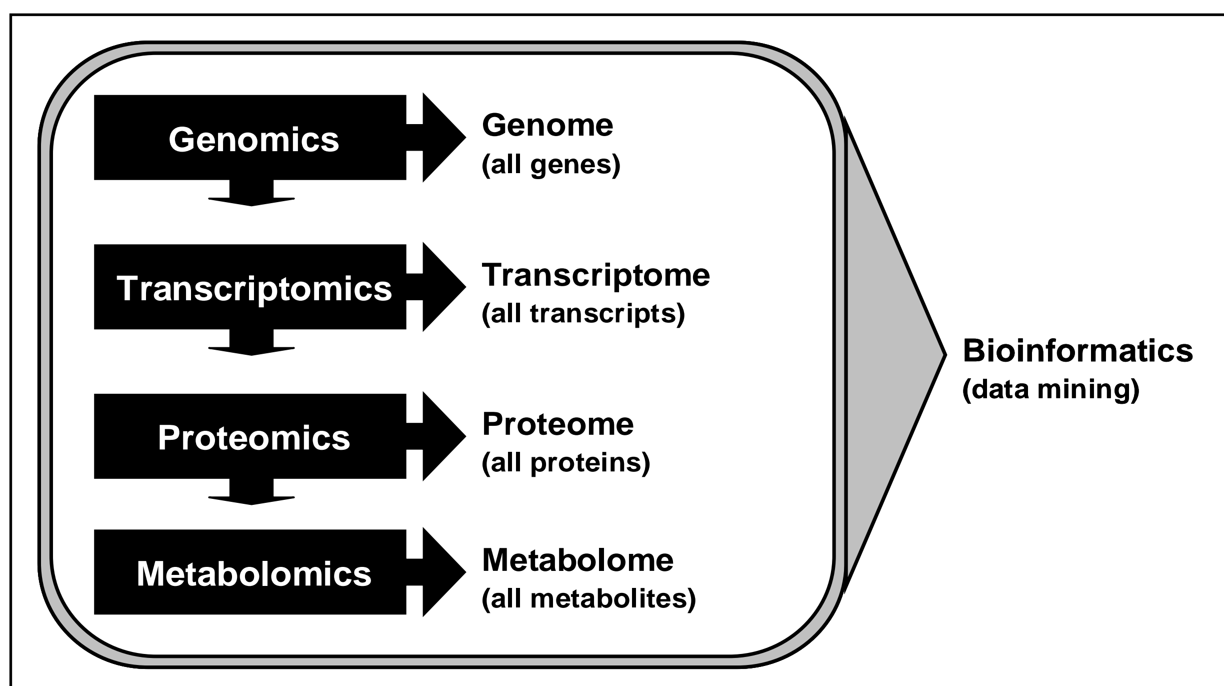


Figure 2.2. A diagram showing various '-omics' technologies (i.e. genomics, transcriptomics, proteomics and metabolomics). Genomics deals with characterising the full complement of genes of an organism. Transcriptomics or genome-wide expression profiling is concerned with the global analysis of gene expression. Proteomics deals with examining the complete set of proteins in a cell. Metabolomics attempts to identify and characterise the metabolome (i.e. the collection of metabolites or all metabolic compounds present in the cell of an organism). Bioinformatics provides computational tools for integrating and analysing huge datasets obtained from '-omics' experimental work.

2.2.1 Genomics

The term genome refers to the complete set of genes inside the cell of any particular organism. Historically, the origin of the word 'genome' is attributed to a German botanist Hans Winkler who coined this term in the 1920s by amalgamating 'gene' and the syllable '-ome' (Petsko, 2002). According to one etymological analysis, the suffix 'ome' is derived from the Sanskrit OM, which means "completeness and fullness" (Lederberg & McCray, 2001). Genomics was later introduced by Victor McKusick and Frank Ruddle three decades ago as the title for the new journal they co-founded in 1987, which focused on linear gene mapping, DNA sequencing and comparison of genomes from different species (McKusick & Ruddle, 1987).

Generally, the genome is usually the starting point of any system-wide analysis, and any changes resulting from the primary genome sequence are reflected in the phenotype of a particular organism (Rossouw & Bauer, 2009). The advent of high-throughput sequencing technologies has now made it possible that more and more sequences of bacterial genomes are generated and placed in the currently established databases. To date, more than 20 complete LAB genomes have been sequenced, annotated and made publicly available (www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Of these, eleven genome sequences are from LAB species associated with wine (Table 2.1).

In the past, research on genetics of industrially important LAB was elucidated by using single-gene molecular approaches. Although these advances shed more light on the genetic variability between LAB strains and/or species, the availability of genome sequences of LAB species holds a greater potential to improve our current knowledge on the fermentative capabilities of LAB (Zhu *et al*., 2009). From the comparative analyses of bacterial genomes, it has been revealed that the genomes of bacterial strains of the same species are significantly divergent from one another (Bon *et al*., 2009; Borneman *et al*., 2010). According to Tettelin *et al.* (2008), these analyses suggest that a bacteria species can be described by its pan-genome, which refers to a sum of a core genome (conserved genome) containing genes shared by all strains and the dispensable genome composed of genes present in some but not all strains as well as the genes that are unique to each strain (Tettelin *et al*., 2008).

2.2.1.1 Genome features

In 2002, the Lactic Acid Bacteria Genome Consortium partnered with the Joint Genome Institute (US Department of Energy) to undertake the complete genome sequencing of *O. oeni* PSU-1 (GenBank accession no. CP000411) using the Whole Genome Shotgun sequencing approach (Mills *et al*., 2005). This strain is currently employed commercially to carry out MLF in wines (Beelman *et al*., 1980), and it was originally isolated in 1972 from a spontaneous MLF in an experimental wine made in Penn State University (Beelman *et al*., 1977). Another *O. oeni* strain ATCC BAA-1163 (accession no. AAUV00000000) also had its genome sequenced. This strain was isolated by Aline Lonvaud-Funel and co-workers (unpublished data) from Bordeaux wine. Very recently, an Australian strain of *O. oeni* (AWRI B429) was also chosen for full genome sequencing and deposited at DDBJ/EMBL/GenBank databases under the accession number ACSE00000000. Together with ATCC BAA-1163, the AWRI B429 strain was sequenced using the shotgun

sequencing approach. The general genome features of all the sequenced LAB species generally found in musts and wine are presented in Table 2.1.

From the comparative analysis, the genomes of LAB are relatively smaller, with sizes ranging from 1,753 Kbp for *O. oeni* ATCC BAA-1163 (currently identified in draft) to 3,308 Kbp for *L. plantarum* WCFS1. The genome of *Lb. plantarum* WCFS1 strain is the largest genome reported so far. It's G+C content is 44.4%. The WCFS1 genome also encodes the complete pathways for amino acid biosynthesis, with the exception of branched-chain amino acids (Mayo *et al.*, 2008). The genome of *Lactobacillus casei* BL23 is the second largest after WCFS1 and JDM1, with the size of 3,079 Kbp (Cai *et al.*, 2009). On the other hand, the complete genome size of the three sequenced plasmid-free *O. oeni* strains (PSU-1, ATCC BAA-1163 and AWRI B429) ranges from 1,753 – 1,927 Kbp, with a G+C content of 37.9% (Mills *et al.*, 2005; Klaenhammer *et al.*, 2005; Borneman *et al.*, 2010). This genome size is at the lower extreme of the range described for LAB, and this could reflect the huge requirement for growth factors as well as the specific adaptation to the ecologically restricted niche of fermenting grape juice and wine (Zé-zé *et al.*, 1998, 2000; Mills *et al.*, 2005). The number of protein-encoding genes also vary from 1,398 for *O. oeni* ATCC BAA-1163 to 3,015 for *L. casei* BL23, indicating substantial gene loss or gene gain events during evolution (Pfeiler & Klaenhammer, 2007; Zhu *et al.*, 2009). For example, during the divergence of *Lactobacillales* from their ancestor in the bacilli, about 600–1,200 genes were lost, including the genes encoding biosynthetic enzymes. Conversely, this divergence was marked by gene gains in the LAB, which reflected a shift towards a nutrient-rich lifestyle during specific niche adaptations (Pfeiler & Klaenhammer, 2007). It is also believed that some of the genes shaping the genomes are acquired as a result of horizontal gene transfer (Kleerebezem *et al.*, 2003).

Borneman *et al.* (2010) also applied an array-based comparative genome hybridization (aCGH) approach in order to investigate the genomic diversity across ten naturally isolated wine strains of *O. oeni* relative to PSU-1, a commercially available strain whose complete genome was sequenced previously (Mills *et al.*, 2005). Among the strains tested, AWRI B429 was chosen for genome sequencing. Based on aCGH analysis, AWRI B429 displayed large deletions spanning the PSU-1 genome and it possessed a high degree of overall heterogeneity throughout other areas of the genome (Borneman *et al.*, 2010).

Table 2.1. General genome features of wine-associated lactic acid bacteria [a]

General features	LBREV [b]	LCAS1	LCAS2	LPLJ	LPLW	LSAK	LMES	AWRI	OE-BAA	PSU-1	PPENT
Sequencing status	Finished	Finished	Finished	Finished	Finished	Finished	Finished	Draft	Draft	Finished	Finished
Genome size (Kbp)	2,291	2,895	3,079	3,197	3,308	1,884	2,038	1,927	1,753	1,780	1,832
G+C content (mol%)	46.2%	46.6%	46.3%	44%	44.5%	41.3%	37.7%	37.9%	37.9%	37.9%	37.4%
% Coding	84%	82%	84%	83%	83%	86%	88%	88%	71%	82%	87%
Genes	2,314	2,906	3,090	3,029	3,135	1,963	2,073	2,161	1,678	1,864	1,847
Protein coding	2,185	2,748	3,015	2,948	3,007	1,879	1,970	2,161	1,398 [c]	1,691	1,755
Structural RNAs	82	76	75	78	86	84	85	None	3	51	72
Pseudogenes	49	82	None	3	42	None	19	None	277	122	20
Contigs	None	None	None	None	11	None	None	58	62	None	None

[a] Data were collected from the NCBI microbial genome database (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

[b] Abbreviations: **LBREV**, *Lactobacillus brevis* ATCC 367 (CP000416); **LCAS1**, *Lactobacillus casei* ATCC 334 (CP000423); **LCAS2**, *Lactobacillus casei* BL23 (FM177140); **LPLJ**, *Lactobacillus plantarum* JDM1 (CP001617); **LPLW**, *Lactobacillus plantarum* WCFS1 (AL935263); **LSAK**, *Lactobacillus sakei* 23K (CR936503); **LMES**, *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 (CP000414); **AWRI**, *Oenococcus oeni* AWRI B429 (ACSE00000000); **OE-BAA**, *Oenococcus oeni* ATCC BAA-1163 (AAUV00000000); **PSU-1**, *Oenococcus oeni* PSU-1 (CP000411); **PPENT**, *Pediococcus pentosaceus* ATCC 25745 (CP000422).

[c] The genome size of *O. oeni* ATCC BAA-1163 strain is probably underestimated because of the draft status of the genome.

Taken together, genomic sequencing and aCGH analysis were able to display significant genomic variation across *O. oeni* strains (Borneman *et al*., 2010). By aligning the complete genome of PSU-1 (CP000411) with the genomes of ATCC BAA-1163 (AAUV00000000) and AWRI B429 (ACSE00000000), the latter two strains displayed the highest degree of single nucleotide polymorphism divergence relative to PSU-1. In addition, the AWRI B429 strain was predicted to encode approximately 400 open reading frames that were not present in the PSU-1 genome, and of these, two corresponded to two glycosyl hydrolyases specific to AWRI B429 (Borneman *et al*., 2010).

In principle, the aCGH technique was first reported by Kallionemi *et al*. (1992), and has now become widely used as a key method for genome-wide analysis of DNA sequence copy number in a single experiment (Jares, 2006). This technique also offers a quick determination of genome plasticity and genome content of a bacterial strain whose genome sequence is unknown (Mayo *et al*., 2008), and is considered as an alternative to complete genome sequencing of closely related species or strains of the same species (Molenaar *et al*., 2005).

2.2.1.2 Genetics of select metabolic pathways of LAB

The genome of *O. oeni* has been shown to encode a variety of enzymes related to aroma and flavour modification in wine. The genetic locus involved in the MLF capacity (*mle*) has been identified in *O. oeni* and other LAB. Genes for the malolactic enzyme (*mleA*), malate permease (*mleP*) and regulatory protein (*mleR*) were previously cloned and sequenced (Labarre *et al*., 1996a, b). As shown in Figure 2.3, these genes are present in a cluster, with the *mleA* and *mleP* genes transcribed in the same operon, and a regulatory protein (*mleR*) transcribed in the opposite direction. The regulatory protein MleR belongs to the LysR-type regulatory protein family found upstream of the *mleA* gene (Schell, 1993). However, the role of the regulatory protein MleR remains unclear as Labarre *et al*. (1996a) could not detect the induction or repression of the malolactic enzyme by L-malate. According to Mills *et al*. (2005), the genetic structure of the *mle* locus is conserved across wine-related LAB species. An *in silico* analysis of *L. plantarum* WCFS1 and *L. plantarum* JDM1 genomes indicates that the two strains also possess the *mle* locus (*mleR*, *mleS* and *mleP*). In *L. plantarum* and *Lactococcus lactis*, the gene encoding the malate dehydrogenase is named *mleS*.

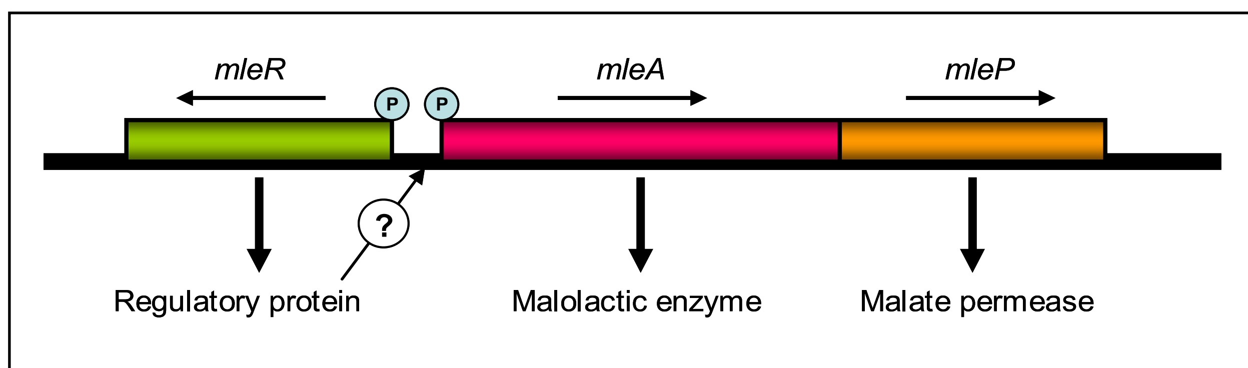


Figure 2.3. Genetic organisation of the *mle* locus of *O. oeni*. The *mleA* and *mleP* genes encoding, respectively, the malolactic enzyme and the malate permease are transcribed on the same operon. Upstream of the operon is another gene encoding an MleR-like protein; this gene is transcribed in the opposite direction. P, promoters of *mleR* and *mle* operon (Labarre *et al*., 1996a).

The presence of genes related to citrate metabolism by wine-associated LAB has also been demonstrated. In *O. oeni* PSU-1 genome, the genes related to citrate utilisation were identified (Mills *et al*., 2005). These genes are organised in the citrate lyase gene cluster (*citR*, *mae*, *maeP*, *citC*, *citD*, *citE*, *citF*, *citX*, *citG*), as diagrammatically illustrated in Figure 2.4. The *maeP* gene encodes a putative citrate transporter, the enzyme involved in the uptake of citrate from the medium. Once inside the cell, citrate is metabolised by a citrate lyase active complex comprising three subunits: γ-subunit (encoded by *citD*), β-subunit (encoded by *citE*) and α-subunit (encoded by *citF*). CitC catalyses the ATP-dependent acetylation of the phosphoribosyl dephospho-CoA group of citrate lyase (Drieder *et al*., 2004). The function of the *citG* product is not yet known in LAB. In the upstream region of *citCDEFG* operon of *O. oeni* PSU-1 is also an open reading frame encoding an NAD-dependent malic enzyme (*mae*) (Bekal-Si Ali *et al*., 1999).

In addition, there are two other genes involved in diacetyl synthesis, which is linked to the downstream metabolism of citrate by citrate-utilising LAB. These genes, *alsD* and *alsS*, have been cloned in *O. oeni* (Garmyn *et al*., 1996). The *alsD* and *alsS* genes encode α-acetolactate decarboxylase and α-acetolactate synthase, respectively. According to Garmyn *et al.* (1996), the two genes are located on the same operon and are constitutively expressed. As shown in Figure 2.4, *L. plantarum* strain also possesses the citrate lyase gene cluster encoding enzymes involved in citrate metabolism.

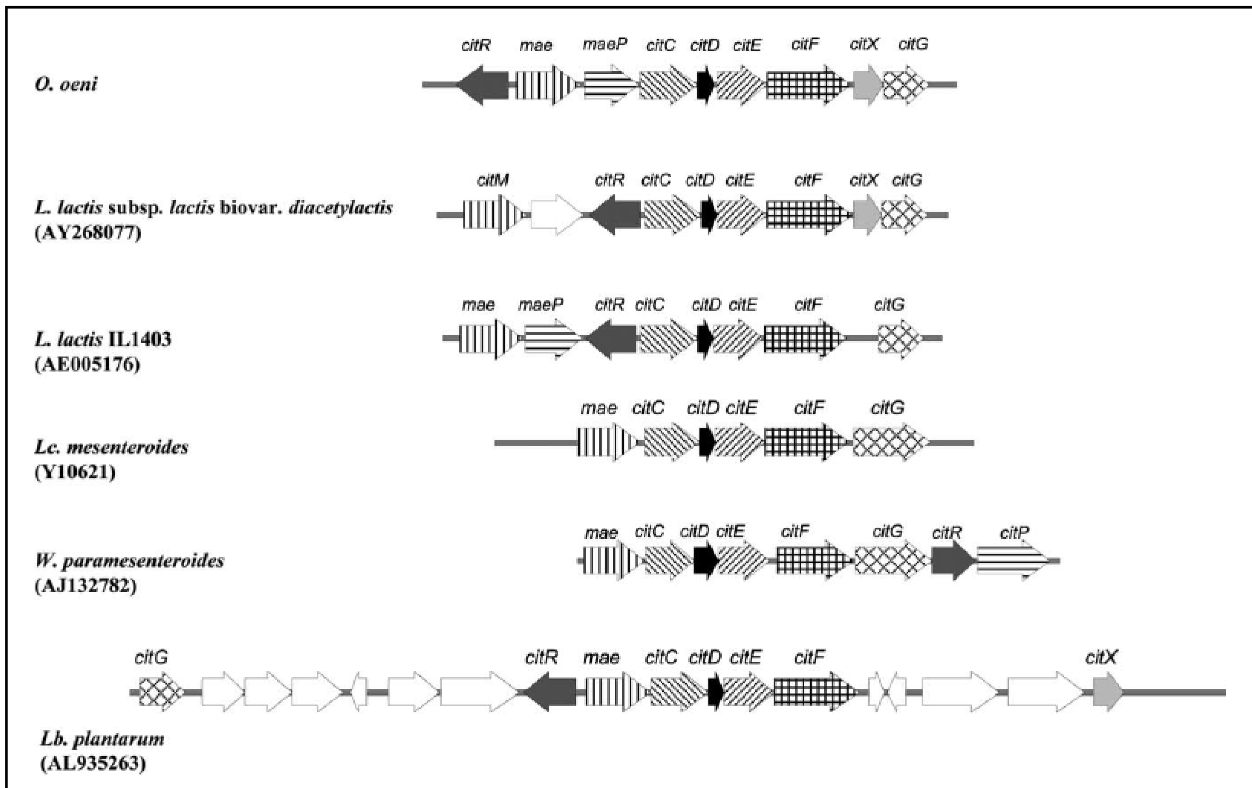


Figure 2.4. Genetic organisation of genes involved in citrate utilisation in various LAB: *Oenococcus oeni* PSU-1 (CP000411), *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* (AY268077), *Lactococcus lactis* IL1403 (AE005176), *Leuconostoc mesenteroides* (Y10621), *Weissella paramesenteroides* (AJ132782) and *Lactobacillus plantarum* WCFS1 (AL935263) (Mills *et al*., 2005).

Quite recently, Michlmayr *et al*. (2010) detected the presence of β-glucosidase-related glycosidase gene in the genome of *O. oeni* ATCC BAA-1163. In addition, the genome sequence of the recently sequenced strain of *O. oeni* AWRI B429 was also found to possess two additional genes encoding glycosyl hydrolyases but not PSU-1 (Borneman *et al*., 2010). According to Sarry & Gunata (2004), the presence of these enzymes might play an important role in the release of desirable aroma components in wine. Previously, Spano *et al*. (2005) also isolated β-glucosidase gene from *O. oeni* and *L. plantarum* strains. This gene was shown to be conserved across LAB species of three different genera (i.e. *Lactobacillus*, *Oenococcus* and *Pediococcus*).

In another study, De las Rivas *et al*. (2009) developed a PCR assay for the detection of LAB with potential to produce volatile phenols. The primers targeting a *pdc* gene encoding phenolic acid decarboxylase were tested against wine-associated LAB strains of six different species. From the PCR detection results, the strains belonging to *L. plantarum*, *Lactobacillus brevis* and *Pediococcus pentosaceus* possessed the *pdc* gene, while those of *O. oeni*, *Lactobacillus hilgardii* and *Leuconostoc mesenteroides* tested negative. De las Rivas *et al*. (2009) also found a correlation between the presence of the *pdc* gene and volatile phenol production, suggesting that the presence of *pdc* gene in wine-associated LAB strains can be used as the preliminary tool for identifying LAB strains with potential to produce volatile phenols.

The presence of genes encoding enzymes involved in arginine metabolism has also been reported. Arginine can be metabolised by wine LAB via the arginine deiminase (ADI) pathway (Liu *et al*., 1996). The ADI pathway comprises three reactions catalysed by arginine deiminase (ADI, encoded by *arcA*), ornithine transcarbamylase (OTC, encoded by *arcB*) and carbamate kinase (CK, encoded by *arcC*). The genes coding for ADI pathway have been identified and characterized in different LAB (Tonon *et al*., 2001; Dong *et al*., 2002; Araque *et al*., 2009). These genes are clustered in an operon-like structure: *arcA*, *arcB* and *arcC* (Tonon *et al*., 2001). A fourth gene encoding a membrane transport protein was also identified (Divol *et al*., 2003). Recently, an additional gene encoding a putative arginyl-tRNA synthetase (*argS2*) was identified in the ADI locus of *O. oeni* (Nehmé *et al*., 2006).

2.2.2 Transcriptomics

Unlike genome sequencing and comparative genomic approaches that focus on DNA (Zhang *et al*., 2010), transcriptomics or global analysis of gene expression (also called genome-wide expression profiling) deals with understanding the regulation of genes under certain environmental conditions. Basically, transcriptomics is the study of the transcriptome, or the complete set of RNA transcripts (mRNA, rRNA, tRNA and other non-coding RNA) produced by the genome at any particular time. The transcriptome can vary with external environmental conditions and reflects the genes that are being actively expressed at any given time, with the exception of mRNA degradation phenomena such as transcriptional attenuation (Ye *et al*., 2001; Horak & Snyder, 2002).

DNA microarrays (also known as DNA chips or gene chips) are an extremely powerful platform in transcriptomics that enable genome-wide analysis of mRNA transcript expression and regulation (Schena *et al*., 1998; Richmond *et al*., 1999). These arrays are constructed by hybridizing hundreds or thousands of single-stranded nucleic acid fragments to a second complementary single strand to generate a double-stranded DNA molecule (Jares, 2006). DNA microarray-based technology was first introduced by Schena *et al*. (1995) who quantitatively monitored gene expression patterns using *Arabidopsis thaliana* as a model organism. As such, the large-scale genome sequencing effort has now made it possible to apply DNA microarrays on which all the genes of an organism are represented, enabling simultaneous assessment of the expression of all these genes (Ramsay, 1998).

Microarray-based technology has been successfully used to examine whole-genome expression profiles of bacteria grown under various environmental conditions (Park *et al*., 2005). By applying DNA microarrays, Molenaar *et al*. (2005) compared the genomic contents of 20 *L. plantarum* strains of various origins relative to *L. plantarum* WCFS1. An array-based genotyping analysis indicated that the gene categories with the most genes conserved in all strains were those involved in biosynthesis or degradation of structural compounds such as proteins, lipids and DNA. On the other hand, the genes involved in sugar transport and catabolism were highly variable between the strains, including regions encoding plantaricin biosynthesis, non-ribosomal peptide biosynthesis and exopolysaccharide biosynthesis. This study therefore showed that there is a high degree of genetic variability amongst *L. plantarum* strains relative to the WCFS1 strain (Molenaar *et al*., 2005).

Recently, Nam *et al*. (2009) described a new array-based technological approach that uses environmental mRNAs (metatranscriptomes) in order to investigate microbial dynamics during kimchi fermentation. This approach involves cDNA synthesis via the selective extraction of metatranscriptomes or via the subtraction of 16S rRNAs and 23S rRNAs to accurately monitor microbial activity and assess real microbial diversity. From the metatranscriptomic analysis, this approach was able to reveal a more divergent

microbial population and more accurately portrayed these changes in microbial activity (Nam *et al.*, 2009).

In conjunction with DNA microarrays, transcription profiling results from high-throughput profiling analysis must usually be verified by quantitative PCR methods. In such cases, quantitative real-time PCR is a reliable method which can be used to provide a quantitative analysis of gene expression. Gene-specific primer design is a key step in obtaining reliable results from quantitative PCR study (Chen *et al.*, 2005; De Vos *et al.*, 2004). A couple of studies on targeted expression analysis of genes encoding enzymes of biotechnological relevance in *O. oeni* strains have been reported elsewhere. By applying reverse transcription real-time quantitative PCR (RT-qPCR), Augagneur *et al.* (2007) investigated the dual effect of organic acids (L-malic and citric acids) on the transcription of three genes (*maeP*, *yaeP* and *mleP*) encoding organic acid transporters in *O. oeni*; these genes were tested under various pH conditions, including low pH that is more relevant to winemaking. From the expression profile analysis, it was observed that there was a substantial increase in the abundance of mRNA encoding MleP protein derived from cells incubated with L-malic acid at various pH levels. In addition, the relative *yaeP* transcript level increased at higher pH and in the presence of citrate. On the other hand, the expression level of the *maeP* gene was not induced by citrate or pH.

Recently, Olguín *et al.* (2009) investigated the expression of genes related to citrate/pyruvate metabolism by *O. oeni* under the effect of different wine stress factors, such as the presence of ethanol and low pH. The expression analysis of these genes was performed by means of RT-qPCR. Regarding citrate uptake and utilisation, the transcription results indicated that the citrate pathway genes were over-expressed in the presence of ethanol than when ethanol was absent, and that the expression of these genes was little affected by pH. These genes included *maeP* (encoding the putative citrate transporter), *citI* (encoding the *cit* operon activator) and *citE* (encoding citrate lyase β-subunit). While the expression of the *alsS* gene varied occasionally, the *alsD* gene expression was increased during MLF. The latter two genes code for α-acetolactate decarboxylase and α-acetolactate synthase enzymes in diacetyl/acetoin pathway, respectively (Olguín *et al.*, 2009).

Several other studies have also sought to elucidate the response mechanisms by which *O. oeni* adapts to the stressful wine environment during fermentation. When inoculated into wine, *O. oeni* becomes exposed to many environmental stresses such as those derived from low pH, high ethanol content, nutrient depletion, etc. In order for it to survive these harsh conditions, *O. oeni* needs to develop certain adaptive mechanisms. The synthesis of a variety of stress response proteins, such as heat-shock proteins, chaperons and ATP-dependent proteases, is one of the mechanisms involved in the adaptation of *O. oeni* to stress. Studies on the expression of genes encoding stress response proteins have been reported by several authors (Coucheney *et al*., 2005; Desroche *et al*., 2005; Capozzi *et al*., 2010; Olguín *et al*., 2010). In a previous study, Coucheney *et al*. (2005) observed a good correlation between the expression level of the *hsp18* gene encoding the small heat-shock protein (Lo18) of *O. oeni* and its ability to grow and perform MLF. These results are in agreement with the findings of other authors (Capozzi *et al*., 2010; Olguín *et al*., 2010) who recently evaluated the expression level of the *hsp18* gene of the selected *O. oeni* strains using RT-qPCR approach. The latter authors observed the highest expression level of several stress response genes in *O. oeni* strains showing the best MLF performances. The highest expression of *hsp18* gene suggests that this gene can be used as a tool to evaluate the ability of *O. oeni* strains to survive in wine after direct inoculation and to perform MLF (Capozzi *et al*., 2010). There are other stress response genes that have also been investigated, and whose expression patterns were analyzed quantitatively. These genes include *groES*, *grpE*, *ctsR*, *clpL*, *clpP*, *clpX*, *rmlB*, *trxA*, etc. (Desroche *et al*., 2005; Olguín *et al*., 2010).

2.2.3 Proteomics

While genomics deals with the genetic make-up of a particular organism, proteomics is aimed at examining the global protein expression in cells (Park *et al*., 2005) or outside the cell (exoproteome). The term proteomics refers to the study of the complete set of proteins in a cell, and it includes the identification of proteins and description of their function (Wilkins *et al*., 1996). In order to fully characterize a proteome (i.e. a complete set of proteins), it is required that high-throughput proteomics methodologies be developed. A standard procedure is two-dimensional gel electrophoresis (2D-PAGE), which is employed for separating proteins based on their isoelectric point and molecular mass. Following 2D-

PAGE strategy is mass spectrometry (MS) for the identification and analysis of separated proteins (Gygi & Aebersold, 2000; Nie *et al*., 2008).

Although 2D-PAGE has widely been used as a tool for proteome analysis, this approach is generally marred by some limitations. One of the limitations is that 2D-PAGE can only allow the detection of a few hundred proteins after their separation, thus covering only "low-complexity" proteomes (Zhang *et al*., 2010). In addition, this approach is limited by the difficulty of purifying proteins from 2D gels, as well as the lack of reproducibility derived from inherently limited resolution of 2D-PAGE (Choe & Lee, 2003).

In overcoming these limitations, there have been improvements in the development of proteomics methodologies, such as isotope-coded affinity tags (ICAT), isobaric tag for relative and absolute quantification (iTRAQ) (Yan et al., 2008) or label-free comparative quantitative proteomics (Haqqani *et al*., 2008). These quantitative technologies allow for an accurate proteome measurement (Zhang *et al*., 2010), thus making proteomic approach a powerful tool in exploring cellular metabolism (Park *et al*., 2005).

Applying the proteomic approach, Silveira *et al*. (2004) examined the proteome of *O. oeni*, focusing mainly on the site-specific location of proteins involved in ethanol adaptation, including cytoplasmic, membrane-associated and integral membrane proteins. From the proteomic analysis, it was shown that the presence of ethanol triggered alterations in protein patterns of *O. oeni* cells. Among the total number (n = 28) of proteins analysed, 50% were identified as proteins with assigned function involved in a variety of cellular processes. The findings from this study provided evidence for an active ethanol adaptation response of *O. oeni* at the cytoplasmic and membrane protein levels (Silveira *et al*., 2004). Apart from examining the ethanol stress factor, Zapparoli (2004) also evaluated the stress resistance of *O. oeni* cells undergoing starvation during the stationary growth phase. The total protein analysis using 2D-PAGE highlighted differential protein expression patterns in cell cultures aged differentially, and the changes in expression profiles were associated with stress resistance during starvation conditions (Zapparoli, 2004).

In another study, a proteomic approach was used to study biogenic amine production and protein biosynthesis by two *Lactobacillus* strains with known potential to produce

histamine, putrescine and cadaverine (Pessione *et al*., 2005). The two strains, *Lactobacillus* sp. 30a (ATCC 33222) originating from the horse gastrointestinal tract and a *Lactobacillus* sp. strain (w53) isolated from amine-contaminated wine, carried genetic determinants for histidine decarboxylase (HDC) and ornithine decarboxylase (ODC). Proteomic analyses revealed the close dependence of HDC and ODC biosynthesis on the presence of high content of free amino acids in the growth medium (Pessione *et al*., 2005). This proteomic study was the first to report on the proteomic analysis of amine-producing bacteria and it holds promise for evaluating the potential of other wine-associated LAB to spoil wines by producing unwanted compounds such as biogenic amines.

In examining the physiological response of LAB to the presence of tannins in the growth media, the effect of tannic acid on wine *L. hilgardii* strain was examined by a combination of physiologic and proteomic approaches (Bossi *et al*., 2007). From this work, it was demonstrated that there was a decrease in the intensity of proteins on 2D maps, suggesting the possible role of tannins in the inhibition of the bacterial survival and growth in a wine environment (Bossi *et al*., 2007). Following this study, the effect of tannic acid was also evaluated in a *L. plantarum* wine strain using proteomic analysis of starved cells grown in the presence of tannic acid or glucose as a carbon source (Cecconi *et al*., 2009a). In conjunction with the physiological characterisation, a comparative 2D-PAGE analysis of total proteins extracted from the bacterial cells was performed. The results indicated that the growth rates of *L. plantarum* grown in the presence of tannic acid were reduced, but it was observed that during the stationary phase cells maintained a higher viability than glucose-grown cells. Additionally, the proteomic data suggested that tannic acid does not have global effect on protein expression, but rather alters the expression of a relatively low number of proteins involved in important cellular and metabolic pathways, such as glycolysis, amino acid metabolism, translation and protein folding (Cecconi *et al*, 2009a).

Recently, Cecconi *et al*. (2009b) applied a comparative proteomic approach to assess the response of *O. oeni* during the pre-inoculation phase. Using the freeze-dried culture of a commercial strain of *O. oeni* (Lalvin VP41), comparisons were made between acclimated and non-acclimated cells to examine the importance of cell acclimation prior to inoculation into wine. The results revealed the different physiological status between acclimated and

non-acclimated cells, suggesting their different behaviour in wine (Cecconi *et al.*, 2009b). Finally, Folio *et al.* (2008) identified a novel extracellular protein of *O. oeni* ATCC BAA-1163 strain with protease activity, which was named EprA. The proteomic analysis of this bacterial strain, which was cultured in two media with different nitrogen concentrations, indicated that the protein profiles shared similarities between the two nitrogen environments.

2.2.4 Metabolomics

In analogy to genomics, transcriptomics and proteomics, metabolomics is one of the newest 'omics' disciplines to be developed with the aim of understanding the global systems biology (Rochfort, 2005). This '-omics' approach deals with the identification and characterisation of the metabolome, herein referred to as the collection of all metabolic compounds (metabolites) present in the cell of an organism under a given set of conditions. These small molecules can include peptides, amino acids, nucleic acids, carbohydrates, organic acids, polyphenols, esters, or any other chemical component that can be used or synthesised by microbial cells (Wishart, 2008).

There are a number of analytical platforms that are currently employed for identifying and quantifying cellular metabolic products. These include nuclear magnetic resonance (NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS), capillary electrophoresis-mass spectrometry (CE-MS), liquid chromatography-mass spectrometry (LC-MS), high-pressure liquid chromatography (HPLC), direct-injection mass spectrometry (DIMS) and Fourier transform infrared (FT-IR) spectroscopy. As such, an ideal analytical tool for analyzing extracted metabolites should be high-throughput, sensitive, robust, reproducible, have a wide dynamic range, and unbiased towards particular classes of metabolites (Singh, 2006; Lenz & Wilson, 2007). However, a major drawback associated with these technologies is that each analysis is targeted to a specific class of metabolites or is only capable of detecting a small number of metabolites. For a true non-targeted approach, one has to run several of these methods in order to be able to cover a wide range of metabolites (Mendes, 2002). The in-depth descriptions of these analytical tools are given in two recent reviews by Dunn & Ellis (2005) and Lenz & Wilson (2007).

Until recently, metabolomics has been extensively applied to diagnostics, drug research and nutrition (Wishart, 2008). However, only a few studies on metabolic profiling have been undertaken in other research disciplines such as wine fermentation. As such, wine is also composed of a multitude of chemical components, most of which are produced as secondary metabolic end-products of fermentation by wine microbiota. Previously, NMR-based metabolomic approach was applied in wine yeast strains to monitor wine fermentation and evaluate the fermentative traits of yeast strains (Son *et al*., 2009a, b).

On the other hand, metabolomic profiling of wine LAB is also documented. The use of various analytical tools has enabled researchers to study variations on the volatile aroma and flavour composition of wines resulting from MLF (Laurent *et al*., 1994; De Revel *et al*., 1999; Maicas *et al*., 1999; Lloret *et al*., 2002; Fernandes *et al*., 2003; Ugliano & Moio, 2005; Boido *et al*., 2002, 2009). Through the results obtained, the latter authors noted differences in the concentration of volatile chemical components in wines having undergone MLF, and this was also confirmed by sensorial differences between wines. Differences in metabolic compound profiles of wines produced as a consequence of MLF were also shown to be strain-dependent (Lee *et al*., 2009a, b).

Recently, Boido *et al*. (2009) studied the influence of two *O. oeni* strains on the volatile compounds in Tannat wines as well as the effect of wine ageing in bottle on the aroma compounds produced during MLF. From the metabolite profile analysis, modifications in the concentration of acetates, ethyl esters and other secondary metabolites during MLF were observed; some of these compounds impart fruity aromas in wine. In addition, a decrease in the concentration of some acetates and ethyl esters in wine after bottle ageing was observed, and these changes could have implications on the changes in fruity aromas (Boido *et al*., 2009).

In another study, the influence of five commercial *O. oeni* strains on fermentative behaviours and variations of metabolites during MLF was investigated (Lee *et al*., 2009a). MLF behaviours and metabolic variations were evaluated through a combination of ^{1}H NMR- and GC-MS-based metabolomic profiling in conjunction with multivariate statistical analysis. The results revealed different malolactic behaviours, thus contributing to variations in the secondary metabolites rather than the primary metabolites.

Additionally, there were also differences observed between wines produced with different strains (Lee *et al.*, 2009a). In a follow-up study, Lee *et al.* (2009b) compared the fermentative behaviour and metabolic effects of a wine *L. plantarum* strain with those of a commercial *O. oeni* strain through ^{1}H NMR- and GC-based metabolic profiling. From the findings, it was observed that higher levels of primary metabolites were more pronounced in wines fermented by *L. plantarum* compared with those by *O. oeni*. The results obtained from this study suggested that different genera of LAB have an effect on both the primary and secondary metabolites in wine.

Although major advances have been made with respect to metabolomic profiling, the technologies used are less mature and most of the studies done thus far are not sufficiently comprehensive. In addition, the measurement accuracy of metabolites needs further improvements (Fiehn, 2001; Kell, 2004). Given the wide dynamic and chemical range of low molecular weight compounds in a biological sample, it has not yet been possible to perform a global metabolome analysis within a single analytical platform (Singh, 2006). However, the studies conducted until this far holds a greater promise that metabolomics could be a powerful tool in deciphering microbial metabolism (Cascante & Marin, 2008). Furthermore, there are a number of advantages that the metabolomics discipline display: it provides insights into metabolic profiles, it provides means for validating *in silico* pathways constructed based on available genome sequences, and it provides valuable information about how proteins function to produce energy and materials in the cell given that metabolites are downstream of all genome and proteome regulatory structures (Phelps *et al.*, 2002; Park *et al.*, 2005; Oldiges *et al.*, 2007).

2.2.5 Bioinformatics

The term *bioinformatics* was coined in the late 1970s by Paulien Hogeweg and Ben Hesper to refer to the study of informatic processes in biotic systems (Hogeweg, 1978; Hogeweg & Hesper, 1978). The terms *bioinformatics* and *computational biology* are often used interchangeably. According to the definition by the National Institutes of Health in Bethesda, *bioinformatics* refers to "research, development, or application of computational tools and approaches for expanding the use of biological, medical, behavioural or health data, including those to acquire, store, organise, archive, analyse, or visualise such data." *Computational biology*, on the other hand, involves "the development and application of

data-analytical and theoretical methods, mathematical modeling and computational simulation techniques to the study of biological, behavioural, and social systems."

The advent of high-throughput '-omics' technologies became the major impetus towards the development of bioinformatics as a discipline which enables the integration and analysis of the enormous quantities of datasets generated by different '-omics' technology platforms, such as genome sequencing, transcriptomics, proteomics and metabolomics. The field of bioinformatics relies heavily on the use of Internet as a place to access sequence data, to access softwares that are useful to analyse molecular data, and as a place to integrate different kinds of resources and information relevant to biology (Pevsner, 2003). There are multitudes of relevant online bioinformatics resources which enable genome data visualisation and also to carry out comparative genomic analysis. Siezen *et al*. (2004) listed some of the useful websites that are relevant to bioinformatics.

The most commonly used databases containing genome and protein sequences, obtained mostly from individual laboratories and large-scale sequencing facilities, are GenBank of the National Center for Biotechnology Information (NCBI), the European Molecular Biology Laboratory database (EMBL) of the European Bioinformatics Institute (EBI), and the DNA Database of Japan (DDBJ). These databases are centralised and publicly accessible. They are also regarded as the most comprehensive databases for nucleotide sequences and supporting biological annotations. All three databases share sequence data on a daily basis (Pevsner, 2003; Chen *et al*., 2005).

Like other functional genomics technologies, metabolomics also generates enormous quantities of datasets that it would be near-impossible to analyse without the use of currently available bioinformatics tools. A series of pathway databases and their associated software exist on the Internet. These databases catalogue biochemical compounds and their properties, enzymes, reactions, regulatory interactions and pathways (Mendes, 2002). Two of the major pathway databases in the public domain are BioCYC and KEGG (Chen *et al*., 2005). The KEGG (Kyoto Encyclopedia of Genes and Genomes) database, for example, encompasses genome projects, pathway databases, biochemical compounds, and reactions (Kanehisa *et al*., 2004). However, there are also other

established databases available for *in silico* construction of model pathways, and these include ExPASy, EMP, BRENDA and PathDB (Mendes, 2002; Park *et al*., 2005).

2.3 LINKING MULTIPLE '-OMICS' TECHNOLOGIES

As long as the whole genome sequence of the target organism is known, it is now feasible to monitor and examine global gene expression by DNA microarray analysis (Park *et al*., 2005). This was demonstrated recently by Borneman *et al*. (2010) who investigated the diversity in *O. oeni* strains by high-density microarray comparative genome hybridization. However, there has not been a success in finding a significant correlation between protein and mRNA abundances (Gygi *et al*., 1999). According to Park *et al*. (2005), this discrepancy arises from several factors, including protein regulation by post-translational modification, post-transcriptional regulation of protein synthesis, differences in the half-lives of mRNA and proteins, and possible functional requirement for protein binding (Park *et al*., 2005). Amongst various '-omics' platforms, transcriptomics in combination with proteomics remain the most commonly used method, since mRNA levels plus protein abundance and activity data are thought to faithfully represent biological systems (Park *et al*., 2005).

2.4 CONCLUDING REMARKS

An explosion of genome sequence data of various LAB has now enabled the development of various '-omics' technology platforms, such as comparative genomics, transcriptomics, proteomics and metabolomics. However, there is currently no single technique that ought to fulfill all the requirements of an ideal global metabolite profiling tool, and each of the techniques has both advantages and limitations (Lenz & Wilson, 2007). Therefore, the integration of various '-omics' approaches will be an extremely powerful tool in trying to decipher the complexity of biological systems at different levels given that, so far, no study has been able to successfully exploit these integrated approaches.

2.5 LITERATURE CITED

Araque, I., Gil, J., Carreté, R., Bordons, A. & Reguant, C., 2009. Detection of *arc* genes related with the ethyl carbamate precursors in wine lactic acid bacteria. J. Agric. Food Chem. 57, 1841-1847.

Augagneur, Y., Ritt, J.F., Linares, D.M., Remize, F., Tourdot-Maréchal, R., Garmyn & D., Guzzo, J., 2007. Dual effects of organic acids as a function of external pH in *Oenococcus oeni*. Arch. Microbiol. 188, 147-157.

Bartowsky, E.J., 2005. *Oenococcus oeni* and malolactic fermentation – moving into the molecular arena. Aust. J. Grape Wine Res. 11, 174-187.

Bayat, A., 2002. Science, medicine, and the future Bioinformatics. BMJ. 324, 1018-1022.

Beelman, R.B., Gavin III, A., Keen & R.M., 1977. A new strain of *Leuconostoc oenos* for induced malolactic fermentation in eastern wines. Am. J. Enol. Vitic. 28, 159-165.

Beelman, R.B., McArdle, F.J. & Duke, G.R., 1980. Comparison of *Leuconostoc oenos* strains ML-34 and PSU-1 to induce malolactic fermentation in Pennsylvania red table wines. Am. J. Enol. Viticult. 31, 269-276.

Bekal-Si Ali, S., Diviès, C. & Prévost, H., 1999. Genetic organisation of the *citCDEF* locus and identification of *mae* and *clyR* genes from *Leuconostoc mesenteroides*. J. Bacteriol. 181, 4411-4416.

Boido, E., Lloret, A., Medina, K., Carrau, F. & Dellacassa, E., 2002. Effect of β-glycosidase activity of *Oenococcus oeni* on the glycosylated flavour precursors of Tannat wine during malolactic fermentation. J. Agric. Food Chem. 50, 2344-2349.

Boido, E., Medina, K., Farina, L., Carrau, F., Versini, G. & Dellacassa, E., 2009. The effect of bacterial strain and aging on the secondary volatile metabolites produced during malolactic fermentation of Tannat red wine. J. Agric. Food Chem. 57, 6271-6278.

Bon, E., Delaherche, A., Bilhère, E., De Daruvar, A., Lonvaud-Funel, A. & Le Marrec, C., 2009. *Oenococcus oeni* genome plasticity is associated with fitness. Appl. Environ. Microbiol. 75, 2079-2090.

Borneman, A.R., Bartowsky, E.J., McCarthy, J. & Chambers, P.J., 2010. Genotypic diversity in *Oenococcus oeni* by high-density microarray comparative genome hybridization and whole genome sequencing. Appl. Microbiol. Biotechnol. 86, 681-691.

Bossi, A., Rinalducci, S., Zolla, L., Antonioli, P., Righetti, P.G. & Zapparoli, G., 2007. Effect of tannic acid on *Lactobacillus hilgardii* analysed by a proteomic approach. J. Appl. Microbiol. 102, 787-795.

Cai, H., Thompson, R., Budinich, M.F., Broadbent, J.R., Steele, J.L., 2009. Genome sequence and comparative genome analysis of *Lactobacillus casei*: Insights into their niche-associated evolution. Genome Biol. Evol. 1, 239-257.

Capozzi, V., Russo, P., Beneduce, L., Weidmann, S., Grieco, F., Guzzo, J. & Spano, G., 2010. Technological properties of *Oenococcus oeni* strains isolated from typical southern Italian wines. Lett. Appl. Microbiol. 50, 327-334.

Cascante, M. & Marin, S., 2008. Metabolomics and fluxomics approaches. Essays Biochem. 45, 67-82.

Cecconi, D., Cristofoletti, M., Milli, A., Antonioli, P., Rinalducci, S., Zolla, L. & Zapparoli, G., 2009a. Effect of tannic acid on *Lactobacillus plantarum* wine strain during starvation: a proteomic study. Electrophoresis 30, 957-965.

Cecconi, D., Milli, A., Rinalducci, S., Zolla, L. & Zapparoli, G., 2009b. Proteomic analysis of *Oenococcus oeni* freeze-dried culture to assess the importance of cell acclimation to conduct malolactic fermentation in wine. Electrophoresis 30, 2988-2995.

Chen, J., Wu, S. & Davison, D.B., 2005. Applied bioinformatics for drug discovery and development. In: *Handbook of Genome Research. Genomics, Proteomics, Metabolomics, Bioinformatics, Ethical and Legal Issues*. C.W. Sensen (ed.), WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, pp. 353-382.

Choe, L.H. & Lee, K.H., 2003. Quantitative and qualitative measure of intralaboratory two-dimensional protein gel reproducibility and the effects of sample preparation, sample load, and image analysis. Electrophoresis 24, 3500-3507.

Coucheney, F., Desroche, N., Bou, M., Tourdot-Maréchal, R., Dulau, L. & Guzzo, J., 2005. A new approach for selection of *Oenococcus oeni* strains in order to produce malolactic starters. Int. J. Food Microbiol. 105, 463-470.

De las Rivas, B., Rodríguez, H., Curiel, J.A., Landete, J.M. & Muñoz, R., 2009. Molecular screening of wine lactic acid bacteria degrading hydroxycinnamic acids. J. Agric. Food Chem. 57, 490-494.

De Revel, G., Martin, N., Pripis-Nicolau, L., Lonvaud-Funel, A. & Bertrand, A., 1999. Contribution to the knowledge of malolactic fermentation influence on wine aroma. J. Agric. Food Chem. 47, 4003-4008.

De Vos, W.M., Bron, P.A. & Kleerebezem, M., 2004. Post-genomics of lactic acid bacteria and other food-grade bacteria to discover gut functionality. Curr. Opin. Biotechnol. 15, 86-93.

Desroche, N., Beltramo, C. & Guzzo, J., 2005. Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *Oenococcus oeni*. J. Microbiol. Methods 60, 325-333.

Dicks, L.M.T., Dellaglio, F. & Collins, M.D., 1995. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig.] gen. nov., comb. nov. Int. J. Syst. Bacteriol. 45, 395-397.

Divol, B., Tonon, T., Morichon, S., Gindreau, E. & Lonvaud-Funel, A., 2003. Molecular characterization of *Oenococcus oeni* genes encoding proteins involved in arginine transport. J. Appl. Microbiol. 94, 738-746.

Dong, Y., Chen, Y.Y., Snyder, J.A. & Burne, R.A., 2002. Isolation and molecular analysis of the gene cluster for the arginine deiminase system from *Streptococcus gordonii* DL1. Appl. Environ. Microbiol. 68, 5549-5553.

Drieder, D., Bekal, S. & Prévost, H., 2004. Genetic organisation and expression of citrate permease in lactic acid bacteria. Genet. Mol. Res. 3, 273-281.

Du Toit, M., Engelbrecht, L., Lerm, E. & Krieger-Weber, S., 2010. *Lactobacillus*: the next generation of malolactic fermentation starter cultures–an overview. Food Bioprocess Technol. doi: 10.1007/s11947-010-0448-8 (In press).

Dunn, W.B. & Ellis, D.I., 2005. Metabolomics: current analytical platforms and methodologies. Trends Analytical Chem. 24, 285-294.

Fernandes, L., Relva, A.M., Gomes Da Silva, M.D.R. & Costa Freitas, A.M., 2003. Different multidimensional chromatographic approaches applied to the study of wine malolactic fermentation. J. Chromatogr. A 995, 161-169.

Fiehn, O., 2001. Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. Comp. Funct. Genomics 2, 155-168.

Folio, P., Ritt, J.-F., Alexandre, H. & Remize, F., 2008. Characterization of EprA, a major extracellular protein of *Oenococcus oeni* with protease activity. Int. J. Food Microbiol. 127, 26-31.

Garmyn, D., Monnet, C., Martineau, B., Guzzo, J., Cavin, J.-F. & Divies, C., 1996. Cloning and sequencing of the gene encoding α-acetolactate decarboxylase from *Leuconostoc oenos*. FEMS Microbiol. Lett. 145, 445-450.

Garvie, E.I., 1967. *Leuconostoc oenos* sp. nov. J. Gen. Microbiol. 48, 431-438.

Gygi, S.P. & Aebersold, R., 2000. Mass spectrometry and proteomics. Curr. Opin. Chem. Biol. 4, 489-494.

Gygi, S.P., Rochon, Y., Franza, B.R. & Aebersold, R., 1999. Correlation between protein and mRNA abundance in yeast. Mol. Cell Biol. 19, 1720-1730.

Haqqani, A.S., Kelly, J.F. & Stanimirovic, D.B., 2008. Quantitative protein profiling by mass spectrometry using label-free proteomics. Methods Mol. Biol. 439, 241-256.

Hogeweg, P., 1978. Simulating the growth of cellular forms. Simulation 31, 90-96.

Hogeweg, P. & Hesper, B., 1978. Interactive instruction on population interactions. Comput. Biol. Med. 8, 319-27.

Horak, C.E. & Snyder, M., 2002. Global analysis of gene expression in yeast. Funct. Integr. Genomics 2, 171-180.

Jares, P., 2006. DNA Microarray applications in functional genomics. Ultrastruct. Pathol. 30, 209-219.

Kallioniemi, A., Kallioniemi, O.P., Sudar, D., Rutovitz, D., Gray, J.W., Waldman , F. & Pinkel, D., 1992. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258, 818-821.

Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y. & Hattori, M., 2004. The KEGG resource for deciphering the genome. Nucleic Acids Res. 32, D277-D280.

Kell, D.B., 2004. Metabolomics and systems biology: making sense of the soup. Curr. Opin. Microbiol. 7, 296-307.

Klaenhammer, T.R., Barrangou, R., Buck, B.L., Azcaráte-Peril, M.A. & Altermann, E., 2005. Genomic features of lactic acid bacteria effecting bioprocessing and health. FEMS Microbiol. Rev. 29, 393-409.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Lee, R., Tarchini, R., Peters, S.A., Sandbrink, H.M., Fiers, M.W.E.J., Stiekema, W., Lankhorst, R.M.K., Bron, P.A., Hoffer, S.M., Groot, M.N.N., Kerkhoven, R., de Vries, M., Ursing, B., de Vos, W.M. & Siezen, R.J., 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. 100, 1990-1995.

Kunkee, R.E., 1991. Some roles of malic acid in the malolactic fermentation in wine making. FEMS Microbiol. Rev. 88, 55-72.

Labarre, C., Divies, C. & Guzzo, J., 1996a. Genetic organization of the mle locus and identification of a *mleR*-like gene from *Leuconostoc oenos*. Appl. Environ. Microbiol. 62, 4493-4498.

Labarre, C., Guzzo, J., Cavin, J.-F. & Divies, C., 1996b. Cloning and characterization of the genes encoding the malolactic enzyme and the malate permease of *Leuconostoc oenos*. Appl. Environ. Microbiol. 62, 1274-1282.

Laurent, M.-H., Henick-Kling, T. & Acree, T.E., 1994. Changes in the aroma and odor of Chardonnay wine due to malolactic fermentation. Vit. Enol. Sci. 49, 3-10.

Lederberg, J. & McCray, A.T., 2001. 'Ome sweet 'omics: -- A genealogical treasury of words. The Scientist 15, 8.

Lee, J.E., Hong, Y.S. & Lee, C.H., 2009a. Characterization of fermentative behaviors of lactic acid bacteria in grape wines through ^{1}H NMR and GC-based metabolic profiling. J. Agric. Food Chem. 57, 4810-4817.

Lee, J.E., Hwang, G.S., Lee, C.H. & Hong, Y.S., 2009b. Metabolomics reveals alterations in both primary and secondary metabolites by wine bacteria. J. Agric. Food Chem. 57, 10772-10783.

Lenz, E.M. & Wilson, I.D., 2007. Analytical strategies in metabonomics. J. Proteome Res. 6, 443-458.

Lerm, E., Engelbrecht, L. & Du Toit, M., 2010. Malolactic Fermentation: The ABC's of MLF. S. Afr. J. Enol. Vitic. 31, 186-212.

Lin, J. & Qian, J., 2007. Systems biology approach to integrative comparative genomics. Expert Rev. Proteomics 4, 107-119.

Liu, S.-Q., 2002. Malolactic fermentation in wine - beyond deacidification. J. Appl. Microbiol. 92, 589-601.

Liu, S.-Q., Pritchard, G.G., Hardman, M.J. & Pilone, G.J., 1996. Arginine catabolism in wine lactic acid bacteria: is it via the arginine deiminase pathway or the arginase-urease pathway? J. Appl. Bacteriol. 81, 486-492.

Lloret, A., Boido, E., Lorenzo, D., Medina, K., Carrau, F., Dellacassa, E. & Versini, G., 2002. Aroma variation in Tannat wines: effect of malolactic fermentation on ethyl lactate level and its enantiomeric distribution. Ital. J. Food Sci. 14, 175-180.

Maicas, S., Gil, J.V., Pardo, I. & Ferrer, S., 1999. Improvement of volatile composition of wines by controlled addition of malolactic bacteria. Food Res. Int. 32, 491-496.

Mayo, B, van Sinderen, D. & Ventura, M, 2008. Genome analysis of food grade lactic acid-producing bacteria: from basics to applications. Curr. Genomics 9, 169-183.

McKusick, V.A. & Ruddle, F.H., 1987. Toward a complete map of the human genome. Genomics 1, 103-106.

Mendes, P., 2002. Emerging bioinformatics for the metabolome. Brief. Bioinformatics 3, 134-145.

Michlmayr, H., Schümann, C., Wurbs, P., Barreira Braz da Silva, N.M., Rogl, V., Kulbe, K.D. & del Hierro, A.M., 2010. A β-glucosidase from *Oenococcus oeni* ATCC BAA-1163 with potential for

aroma release in wine: cloning and expression in *E. coli*. World J. Microbiol. Biotechnol. 26, 1281-1289.

Mills, D.A., Rawsthorne, H., Parker, C., Tamir, D. & Makarova, K., 2005. Genomic analysis of *Oenococcus oeni* PSU-1 and its relevance to winemaking. FEMS Microbiol. Rev. 29, 465-475.

Molenaar, D., Bringel, F., Schuren, F.H., de Vos, W.M., Siezen, R.J. & Kleerebezem, M., 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. J. Bacteriol. 187, 6119-6127.

Nam, Y.-D., Chang, H.-W., Kim, K.-H., Roh, S.W. & Bae, J.-W., 2009. Metatranscriptome analysis of lactic acid bacteria during kimchi fermentation with genome-probing microarrays. Int. J. Food Microbiol. 130, 140-146.

Nehmé, B., Ganga, M.A. & Lonvaud-Funel, A., 2006. The arginine deiminase locus of *Oenococcus oeni* includes a putative arginyl-tRNA synthetase ArgS2 at its 3′-end. Appl. Microbiol. Biotechnol. 70, 590-597.

Nie, L., Wu, G. & Zhang, W., 2008. Statistical application and challenges in global gel-free proteomic analysis by mass spectrometry. Crit. Rev. Biotechnol. 28, 297-307.

Oldiges, M., Lütz, S., Pflug, S., Schroer, K., Stein, N. & Wiendahl, C., 2007. Metabolomics: current state and evolving methodologies and tools. Appl. Microbiol. Biotechnol. 76, 495-511.

Olguín, N., Bordons, A. & Reguant, C., 2009. Influence of ethanol and pH on the gene expression of the citrate pathway in *Oenococcus oeni*. Food Microbiol. 26, 197-203.

Olguín, N., Bordons, A. & Reguant, C., 2010. Multigenic expression analysis as an approach to understanding the behaviour of *Oenococcus oeni* in wine-like conditions. Int. J. Food Microbiol. 144, 88-95.

Park, S.J., Lee, S.Y., Cho, J., Kim, T.Y., Lee, J.W., Park, J.H. & Han, M.-J., 2005. Global physiological understanding and metabolic engineering of microorganisms based on omics studies. Appl. Microbiol. Biotechnol. 68, 567-579.

Pasteur, L., 1866. Etudes sur le vin. Imprimerie impériale, Paris.

Pessione, E., Mazzoli, R., Giuffrida, M.G., Lamberti, C., Garcia-Moruno, E., Barello, C., Conti, A. & Giunta, C., 2005. A proteomic approach to studying biogenic amine producing lactic acid bacteria. Proteomics 5, 687-698.

Petsko, G.A., 2002. No place like Ome. Genome Biol. 3, 1-2.

Pevsner, J., 2003. Bioinformatics and functional genomics. John Wiley & Sons, Inc., Hoboken, New Jersey. pp. 753.

Pfeiler, E.A. & Klaenhammer, T.R., 2007. The genomics of lactic acid bacteria. Trends Microbiol. 15, 546-553.

Phelps, T.J., Palumbo, A.V. & Beliaev, A.S., 2002. Metabolomics and microarrays for improved understanding of phenotypic characteristics controlled by both genomics and environmental constraints. Curr. Opin. Biotechnol. 13, 20-24.

Ramsay, G., 1998. DNA chips: state-of-the-art. Nat. Biotechnol. 16, 40-53.

Richmond, C.S., Glasner, J.D., Mau, R., Jin, H. & Blattner, F.R., 1999. Genome-wide expression profiling in *Escherichia coli* K-12. Nucleic Acids Res. 27, 3821-3835.

Rochfort, S., 2005. Metabolomics reviewed: a new "omics" platform technology for systems biology and implications for natural products research. J. Nat. Prod. 68, 1813-1820.

Rossouw, D. & Bauer, F.F., 2009. Wine science in the omics era: the impact of systems biology on the future of wine research. S. Afr. J. Enol. Vitic. 30, 101-109.

Sarry, J.E. & Gunata, Z., 2004. Plant and microbial glycoside hydrolases: volatile release from glycosidic aroma precursors. Food Chem. 87, 509-521.

Schell, M.A., 1993. Molecular biology of the LysR family of transcriptional regulators. Annual Rev. Microbiol. 47, 597-626.

Schena, M., Heller, R.A., Theriault, T.P., Konrad, K., Lachenmeier, E. & Davis, R.W., 1998. Microarrays: biotechnology's discovery platform for functional genomics. Trends Biotechnol. 16, 301-306.

Schena, M., Shalon, D., Davis, R.W. & Brown, P.O., 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270, 467-470.

Siezen, R.J., van Enckevort, F.H.J., Kleerebezem, M. & Teusink, B., 2004. Genome data mining of lactic acid bacteria: the impact of bioinformatics. Curr. Opin. Biotechnol. 15, 105-115.

Silveira, M.G., Baumgärtner, M., Rombouts, F.M. & Abee, T., 2004. Effect of adaptation to ethanol on cytoplasmic and membrane protein profiles of *Oenococcus oeni*. Appl. Environ. Microbiol. 70, 2748-2755.

Singh, O.V., 2006. Proteomics and metabolomics: the molecular make-up of toxic aromatic pollutant bioremediation. Protomics 6, 5481-5492.

Son, H.S., Hwang, G.S., Kim, K.M., Kim, E.Y., van den Berg, F., Park, W.M., Lee, C.H. & Hong, Y.S., 2009a. ^{1}H NMR-based metabolomic approach for understanding the fermentation behaviors of wine yeast strains. Anal. Chem. 81, 1137-1145.

Son, H.S., Hwang, G.S., Park, W.M., Hong, Y.S. & Lee, C.H., 2009b. Metabolomic characterization on malolactic fermentation and fermentative behaviors of wine yeasts in grape wine. J. Agric. Food Chem. 57, 4801-4809.

Spano, G., Rinaldi, A., Ugliano, M., Moio, L., Beneduce, L. & Massa, S., 2005. A β-glucosidase gene isolated from wine *Lactobacillus plantarum* is regulated by abiotic stresses. J. Appl. Microbiol. 98, 855-861.

Swiegers, J.H., Bartowsky, E.J., Henschke, P.A. & Pretorius, I.S., 2005. Yeast and bacterial modulation of wine aroma and flavour. Aust. J. Grape Wine Res. 11, 139-173.

Tang, Y.J., Martin, H.G., Myers, S., Rodriguez, S., Baidoo, E.E. & Keasling, J.D., 2009. Advances in analysis of microbial metabolic fluxes via ^{13}C isotopic labeling. Mass Spectrom. Rev. 28, 362-375.

Tettelin, H., Riley, D., Cattuto, C. & Medini, D., 2008. Comparative genomics: the bacterial pan-genome. Curr. Opin. Microbiol. 11, 472-477.

Tonon, T., Bourdineaud, J.-P. & Lonvaud-Funel, A., 2001. The *arcABC* gene cluster encoding the arginine deaminase pathway of *Oenococcus oeni*, and arginine induction of a CRP-like gene. Res. Microbiol. 152, 653-661.

Ugliano, M. & Moio, L., 2005. Changes in the concentration of yeast-derived volatile compounds of red wine during malolactic fermentation with four commercial starter cultures of *Oenococcus oeni*. J. Agric. Food Chem. 53, 10134-10139.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H. & Lee, T.H., 1985. Occurrence and growth of lactic acid bacteria in wine: a review. Am. J. Enol. Vitic. 36, 302-313.

Wilkins, M.R., Pasquali, C., Appel, R.D., Ou, K., Golaz, O., Sanchez, J.C., Yan, J.X., Gooley, A.A., Hughes, G., Humphery-Smith, I., Williams, K.L. & Hochstrasser, D.F., 1996. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. Biotechnol. (NY) 14, 61-65.

Wishart, D.S., 2008. Metabolomics: applications to food science and nutrition research. Trends Food Sci. Technol. 19, 482-493.

Yan, W., Hwang, D. & Aebersold, R., 2008. Quantitative proteomic analysis to profile dynamic changes in the spatial distribution of cellular proteins. Methods Mol. Biol. 432, 389-401.

Ye, R.W., Wang, T., Bedzyk, L. & Croker, K.M., 2001. Applications of DNA microarrays in microbial systems. J. Microbiol. Methods 47, 257-272.

Zapparoli, G., 2004. Colony dimorphism associated with stress resistance in *Oenococcus oeni* VP01 cells during stationary growth phase. FEMS Microbiol. Lett. 239, 261-265.

Zé-Zé, L., Teneiro, R., Brito, L., Santos, M.A. & Paveia, H., 1998. Physical map of the genome of *Oenococcus oeni* PSU-1 and localization of genetic markers. Microbiol. 144, 1145-1156.

Zé-Zé, L., Teneiro, R. & Paveia, H., 2000. The *Oenococcus oeni* genome: physical and genetic map of strain GM and comparison with the genome of a 'divergent' strain, PSU-1. Microbiol. 146, 3195-3204.

Zhang, W., Li, F. & Nie, L., 2010. Integrating multiple 'omics' analysis for microbial biology: application and methodologies. Microbiol. 156, 287-301.

Zhu, Y., Zhang, Y. & Li, Y., 2009. Understanding the industrial application potential of lactic acid bacteria through genomics. Appl. Microbiol. Biotechnol. 83, 597-610.

CHAPTER 3

RESEARCH RESULTS I

Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines

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ORIGINAL ARTICLE

Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines

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Abstract

Aims: The objective of this study was to investigate the presence of genes coding for enzymes of oenological relevance in wine *Lactobacillus* strains isolated from South African grape and wine samples during the 2001 and 2002 harvest seasons.

Methods and Results: A total of 120 wine lactobacilli isolates belonging to *Lactobacillus plantarum*, *Lactobacillus hilgardii*, *Lactobacillus brevis*, *Lactobacillus pentosus*, *Lactobacillus paracasei*, *Lactobacillus sakei* and *Lactobacillus paraplantarum* were genetically screened for enzyme-encoding genes using PCR with primers specific for β-glucosidase, protease, esterase, citrate lyase and phenolic acid decarboxylase. The results of PCR screening showed that the *Lactobacillus* strains possessed different combinations of enzymes and that some strains did not possess any of the enzymes tested. Confirmation analysis with gene sequencing also showed high similarity of genes with those available in GenBank database.

Conclusion: In this study, we have demonstrated the existence of genes coding for wine-related enzymes in wine lactobacilli that could potentially hydrolyse wine precursors to positively influence wine aroma.

Significance and Impact of the Study: An expansion of knowledge on the genetic diversity of wine-associated lactic acid bacteria will enable the selection of novel malolactic fermentation starter cultures with desired oenological traits for the improvement of the organoleptic quality of the wine, and hence wine aroma.

Introduction

Malolactic fermentation (MLF) in wine is a secondary fermentation process that usually occurs after alcoholic fermentation, although it may also occur during alcoholic fermentation. This process involves the decarboxylation of L-malic acid to L-lactic acid and carbon dioxide, and it is normally conducted by lactic acid bacteria (LAB) belonging to the four genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus* (Wibowo *et al.* 1985). Among these genera, *Oenococcus oeni* is most frequently associated with MLF because of its adaptation to survive very harsh winemaking conditions of low pH (Drici-Cachon *et al.* 1996), high ethanol concentration (Davis *et al.* 1988) and the presence of sulfur dioxide (Henick-Kling 1988).

Apart from wine deacidification, wine LAB can positively alter the chemical composition of wine through the metabolism of precursors present in wine during fermentation. A wide range of secondary modifications are of great importance for the taste and flavour improvement of wine, and these include citrate metabolism, amino acid metabolism, metabolism of polysaccharides, metabolism of polyols, catabolism of aldehydes, hydrolysis of glycosides, synthesis and hydrolysis of esters, and degradation of phenolic acids, lipolysis, proteolysis and peptidolysis (Liu 2002; Matthews *et al.* 2004). These reactions rely on the hydrolytic action of enzymes, and the metabolites formed as a result of bacterial enzymatic activity can positively alter the sensory properties of wine. Some of the enzymes of major interest in wine aroma include

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β-glucosidases, proteases, esterases, citrate lyases and phenolic acid decarboxylases (Liu 2002; Matthews *et al.* 2004).

Enzymes in winemaking provide a broader range of biotechnological benefits, among which the formation of wine aroma has become the major focus area of interest in recent years. The enzymes not only are involved in flavour enhancement but can also assist in improving the colour of red wines and solve the problems associated with wine filtration. It is therefore crucial to ascertain the potential of microbes, more specifically wine LAB, to possess enzymes which confer desirable traits in wine with respect to the formation of aroma. Although the presence of a broad range of enzymes in yeasts (Rosi *et al.* 1994; Strauss *et al.* 2001) and bacteria (Liu 2002; Matthews *et al.* 2004) has been documented, very few studies have taken a step ahead to genetically elucidate the potential of wine LAB to possess a wide range of genes coding for enzymes of interest in winemaking.

The enzyme and its genetic determinants that have been best studied in wine LAB is β-glucosidase. 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. A recent study (Spano *et al.* 2005) has shown that different LAB species such as *Lactobacillus plantarum*, *O. oeni*, *Pediococcus damnosus*, *Lactobacillus paraplantarum* and *Lactobacillus pentosus* have the coding gene sequence and they were highly homologous. The expression of the β-glucosidase gene of *Lact. plantarum* was evaluated under different wine conditions, and results showed that it is regulated by factors such as temperature, ethanol and pH.

A recent study by de las Rivas *et al.* (2009) has demonstrated the existence of the *pdc* gene encoding a phenolic acid decarboxylase in wine LAB strains. In addition, the presence of wine-related enzyme-encoding genes in wine LAB was further confirmed by Olguín *et al.* (2009), who investigated the expression patterns of genes related to citrate utilization in *O. oeni* PSU-1 strain under the conditions simulating those of winemaking. The results shown by these authors give an indication that wine LAB have the potential to possess genes coding for enzymes of interest in winemaking, with particular attention devoted to those related to wine aroma formation. Nevertheless, this area of wine LAB genomics still merits further studies with intent to detect the presence of other enzyme-encoding genes of particular relevance in winemaking.

This is the main reason that, in this study, we investigated the presence of genes coding for β-glucosidase, protease, esterase, citrate lyase (α-, β- and γ-subunits) and phenolic acid decarboxylase in wine lactobacilli. The large presence of lactobacilli in South African wines stimulated our interest to genetically characterize the isolated strains of *Lactobacillus* belonging to *Lact. plantarum*, *Lactobacillus hilgardii*, *Lactobacillus brevis*, *Lact. pentosus*, *Lactobacillus paracasei*, *Lactobacillus sakei* and *Lact. paraplantarum* for the latter enzymes. Genes codings for the different enzymes tested were identified through PCR detection with enzyme-specific primers, and the amplified DNA fragments were subsequently sequenced for comparative sequencing analysis.

Materials and methods

Bacterial isolates and culture conditions

All bacterial isolates used in this study form part of the culture collection of the Institute for Wine Biotechnology and were isolated during the 2001 and 2002 harvest seasons from five different commercial wineries situated in the Western Cape, South Africa. All strains were routinely grown at 30°C on de Mann-Rogosa-Sharpe (MRS; Biolab, Wadeville, South Africa) medium. *Escherichia coli* DH5α cells were used as a host for cloning experiments, and they were grown with aeration in Luria–Bertani (LB; Biolab) broth at 37°C (Sambrook *et al.* 1989). For the selection of *E. coli* positive transformants, 100 μg ml^{-1} of ampicillin was incorporated in the LB medium. The plasmid pLOCPAD was provided by Prof. J.-F. Cavin (Université de Bourgogne, Dijon, France) and was used as the positive control for PCR screening of lactobacilli for phenolic acid decarboxylase gene. All solid media contained 2% (w/v) agar.

Molecular detection of genes

To detect the presence of β-glucosidase, protease, esterase, citrate lyase and phenolic acid decarboxylase genes from different wine *Lactobacillus* species, the isolates were screened using colony PCR. Bacterial isolates were first grown on MRS agar plates, after which one colony from each plate was applied directly to PCR using specific primer sets (Table 1). Nucleotide gene sequences used for designing the primers were extracted from the Integrated Microbial Genomes database (http://img.jgi.doe.gov of the DOE Joint Genome Institute). All the primers used in this study were synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa).

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Table 1 The list of primers used in this study

Primer name	Primer sequence (5′–3′)	Application	References
BGL-1	GTGACTATGGTAGAGTTTCC – fwd	β-Glucosidase gene	Spano *et al.* 2005
BGL-2	TCAAAACCCATTCCGTTCCCCA – rev	β-Glucosidase gene	Spano *et al.* 2005
Prt-1	GCATGGCTAATAAATCATTAATCAAAG – fwd	Serine protease HtrA gene	This work
Prt-2	GCTTAGTTACTTTGTTTAGTTAACGTTTTG – rev	Serine protease HtrA gene	This work
Est-1	GCTAATTTGTAACCGTATCCGCC – fwd	Putative esterase gene	This work
Est-2	CGCGCATGTTAACTTTTAGTAGAAC – rev	Putative esterase gene	This work
citD-f	ATGGAAATTAARAMAACKGCAKTMGC – fwd	Citrate lyase (gamma subunit) gene	This work
citD-r	GCYGCYGTAATRGTYGKYGCYTTWAT – rev	Citrate lyase (gamma subunit) gene	This work
citE-1	TTACGBCGSACRATGATGTTTGT – fwd	Citrate lyase (beta subunit) gene	This work
citE-2	TATTTTTCAATGTAATTDCCCTCC – rev	Citrate lyase (beta subunit) gene	This work
citF-a	ATGGYATGACRATTTCWTTYCAYCAYCA – fwd	Citrate lyase (alpha subunit) gene	This work
citF-b	ATCAATVAHBSWRCCRTCRCGRTAYTC – rev	Citrate lyase (alpha subunit) gene	This work
PAD-1	AARAAYGAYCAYACYRTTGATTACC – fwd	Phenolic acid decarboxylase gene	This work
PAD-3	TTCTTCWACCCAYTTHGGGAAGAA – rev	Phenolic acid decarboxylase gene	This work

For PCR experiments, each colony was added to a 50-μl PCR mixture containing 1·25 U Supertherm polymerase (Hoffman-La Roche, Basel, Switzerland), 0·4 μmol l^{-1} of each primer, 1·5 mmol l^{-1} $MgCl_2$, 0·25 mmol l^{-1} dNTP mix and 1× PCR buffer. The reaction mixture was subjected to PCR using T3 Thermocycler (Biometra® GmbH, Göttingen, Germany) through the temperature profiles indicated in Table 2. PCR products were analysed by electrophoresis on agarose (Sigma-Aldrich, St Louis, MO, USA) gels containing 0·2 μg ml^{-1} ethidium bromide. Gels were run for *c.* 60 min at 80 V in 1× TAE (100 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA, pH 8·0, 20 mmol l^{-1} acetate) buffer. DNA fragments were visualized by UV transillumination and documented with Alpha Imager (Alpha Innotech Corporation, San Leandro, CA, USA). Lambda DNA digested with *Eco*RI and *Hin*dIII (Fermentas, Vilnius, Lithuania) was used as the standard molecular weight marker, and a 100-bp molecular weight marker XIV (Roche Diagnostics, Mannheim, Germany) was used for the expected low band size fragments.

DNA preparation and gene amplification

Among the isolates that possessed all five enzyme genes, ten were selected from which genomic DNA was extracted and used as template to amplify the coding sequences of the respective genes. The selected isolates belonged to *Lact. plantarum*, *Lact. paracasei*, *Lact. hilgardii*, *Lact. brevis* and *Lact. pentosus*. Isolation of chromosomal DNA was performed by phenol extraction as suggested by Vaquero *et al.* (2004) and modified by precipitating chromosomal DNA by adding two volumes of cold ethanol and one-tenth volume of 3 mol l^{-1} sodium acetate solution. The precipitated DNA was washed with 70% ethanol, dried in a speedy vacuum and dissolved in 100 μl of 1× TE buffer containing 5 μl of RNase (10 mg ml^{-1}) (Sigma-Aldrich).

Table 2 Thermal cycling conditions used for the PCR detection of genes coding for β-glucosidase, protease, esterase, citrate lyase and phenolic acid decarboxylase

		Main cycling conditions					
Primer pair	T_{Di} (°C), time (min)	Number of cycles	T_D (°C), time	T_A (°C), time	T_E (°C), time	T_{Ef} (°C), time (min)	References
BGL-1/BGL-2	94, 5	30	94, 1 min	50, 40 s	72, 1·2 min	72, 10	Spano *et al.* 2005
Prt-1/Prt-2	94, 5	30	94, 1 min	55, 30 s	72, 1 min	72, 10	This work
Est-1/Est-2	94, 5	30	94, 1 min	53, 30 s	72, 1 min	72, 10	This work
citD-f/citD-r	94, 3	35	94, 45 s	54, 30 s	72, 1 min	72, 5	This work
citE-1/citE-2	94, 3	35	94, 30 s	54, 1 min	72, 1 min	72, 10	This work
citF-a/citF-b	94, 5	35	94, 1 min	49, 45 s	72, 1 min	72, 10	This work
PAD-1/PAD-2	94, 2	35	94, 40 s	50, 1 min	72, 30 s	72, 5	This work

T_{Di}, initial denaturation temperature; T_D, denaturation temperature; T_A, annealing temperature; T_E, extension temperature; T_{Ef}, final extension temperature.

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DNA samples were then incubated at 65°C for 4 min before storage at −20°C.

The quantification of DNA was performed spectrophotometrically using a NanoDrop® ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA). To isolate the genes from the selected isolates, the same sets of PCR primers and thermal cycling conditions were employed as described earlier, except that 10 ng of the extracted DNA and 1·25 U *TaKaRa Ex Taq* DNA polymerase (TaKaRa Biomedicals, Shiga, Japan) were used while $MgCl_2$ was not incorporated. PCR amplification products were purified with QIAquick® PCR Purification kit (Qiagen, Milan, Italy) and cloned into the pGEM-T® Easy vector (Promega, Madison, WI, USA) according to the manufacturers' instructions. After transformation into *E. coli*, the PCR fragments were purified and sent for sequencing.

DNA sequencing and analysis

All sequencing reactions were performed by Inqaba Biotechnical Industries and the Central Analytical Facility (Stellenbosch University, South Africa). DNA sequencing was performed on both strands by using universal primers (T7 and SP6). Nucleotide sequence data were assembled, and multiple sequence alignment was carried out with the biological sequence alignment editor (BioEdit program; Ibis Therapeutics, Carlsbad, CA, USA). The basic local alignment search tool (*BLASTn*) of the National Center of Biotechnology Information (NCBI) was used for searching homologous nucleotide sequences (Altschul *et al.* 1990).

Results

Genetic screening

PCR screening with the designed primers resulted in single gene products of 1392 bp for β-glucosidase, 1020 bp for esterase, 1263 bp for protease, 245 bp for citrate lyase γ-subunit, 897 bp for citrate lyase β-subunit, 1331 bp for citrate lyase α-subunit and 210 bp for phenolic acid decarboxylase (partial sequence) (Fig. 1). The distribution of the enzymes within the lactobacilli tested is presented in Table 3: 41% were positive for β-glucosidase, 58% for protease, 59% for esterase, 60% for citrate lyase γ-subunit, 68% for citrate lyase β-subunit, 62% for citrate lyase α-subunit and 75% for phenolic acid decarboxylase. Of all the lactobacilli isolates, 32 possessed genes for all five enzymes evaluated in this study. In addition, β-glucosidase genes from six isolates yielded PCR fragments of 1308 bp (Fig. 1a), when compared to the rest with 1392 bp. Among these, two isolates *Lact. plantarum* 113·1 and *Lact. brevis* 116·3 were selected for comparative sequence alignments.

Comparative analysis of gene sequences

Among the isolates that possessed all five genes, ten were selected, and the genes encoding the corresponding enzymes were sequenced. An analysis of β-glucosidase gene sequences showed that these sequences were highly homologous to those of *Lact. plantarum* (GenBank

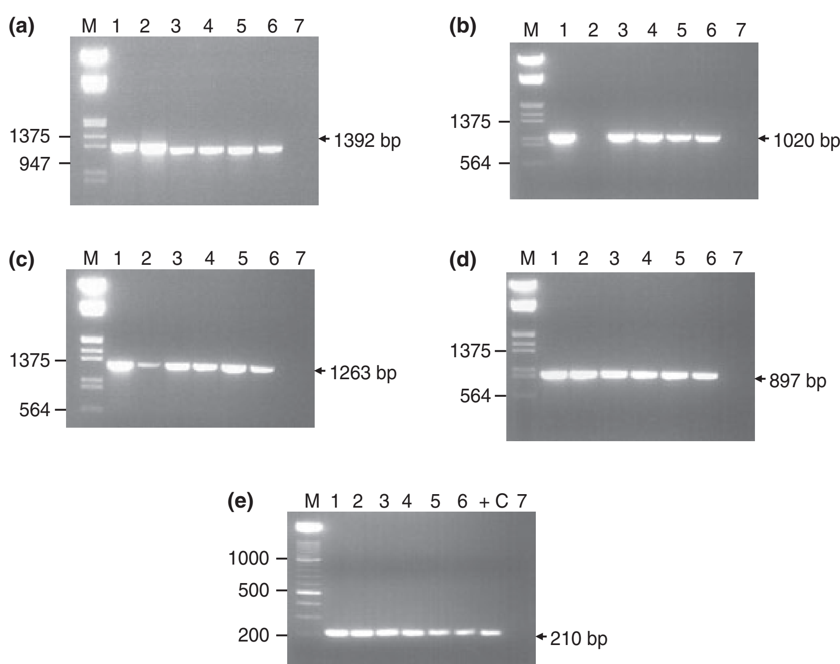

Figure 1 PCR amplifications showing the presence of β-glucosidase genes (a), esterase genes (b), serine protease HtrA genes (c), citrate lyase genes (d) and phenolic acid decarboxylase genes (e). M is the standard molecular weight marker. Strains: lane 1 – *Lactobacillus hilgardii* 87·1; lane 2 – *Lactobacillus paraplantarum* 107·1; lane 3 – *Lactobacillus plantarum* 120·1; lane 4 – *Lactobacillus paracasei* 146·1; lane 5 – *Lactobacillus brevis* 116·3; lane 6 – *Lact. plantarum* 113·1; lane 7 – negative control; +C – pLOCPAD (positive control for phenolic acid decarboxylase gene).

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Table 3 Detection of enzyme-encoding genes with enzyme-specific primers using colony PCR

Species	*βgl*	*prt*	*estA*	*citD*	*citE*	*citF*	*pad*
Lactobacillus spp.	120* (49†)	120 (69)	120 (71)	120 (72)	120 (81)	120 (74)	120 (90)
Lactobacillus plantarum	84 (34)	84 (57)	84 (59)	84 (59)	84 (67)	84 (61)	84 (72)
Lactobacillus hilgardii	02 (02)	02 (02)	02 (02)	02 (01)	02 (02)	02 (02)	02 (02)
Lactobacillus brevis	11 (03)	11 (03)	11 (03)	11 (03)	11 (03)	11 (02)	11 (04)
Lactobacillus paracasei	10 (04)	10 (04)	10 (05)	10 (05)	10 (05)	10 (05)	10 (06)
Lactobacillus pentosus	10 (04)	10 (01)	10 (01)	10 (02)	10 (02)	10 (01)	10 (03)
Lactobacillus sakei	01 (00)	01 (01)	01 (01)	01 (00)	01 (00)	01 (01)	01 (01)
Lactobacillus paraplantarum	02 (02)	02 (01)	02 (00)	02 (02)	02 (02)	02 (02)	02 (02)

Bgl, β-glucosidase; *prt*, protease; *estA*, esterase; *citD*, citrate lyase γ-subunit; *citE*, citrate lyase β-subunit; *citF*, citrate lyase α-subunit; *pad*, phenolic acid decarboxylase.
*The total number of isolates tested.
†The number of positive strains.

accession no. AY489109) and *O. oeni* (accession no. AY489108) with the highest nucleotide sequence identity of 99%. Additionally, β-glucosidase genes from the two other selected isolates (*Lact. plantarum* 113·1 and *Lact. brevis* 116·3) that possessed smaller fragments were sequenced, and the alignment results showed that the genes from these isolates possessed the sequences with 84 bp missing (Fig. 2). Nevertheless, nucleotide sequences for these two isolates were highly similar to β-glucosidase gene sequences of *O. oeni* and *Lact. plantarum* WCFS1 available in GenBank database.

Similar trends of nucleotide sequence homology were also observed for esterase, protease, citrate lyase and phenolic acid decarboxylase genes from the isolates investigated. The nucleotide sequences of esterase genes exhibited 99% identity with the esterase gene from *Lact. plantarum* WCFS1 strain (AL935258), with minor differences noticeable in a few nucleotides (data not shown). Furthermore, analysis of the protease gene sequences showed that these genes belong to the class of serine protease HtrA enzymes. Protease genes exhibited a significant homology with serine protease HtrA genes similar to those previously identified in *Lact. plantarum* WCFS1 (accession no. AL935252; 99% identity) and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 (accession no. CR954253; 73% identity). Protease genes were also homologous to a lesser extent to trypsin-like serine protease genes with post-synaptic density protein, disc large and zo-1 (PDZ) domain, and this was the case for protease genes from *Lact. brevis* ATCC 367 (accession no. CP000416; 69% identity), *Lact. delbrueckii* ssp. *bulgaricus* ATCC BAA-365 (accession no. CP000412; 73% identity) and *Pediococcus pentosaceus* ATCC 25745 (accession no. CP000422; 70% identity) (data not shown).

The nucleotide sequence alignment of the α-subunit of citrate lyase genes also showed that these genes are highly similar to those of *Lact. plantarum* WCFS1 (accession no. AL935255; 98–99% identity), *Lactobacillus reuteri* (accession no. DQ233691; 74% identity), *Lactobacillus acidophilus* NCFM (accession no. CP000033; 74% identity), *O. oeni* PSU-1 (accession no. CP000411; 72% identity), *Lactobacillus helveticus* DPC 4571 (accession no. CP000517; 72% identity), *Leuconostoc citreum* KM20 (accession no. DQ489736; 71% identity), *Lactobacillus casei* ATCC 334 (accession no. CP000423; 66% identity), *Lact. casei* BL23 (accession no. FM177140; 66% identity) and *Lact. sakei* strain 23K (accession no. CR936503; 66% identity) (data not shown).

The nucleotide gene sequences of citrate lyase (β-subunit) were similar to those of *Lact. plantarum* WCFS1 (accession no. AL935255; 97–99% identity), *Lact. reuteri* (accession no. DQ233692; 73% identity), *O. oeni* PSU-1 (accession no. CP000411; 71% identity), *Leuconostoc mesenteroides* (accession no. Y10621; 71–72% identity), *Leuc. citreum* KM20 (accession no. DQ489736; 69–70% identity), *Lact. sakei* strain 23K (accession no. CR936503; 65–66% identity) and *Lact. casei* ATCC 334 (accession no. CP000423; 66–67% identity) (data not shown).

The sequences of γ-subunit of citrate lyase showed similarity to those of *Lact. plantarum* WCFS1 (accession no. AL935255; 97% identity), *O. oeni* PSU-1 (accession no. CP000411; 67% identity), *Leuc. citreum* KM20 (accession no. DQ489736; 80% identity), *Lact. helveticus* DPC 4571 (accession no. CP000517; 71% identity) and *Lact. acidophilus* NCFM (accession no. CP000033; 81% identity) (data not shown).

Similar results of nucleotide similarity were also observed for the partial phenolic acid decarboxylase genes which were highly homologous to *p*-coumaric acid decarboxylase (*pdc*) or phenolic acid decarboxylase genes (*pad*) of *Lact. plantarum* WCFS1 (accession no. AL935262; 98% identity), *Lact. plantarum* (accession no. AF257163; 98% identity), *Lactobacillus fermentum* (accession no. AF257162; 98% identity), *Lact. pentosus* (accession no. AF257161; 98% identity), *Lact. paracasei* (accession no. AF257160; 98% identity), *Lactobacillus crispatus* (accession no. AF257159; 98% identity), *Ped. pentosaceus* (accession no. AJ276891; 80% identity), *Ped. pentosaceus* ATCC 25745

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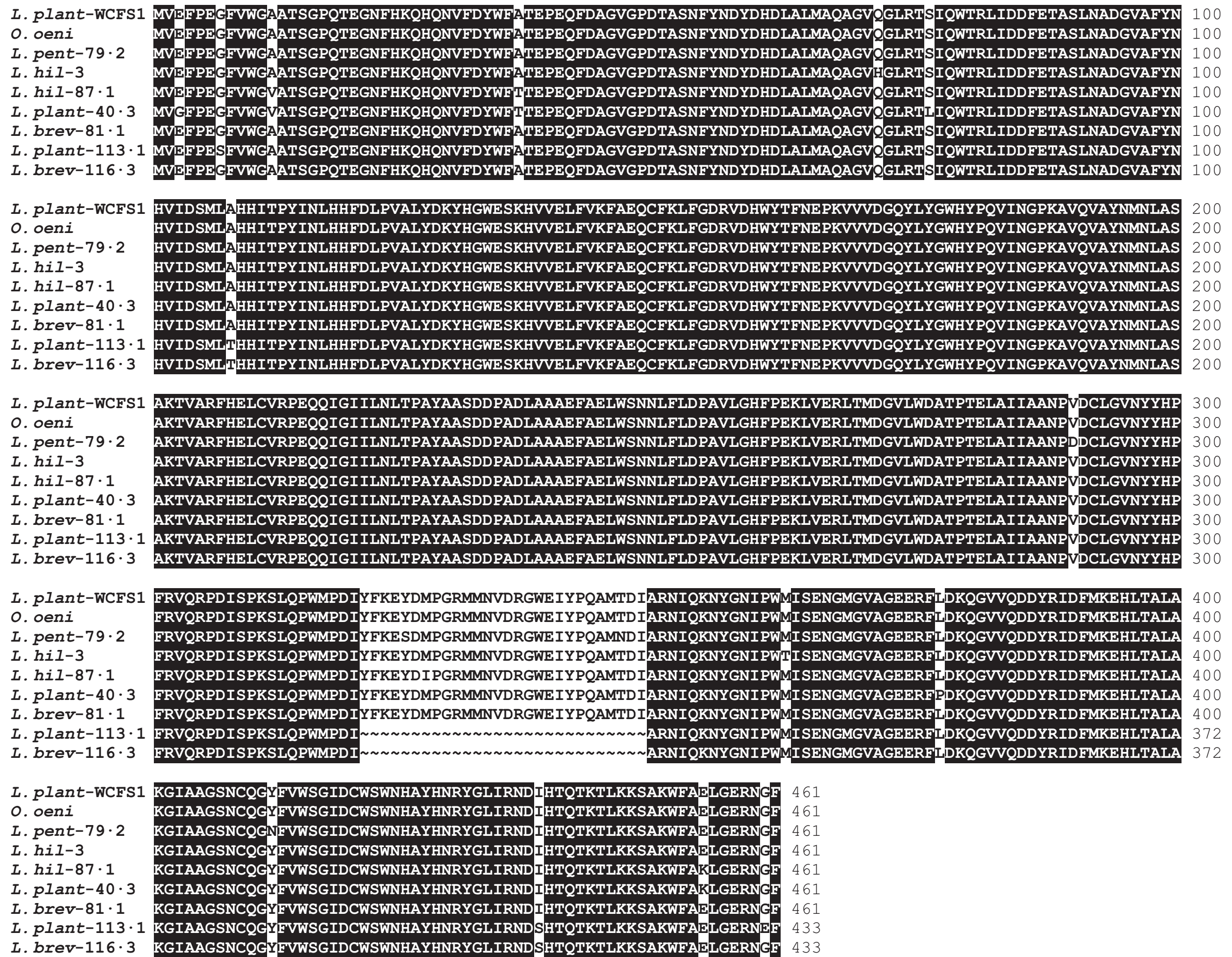

Figure 2 Amino acid sequence alignments of *β*-glucosidase genes from *Lactobacillus plantarum* WCFS1, *Oenococcus oeni*, *Lactobacillus pentosus* 79·2, *Lactobacillus hilgardii* 3, *Lact. hilgardii* 87·1, *Lact. plantarum* 40·3, *Lact. plantarum* 113·1, *Lactobacillus brevis* 81·1 and *Lact. brevis* 116·3. Gene sequences of *Lact. plantarum* WCFS1 (accession no. AY489109) and *O. oeni* (accession no. AY489108) were extracted from GenBank database. Highlighted residues indicate regions that are highly conserved.

(accession no. CP000422; 79% identity), *Lact. sakei* strain 23K (accession no. CR936503; 78% identity), *Lact. brevis* ATCC 367 (accession no. CP000416; 78% identity) and *Lact. hilgardii* (accession no. AF257158; 77% identity) (data not shown).

Discussion

We have demonstrated the existence of different enzyme-encoding genes in several species of *Lactobacillus* associated with winemaking. To our knowledge, this is the first study to investigate the presence of a wide range of the genes coding for different enzymes in *Lactobacillus* species isolated from South African wines. While the enzymes of dairy LAB have received much attention, it has been speculated that wine LAB have the potential to produce enzymes related to the production of aroma in wine (Liu 2002; Matthews *et al.* 2004), and we have now shown using molecular methods that indeed the LAB have a diverse array of enzyme-encoding genes.

In our study, we tested different *Lactobacillus* species for the genes encoding *β*-glucosidase, protease, esterase, citrate lyase (*α*-, *β*- and *γ*-subunits) and phenolic acid decarboxylase. For confirmation, purified PCR fragments from the selected strains were transformed into *E. coli* DH5*α* and subsequently sequenced using universal primers T7 and SP6. The sequencing of the genes allowed us to study the homology patterns of gene sequences between different wine LAB species. The GenBank database was employed as a tool to search for genes similar to those that we sequenced. From the alignment results, it was noteworthy that our gene sequences showed high degrees of similarity to those available in GenBank database. Through PCR detection and sequencing of the

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amplified DNA fragments, we were able to obtain either partial (phenolic acid decarboxylase) or the entire nucleotide sequences of the genes (β-glucosidase, protease, esterase and three citrate lyase subunits). An analysis of these sequences indicated that our gene sequences possessed regions that were highly conserved between species.

Although we genetically tested and subsequently sequenced a limited number of isolates, the results of gene sequence 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 *Lact. plantarum*, *O. oeni*, *Lact. paraplantarum* and *Ped. damnosus*. In our study, the β-glucosidase-encoding gene was only found in a minority of strains (49 of 120), suggesting that this gene is not essential for survival in wine. It is however present in all species tested (except *Lact. sakei*, but only one strain was considered in this study), and the sequences revealed high homologies between species. This could suggest that horizontal gene transfer has occurred. Furthermore, two strains belonging to two different species revealed an identical gap in the nucleotide sequence of their β-glucosidase-encoding gene. This rare event reinforces the hypothesis that horizontal gene transfer occurred between species of lactobacilli. This gap might influence the activity of the enzyme in these particular strains, and this should be further investigated. The presence of β-glucosidase genes from *Lactobacillus* strains that we tested may be beneficial to wine with regard to the formation of wine aroma. By definition, β-glucosidases are enzymes that hydrolyse a bond between glucose and a sugar-bound component. These sugar-bound compounds are nonvolatile and therefore do not contribute to wine aroma (Williams *et al.* 1995). The action of β-glucosidase is thus required for the hydrolysis of these nonaromatic components, thereby liberating the aroma precursors. The activity of glycosidases under oenological conditions was assessed from a range of species belonging to *Lactobacillus*, *Pediococcus* (Grimaldi *et al.* 2005*a*) and *O. oeni* (Grimaldi *et al.* 2005*b*). The results indicated that wine LAB have the potential to impart desirable traits in wine aromatic composition.

Although we did not assess enzyme activity from any of the strains that contained an esterase-encoding gene, it was shown by Matthews *et al.* (2007) that wine LAB esterases are active under wine conditions. Fifty-nine percent of our strains possessed genes coding for esterase, and this gives an indication that these strains may also be involved in ester synthesis and/or hydrolysis (Liu 2002; Matthews *et al.* 2004). In our study, it was shown that *Lact. plantarum* had a high percentage of strains that possessed esterase genes. As one of the most predominant wine lactobacilli, this bacterium can therefore have an impact on ester levels in wine, and hence on wine aroma.

Apart from malic acid decarboxylation during MLF, citrate can also be degraded by many wine LAB. This compound is one of the major organic acids naturally present in grape juice and wine. The metabolism of citrate by wine LAB results to the production of diacetyl, which is considered the most important aromatic compound that imparts a buttery character in wine (Liu 2002). Many studies on the potential of wine LAB to metabolize citrate have been conducted elsewhere (Martineau and Henick-Kling 1995; Nielsen and Richelieu 1999). Recently, Olguín *et al.* (2009) quantified the expression of genes related to citrate metabolism in *O. oeni* PSU-1 strain. The results indicated that the transcriptional behaviour of the genes differ with respect to stress conditions prevalent in wine. In our study, we have also shown through PCR detection that a large majority of wine lactobacilli possess genes coding for a citrate lyase complex (α-, β- and γ-subunit). This enzyme complex is involved in citrate pathway to convert citrate to pyruvate, an essential metabolic pathway, especially active when other sources of energy (e.g. carbohydrates, malic acid) are depleted. The fact that a large majority of lactobacilli possess the genes (and therefore a potentially active citrate lyase) clearly demonstrates that the presence of this gene aided these bacteria to adapt to wine. It should also be noted that some strains tested do not possess all the subunits necessary to form the citrate lyase complex. Further investigation is needed to test whether the citrate catabolic pathway is still active in these strains.

From the *Lactobacillus* strains that we tested, more than 70% seemed to possess the gene coding for phenolic acid decarboxylase. Very recently, de las Rivas *et al.* (2009) developed a PCR assay for the specific detection of the *pdc* gene encoding a phenolic acid decarboxylase in wine LAB isolated from Spanish grape must and wine. The authors could not detect the *pdc* gene in *Lact. hilgardii*, but our study shows that some strains of this species do possess it. Moreover, de las Rivas *et al.* (2009) also demonstrated the direct link between the presence of the *pdc* gene and the ability to produce volatile phenols from hydroxycinnamic acids. This aspect needs further investigation into our strains. Phenolics are compounds naturally present in grape juice and wine, and they can be degraded microbially into volatile phenols during fermentation (Liu 2002). These volatile phenols can either have a positive or a negative influence on wine aroma depending on their concentration. Although many studies have demonstrated the ability of wine LAB to produce volatile phenols (Cavin *et al.* 1993; Chatonnet *et al.* 1995; Couto *et al.* 2006), more work still needs to be carried out on a molecular

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point of view to elucidate the expression patterns of enzyme-encoding genes in wine, under the conditions pertinent to winemaking. Nevertheless, the presence of the *pdc* gene in a large majority of *Lactobacillus* strains confirms that these bacteria might not only be a source of beneficial enzymes but could also spoil wines by potentially producing compounds responsible for off-flavours.

The findings reported in this work give an indication that wine lactobacilli can be used not only for conducting MLF but also as the potential source of enzymes to impact positively on wine aroma. Although many strains were shown to possess the genes with potential to act on specific substrates, it remains imperative to better understand how these genes are regulated under winemaking conditions, and also to evaluate whether the expressed enzymes are active in wine. This study therefore forms the basis for the selection of potential strains to further evaluate them for gene expression and enzyme activity under certain winemaking parameters such as pH and ethanol.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment tool. *J Mol Biol* **215**, 403–410.

Cavin, J.F., Andioc, V., Etievant, P.X. and Divies, C. (1993) Ability of wine lactic acid bacteria to metabolise phenol carboxylic acids. *Am J Enol Vitic* **44**, 76–80.

Chatonnet, P., Dubourdieu, D. and Boidron, J.N. (1995) The influence of *Brettanomyces/Dekkera* sp. yeasts and lactic acid bacteria on the ethylphenol content of red wines. *Am J Enol Vitic* **46**, 463–468.

Couto, J.A., Campos, F.M., Figueiredo, A.R. and How, T.A. (2006) Ability of lactic acid bacteria to produce volatile phenols. *Am J Enol Vitic* **57**, 166–171.

Davis, C.R., Wibowo, D., Fleet, G.H. and Lee, T.H. (1988) Properties of wine lactic acid bacteria: their potential enological significance. *Am J Enol Vitic* **39**, 137–142.

Drici-Cachon, Z., Guzzo, J., Cavin, F. and Diviès, C. (1996) Acid tolerance in *Leuconostoc oenos.* Isolation and characterisation of an acid-resistant mutant. *Appl Microbiol Biotechnol* **44**, 785–789.

Grimaldi, A., Bartowsky, E. and Jiranek, V. (2005a) Screening of *Lactobacillus* spp. and *Pediococcus* spp. for glycosidase activities that are important in oenology. *J Appl Microbiol* **99**, 1061–1069.

Grimaldi, A., Bartowsky, E. and Jiranek, V. (2005b) A survey of glycosidase activities of commercial wine strains of *Oenococcus oeni*. *Int J Food Microbiol* **105**, 233–244.

Grimaldi, A., McLean, H. and Jiranek, V. (2000) Identification and partial characterization of glycosidic activity of commercial strains of the lactic acid bacterium *Oenococcus oeni*. *Am J Enol Vitic* **51**, 362–369.

Guilloux-Benatier, M., Son, H.S., Bouhier, S. and Feuillat, M. (1993) Activités enzymatiques: glycosidases et peptidase chez *Leuconostoc oenos* au cours de la croissance bactérienne. Influence des macromolécules de levures. *Vitis* **32**, 51–57.

Henick-Kling, T. (1988) Yeast and bacterial control in winemaking. In *Wine Analysis*, **Vol. 6** ed. Linskens, H.F. and Jackson, J.F. pp. 276–316. Heidelberg: Springer Verlag.

Liu, S.-Q. (2002) Malolactic fermentation in wine – beyond deacidification. *J Appl Microbiol* **92**, 589–601.

Mansfield, A.K., Zoecklein, B.W. and Whiton, R. (2002) Quantification of glycosidase activity in selected strains of *Brettanomyces bruxellensis* and *Oenococcus oeni*. *Am J Enol Vitic* **53**, 303–307.

Martineau, B. and Henick-Kling, T. (1995) Formation and degradation of diacetyl in wine during alcoholic fermentation with *Saccharomyces cerevisiae* strain EC 1118 and malolactic fermentation with *Leuconostoc oenos* strain MCW. *Am J Enol Vitic* **46**, 442–448.

Matthews, A., Grimaldi, A., Walker, M., Bartowsky, E., Grbin, P. and Jiranek, V. (2004) Lactic acid bacteria as a potential source of enzymes for use in vinification. *Appl Environ Microbiol* **70**, 5715–5731.

Matthews, A., Grbin, P.R. and Jiranek, V. (2007) Biochemical characterisation of the esterase activities of wine lactic acid bacteria. *Appl Microbiol Biotechnol* **77**, 329–337.

McMahon, H., Zoecklein, B.W., Fugelsang, K. and Jasinsky, Y. (1999) Quantification of glycosidase activities in selected yeasts and lactic acid bacteria. *J Ind Microbiol Biotechnol* **23**, 198–203.

Nielsen, J.C. and Richelieu, M. (1999) Control of flavor development in wine during and after malolactic fermentation by *Oenococcus oeni*. *Appl Environ Microbiol* **65**, 740–745.

Olguín, N., Bordons, A. and Reguant, C. (2009) Influence of ethanol and pH on the gene expression of the citrate pathway in *Oenococcus oeni*. *Food Microbiol* **26**, 197–203.

de las Rivas, B., Rodríguez, H., Curiel, J.A., Landete, J.M. and Munoz, R. (2009) Molecular screening of wine lactic acid bacteria degrading hydroxycinnamic acids. *J Agric Food Chem* **57**, 490–494.

Rosi, I., Vinella, M. and Domizio, P. (1994) Characterization of β-glucosidase activity in yeasts of oenological origin. *J Appl Bacteriol* **77**, 519–527.

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Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Spano, G., Rinaldi, A., Ugliano, M., Moio, L., Beneduce, L. and Massa, S. (2005) A β-glucosidase gene isolated from wine *Lactobacillus plantarum* is regulated by abiotic stresses. *J Appl Microbiol* **98**, 855–861.

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

Vaquero, I., Marcobal, A. and Munoz, R. (2004) Tannase activity by lactic acid bacteria isolated from grape must and wine. *Int J Food Microbiol* **96**, 199–204.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H. and Lee, T.H. (1985) Occurrence and growth of lactic acid bacteria in wine: a review. *Am J Enol Vitic* **36**, 302–313.

Williams, P.J., Cynkar, W., Francis, I.L., Gray, J.D., Hand, P.G. and Coombe, B.G. (1995) Quantification of glycosides in grapes, juices and wines through a determination of glycosyl glucose. *J Agric Food Chem* **43**, 121–128.

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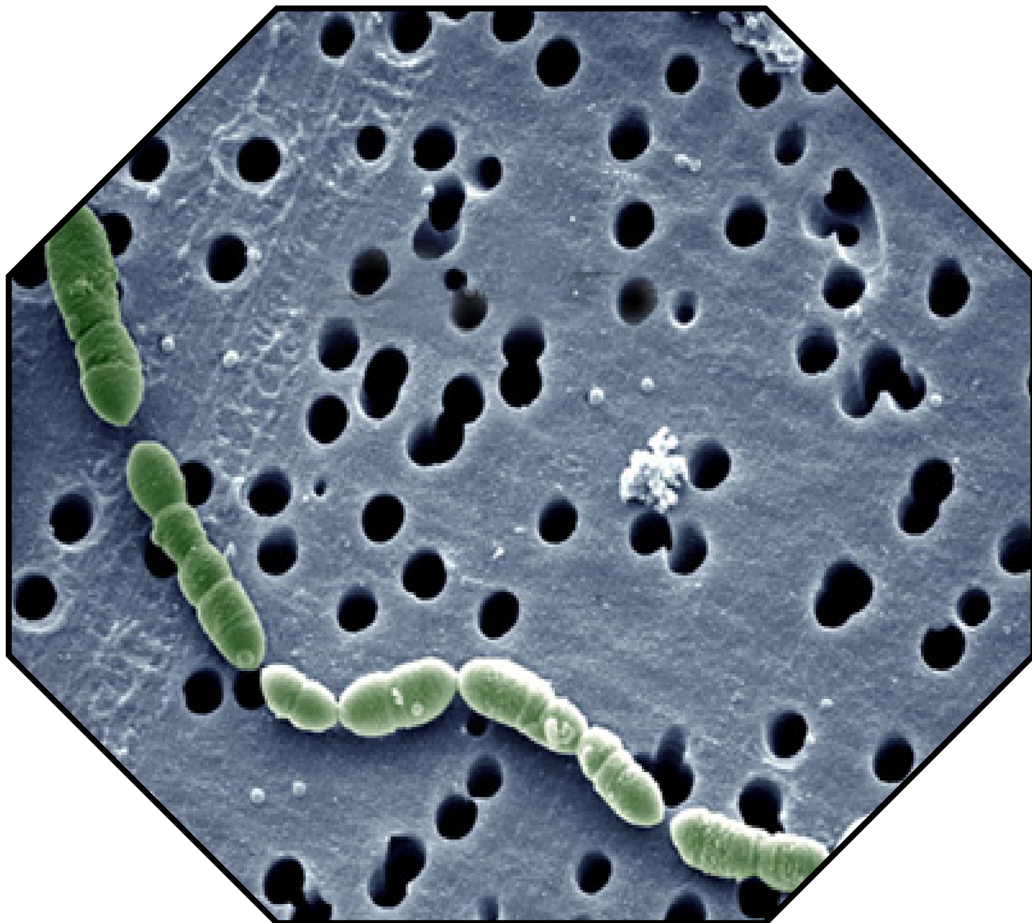
CHAPTER 4

RESEARCH RESULTS II



A survey of genes encoding enzymes of oenological relevance in *Oenococcus oeni* strains of South African wine origin

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CHAPTER 4

A survey of genes encoding enzymes of oenological relevance in *Oenococcus oeni* strains of South African wine origin

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ABSTRACT

Aims: To screen *Oenococcus oeni* strains of South African wine origin for the presence of genes encoding enzymes of oenological relevance using a PCR-based detection approach.

Methods and Results: A total of 24 *O. oeni* strains isolated in South Africa from brandy base wines and from red grape and wine samples were investigated in this study. The reference strains incorporated were NCDO 1894, NCDO 2122 and DSMZ 20252^T. Using a molecular approach, *O. oeni* strains were subjected to PCR analysis with primers targeting the *mleA*, *bgl*, *estA*, *prtP*, *pad*, *citD*, *citE*, *citF*, *maeP*, *alsD*, *alsS*, *metK*, *metB*, *metC*, *gshR*, *arcA*, *arcB* and *arcC* genes. As expected, all the strains possessed all the genes investigated, with the notable exception of *arcB*, *arcC* and *pad*. It is the first time that the *pad* gene encoding a phenolic acid decarboxylase is detected in *O. oeni*, as two of our strains possessed this gene. The identity of PCR-generated fragments, representing all the genes tested for, was confirmed by sequencing. Homology searches were performed by comparing nucleotide sequences of IWBT B040 strain to other DNA sequences available in GenBank database. The highest sequence identities (98–100%) were recorded with those of three other *O. oeni* strains (PSU-1, ATCC BAA-1163 and AWRI B429) published in GenBank. Phylogenetic trees were also constructed based on DNA sequences of the *alsS*, *estA*, *metK* and *mleA* genes, which revealed that all *O. oeni* strains are phylogenetically distinct from other closely related species of the genus *Leuconostoc*.

Conclusions: We have shown using a molecular approach that *O. oeni* strains possess a vast array of enzyme-encoding genes of relevance in winemaking. From this study, it was worth noting that most genes are conserved amongst the South African strains, and a worldwide scale comparison would provide more insights into the genetic diversity of *O. oeni*.

Significance and Impact of the Study: The findings reported in this work provide a better insight on the possible role that *O. oeni* strains can have in modulating wine organoleptic quality with regards to the presence of genes encoding enzymes involved in various metabolic pathways. This study should therefore be followed by the expression analysis of

these genes in order to assess if they are functionally expressed under oenological conditions.

Keywords: *Oenococcus oeni*, wine, enzyme-encoding genes, PCR detection, sequencing

4.1 INTRODUCTION

Oenococcus oeni is the main wine bacterium best adapted to perform malolactic fermentation (MLF). The reduction in wine acidity during MLF derives from the decarboxylation of L-malic acid (malate) to L-lactic acid (lactate) and carbon dioxide. The biological effect of malate conversion relates to increased microbial stabilization (derived from nutrient depletion) as well as improvement of wine sensorial properties (derived from acidity reduction) through the modification of fruit-derived aromas and the production of aroma-active compounds (Kunkee 1998; Lerm *et al*. 2010).

In order for wine-associated lactic acid bacteria (LAB), more specifically *O. oeni*, to impart desirable aroma and flavour attributes in wine, they need a variety of enzymatic activities capable of altering wine chemical profile. The enzymes of *O. oeni* metabolic pathways have been the subject of many studies in recent years. As such, Swiegers *et al*. (2005) described the major metabolic pathways by which the malolactic bacteria modulate flavour-active compounds in wine. Evaluating the presence of certain bacterial metabolic pathway genes coding for enzymes of oenological relevance has also been the subject of many studies. In a previous study, Mills *et al*. (2005) identified the genes encoding enzymes related to citrate utilization in the wine *O. oeni* PSU-1 strain, whose genome was sequenced and made publicly available. These genes are organized in the citrate lyase gene cluster (*citR*, *mae*, *maeP*, *citC*, *citD*, *citE*, *citF*, *citX*, *citG*). Garmyn *et al*. (1996) also cloned and characterized two genes (*alsD* and *alsS*) involved in the synthesis of diacetyl, which imparts a desirable buttery attribute in wine (Bartowsky and Henschke 2004). These two genes are located on the same operon and are constitutively expressed.

The other well-studied enzyme and its genetic determinant is β-glucosidase. In a previous study, Spano *et al*. (2005) detected the β-glucosidase gene in an oenological strain of *O. oeni*; this gene was found to be conserved across various LAB species. Recently, Michlmayr *et al*. (2010) cloned and characterised a β-glucosidase-related glycosidase gene of *O. oeni* ATCC BAA-1163 strain originating from wine. Morever, the genome sequence of a recently sequenced *O. oeni* AWRI B429 strain was also shown to possess two additional genes encoding glycosyl hydrolyases involved in the liberation of aroma compounds from glycosylated wine precursors (Borneman *et al*. 2010). The strains of *O. oeni* have also been evaluated for the presence of *arc* genes (*arcA*, *arcB* and *arcC*) encoding proteins involved in arginine metabolism via the arginine deiminase pathway (Tonon *et al*. 2001; Divol *et al*. 2003). In addition, Knoll *et al*. (2011) cloned and characterized a cystathionine β-/γ-lyase from two oenological strains of *O. oeni*. This enzyme catalyzes the degradation of sulphur-containing amino acids. To our knowledge,

however, the presence of the *pad* gene encoding a phenolic acid decarboxylase has not been detected in any of the *O. oeni* strains tested previously.

In this paper, we report a PCR-based detection of genes encoding enzymes of oenological relevance in *O. oeni* strains isolated from South African wines. In addition, the presence of a putative phenolic acid decarboxylase gene in two *O. oeni* strains was evaluated. To confirm the identity of PCR-generated fragments, purified amplicons of the corresponding genes were sequenced. The genetic biodiversity between the tested *O. oeni* strains and the closest relatives was also studied by constructing phylogenetic trees based on nucleotide gene sequences.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains, media and growth conditions

All *O. oeni* strains investigated in this study form part of the culture collection of the Institute for Wine Biotechnology, Stellenbosch University, South Africa (Table 4.1). Reference strains included were NCDO 1894, NCDO 2122 and DSMZ 20252^T. All strains were routinely grown at 30°C on de Man-Rogosa-Sharpe (MRS; BIOLAB Diagnostics, Wadeville, South Africa) medium supplemented with 20% (v/v) apple juice. The medium was buffered to 5.2 with HCl. Anaerobic conditions were generated by Anaerocult® A (Merck, Darmstadt, Germany). The solid medium contained 2% (w/v) agar. *Escherichia coli* DH5α cells were used as the host for cloning procedures; they were grown in Luria-Bertani medium (BIOLAB Diagnostics) added with an antibiotic (100 μg ml^{-1} of ampicillin).

4.2.2 PCR detection of genes

Genomic DNA was extracted according to the method described by Mtshali *et al*. (2010). To detect the presence of 18 different enzyme-encoding genes in *O. oeni* strains, PCR primer sets listed in Table 4.2 were used. These primers were either taken from the literature (Araque *et al*. 2009; Mtshali *et al*. 2010) or designed in this study based on the nucleotide gene sequences of *O. oeni* PSU-1 (GenBank accession no. CP000411) and ATCC BAA-1163 (accession no. AAUV00000000). All primers were synthesised by the Integrated DNA Technologies, Inc. (Coralville, IA, USA). Reactions were carried out using a Biometra Thermoblock instrument (Biometra® GmbH, Göttingen, Germany) in a 25-μl volume containing 50 ng of template DNA, 0.6 μM of each primer, 1.5 mM $MgCl_2$, 0.25 mM dNTP mix, 1X PCR buffer and 1.25 U of Supertherm polymerase (Southern Cross Biotechnology, Cape Town, South Africa). PCR conditions used for the different primers are shown in Table 4.3. PCR-generated amplicons were checked on agarose gels in 1X

TAE (Tris-acetate-EDTA) buffer containing 0.5 μg ml^{-1} of ethidium bromide and visualized under UV transillumination (Alpha Innotech Corporation, San Leandro, CA, USA).

4.2.3 DNA sequencing

To confirm that the PCR amplification products corresponded to the genes studied, four *O. oeni* strains (IWBT B018, IWBT B040, NCDO 1894 and NCDO 2122), which are representative of each type of wine, were selected for sequencing. PCR assays were performed in 50-μl volumes in the presence of *Ex Taq*™ DNA polymerase (Southern Cross Biotechnology) instead of Supertherm polymerase and cycled as described above. Amplicons were purified using QIAquick® PCR Purification kit (Qiagen, Southern Cross Biotechnology, Cape Town, South Africa) and cloned into pGEM-T® Easy vector (Promega, Madison, USA) according to manufacturers' instructions. Positive transformants were then sequenced by the Central DNA Sequencing Facility, Stellenbosch University, South Africa.

4.2.4 Comparative sequence analysis

After obtaining all the sequence data, the comparative analysis of gene sequences against international databases was performed with BLASTN algorithm of the National Center for Biotechnology Information NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.2.5 Phylogenetic trees

For phylogenetic studies, multiple sequence alignments of nucleotide gene sequences were created using CLUSTAL_X (Thompson et al., 1997) and MEGA v4.1 software (Kumar et al., 2008) by applying default parameters. The sequences were trimmed manually at the same position before being used for further analysis. Phylogenetic trees were constructed using the neighbour-joining algorithm with Kimura's two-parameter distance correction model from the MEGA v4.1 software package. The topologies of the resultant trees were evaluated by bootstrap analysis (Felsenstein, 1985) of 1000 replications.

4.3 RESULTS

4.3.1 Genetic screening of *O. oeni* strains

In this study, a total of 27 *O. oeni* strains were evaluated for the presence of genes encoding enzymes with direct implications on winemaking. The majority of these strains originated from two types of South African wines. Three other representative strains incorporated were of Italian, Japanese and French origin. All PCR assays generated single gene products corresponding to the expected sizes given in Table 4.3. The results of genetic screening (Table 4.4) indicated that the majority of *O. oeni* strains possessed all

the genes tested for, with the exception of the phenolic acid decarboxylase gene that was only present in two strains.

The presence of a *bgl* gene encoding β-glucosidase-related glycosidase was tested with degenerated primers designed after aligning gene sequences of *O. oeni* PSU-1 (accession no. YP_811088) and *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 (accession no. YP_818356). These primers generated PCR fragments with an expected size of 704 bp (internal fragment). PCR detection results indicated that all *O. oeni* strains possessed the *bgl* gene.

The genes (*metB*, *metC*, *metK* and *gshR*) encoding enzymes that act on sulphur-containing amino acids were also tested. The entire population of *O. oeni* strains investigated in this study possessed all these four genes. In addition, the genes related to citrate utilisation and diacetyl synthesis were also detected in all strains during PCR detection assays: *maeP*, *citD*, *citE*, *citF*, *alsD* and *alsS*. To detect the three *arc* genes (i.e. *arcA*, *arcB* and *arcC*) encoding proteins involved in arginine metabolism, the primers described by Araque *et al*. (2009) were employed. Of the entire group of strains tested, all possessed the *arcA* gene encoding arginine deiminase protein. However, the *arcB* (encoding ornithine transcarbamylase) and *arcC* (encoding carbamate kinase) genes were only detected in 23 strains.

4.3.2 Characterization of the *pad* gene in two *O. oeni* strains

In this study, *O. oeni* strains were also tested for the presence of a phenolic acid decarboxylase (*pad*) gene using degenerate primer set previously reported by Mtshali *et al*. (2010). These primers were designed from the gene sequences of various LAB species representing *Bacillus*, *Lactobacillus*, *Pediococcus* and *Lactococcus*. Of the entire group of *O. oeni* strains tested in this study, only two (i.e. IWBT B055 and IWBT B056) yielded PCR amplicons of an expected 210-bp size.

For sequence verification, genomic DNA from the two strains was subjected to PCR with the same set of primers. Purified PCR-generated fragments were sequenced and compared to other published DNA sequences. The obtained nucleotide sequences of the two *O. oeni* strains showed high similarity (>99% identity) to one another. The sequence similarity search was also performed in GenBank database to determine the closest relatives. When the DNA sequence of IWBT B056 strain was compared against PSU-1, ATCC BAA-1163 and AWRI B429 genomes of *O. oeni* strains, the BLAST results yielded no hits. This might suggest that this putative gene is unique to IWBT B055 and IWBT B056.

The *pad* gene sequence of IWBT B056 was also compared to nucleotide sequences of other closely related species. The highest identity was 74–80% between the obtained DNA

sequences and those published in GenBank database. Figure 4.1 shows the alignment of the putative *pad* gene of IWBT B056 strain with other bacterial sequences. Due to high similarity (>99% identity) between the *pad* gene sequences of the two *O. oeni* strains (IWBT B055 and IWBT B056), only one strain was considered in the alignment. From the sequence alignments, it was observed that *Lactobacillus hilgardii* (accession no. AF257158) and *Lactobacillus crispatus* (accession no. AF257159) have one additional nucleotide each in their partial *pad* gene sequences.

4.3.3 Sequence and phylogenetic analyses

Among the group of *O. oeni* strains investigated in this study, four representative strains from each type of wine were selected for comparative analysis of gene sequences. PCR fragments of the corresponding genes were sequenced, and the newly determined nucleotide sequences were compared with each other. The gene sequences of *O. oeni* IWBT B040 were used to search for sequence similarity with database sequences. Table 4.5 shows the percentage identity of gene sequences between IWBT B040 strain and other *O. oeni* strains published in GenBank database. From the percentage identities indicated in the table, it is clear that IWBT B040 is genetically similar (98–100% identity) to three other *O. oeni* strains published in GenBank database.

The construction of phylogenetic trees was inferred by using the neighbour-joining method that uses the distance matrix approach. In order to asses the confidence of phylogenetic relationships, 1000 replicates were conducted. The phylogenetic analyses performed were based on nucleotide sequences of the four randomly selected genes as examples: *alsS*, *estA*, *metK* and *mleA* (Figure 4.2). In the phylogenetic analysis of the four latter genes, it was worth noting that the representative *O. oeni* strains clustered together with other *O. oeni* strains whose gene sequences were extracted from GenBank database. Phylogenetic relationships were also studied by comparing the newly determined sequences with those of closely related species belonging to *Leuconostoc*. The analysis of phylogenies revealed that *O. oeni* strains are distinct from *Leuconostoc* species.

4.3.4 Nucleotide sequences

The nucleotide sequences of *alsD*, *alsS*, *metK*, *metC*, *metB*, *gshR*, *estA*, *prtP* and *maeP* genes (complete open reading frames) from *O. oeni* IWBT B040 strain will be deposited in GenBank/EMBL/DDBJ databases. Accession numbers will be assigned to individual genes once the nucleotides sequences have been deposited to the databases.

4.4 DISCUSSION

One of the most important mechanisms by which malolactic bacteria, more specifically *O. oeni*, contribute to changes in wine organoleptic quality is by producing a wide range of

enzymatic activities capable of degrading precursor components present in wine during fermentation. The presence of genes encoding some of these enzymes has been reported by several authors (Mills *et al*. 2005; Araque *et al*. 2009; Borneman *et al*. 2010; Knoll *et al*. 2011). Using a PCR detection approach, we have also shown that a population of *O. oeni* strains originating from South African wines possess a wide range of genes encoding enzymes of oenological relevance.

A β-glucosidase gene in *O. oeni* strains tested in this study was detected using degenerated primers deduced from nucleotide sequences of *O. oeni* PSU-1 (locus_tag: OEOE_1569) and *Leuc. mesenteroides* subsp. *mesenteroides* ATCC 8293 (locus_tag: LEUM_0875). Previously, Spano *et al*. (2005) also detected a β-glucosidase gene in *O. oeni* strain using the primers deduced from the nucleotide sequences of a putative β-glucosidase gene previously identified on *Lactobacillus plantarum* WCFS1 (accession no. AL935262). However, when these primers were tried in this study, none of the tested strains yielded a signal. An *in silico* analysis of *O. oeni* PSU-1 (accession no. CP000411), ATCC BAA-1163 (AAUV00000000) and AWRI B429 (ACSE00000000) genomes also revealed that these strains do not possess the *O. oeni* β-glucosidase gene identified by Spano *et al*. (2005). Recently, Borneman *et al*. (2010) reported that, in comparison to PSU-1 and ATCC BAA-1163 genomes, the AWRI B429 strain appears to have numerous additional DNA sequences including two genes encoding glycosyl hydrolases, which might play a role in releasing desirable flavour precursors from glycosylated components present in the grape juice (Sarry and Gunata 2004). The presence of the *bgl* gene in a wine-originated strain of *O. oeni* (ATCC BAA-1163) has also been reported by Michlmayr *et al*. (2010).

In relation to the presence of *arc* genes (*arcA*, *arcB* and *arcC*) encoding arginine deiminase (ADI) pathway enzymes, the majority of the tested *O. oeni* strains presented these genes, albeit *arcB* and *arcC* genes were not detected in some strains. Other strains of *O. oeni* have previously been shown to also possess the *arc* genes related to the metabolism of arginine (Tonon *et al*. 2001; Dong *et al*. 2002; Divol *et al*. 2003; Araque *et al*. 2009), which is one of the major amino acids present in wine (Liu 2002). It has also been reported that the presence of *arc* genes and the ability to degrade arginine is strain-dependent (Divol *et al*. 2003; Araque *et al*. 2009). According to Tonon *et al*. (2001), the *arc* cluster (i.e. *arcABC*) is organised in an operon structure with the promoter regions found upstream *arcA* and *arcC*, but not *arcB*. However, some strains may have an additional *arcA* gene of slightly different sequence. In addition, Araque *et al*. (2009) also found a correlation between the presence of only the *arcA* gene and the accumulation of citrulline in the medium, suggesting that the strains not possessing the other two genes (*arcB* and *arcC*) have the genetic potential to spoil wines by forming ethyl carbamate. The latter compound is usually formed in a non-enzymatic reaction when citrulline, or carbamyl phosphate, combines with ethanol (Ough *et al*. 1988).

The absence of the *pad* gene encoding a phenolic acid decarboxylase in *O. oeni* strains was recently demonstrated by De las Rivas *et al*. (2009). These authors developed a PCR assay using degenerated oligonucleotides to test *O. oeni* strains for the *pad* gene. From the PCR detection assays, none of the tested strains yielded the expected PCR product. However, this study has demonstrated the existence of the *pad* gene in two *O. oeni* strains (out of 27 strains tested) of South African wine origin (IWBT B055 and IWBT B056). From our knowledge, this study is the first to report on the presence of a putative (novel) *pad* gene in oenological *O. oeni* strains. Sequence verification was also performed, and the comparative sequence analysis revealed that the *pad* gene from the two strains shares 74–80% identity to other closely related species of the genera *Lactobacillus* and *Pediococcus*. No identity was recorded with database *O. oeni* strains (PSU-1, ATCC BAA-1163 and AWRI B429). These findings could suggest that the *pad* gene in IWBT B055 and IWBT B056 might have been acquired via the horizontal gene transfer from another genus or species. Its presence in only two strains also reinforces the idea that this gene is not essential for *O. oeni* survival.

Among the strains that possessed all the genes tested for, with the exception of the *pad* gene, one strain (IWBT B040) was selected for sequence verification. The nucleotide sequences of the corresponding genes were also compared against DNA sequences of other *O. oeni* strains published in GenBank (Table 4.5). Comparative analysis of gene sequences indicated that there is a high level of genetic similarity between IWBT B040 and other strains of *O. oeni*. These results were also correlated with the phylogenetic relationships between *O. oeni* strains and other closest relatives (Fig. 4.2). The phylogenetic analysis revealed that *O. oeni* strains cluster together, but distinct from closely related species of the genus *Leuconostoc*.

In summary, this work has reported on the presence of genes encoding enzymes of oenological relevance in *O. oeni* strains of South African wine origin. Using a PCR-based detection method, we demonstrated that *O. oeni* strains possess a vast array of enzyme-encoding genes related to winemaking. In addition, a putative *pad* gene encoding phenolic acid decarboxylase was also detected in two *O. oeni* strains, and to our knowledge, this is the first study to report on the presence of this gene in *O. oeni*. However, it remains to be further determined if the two strains could potentially produce volatile phenols in wine. Altogether, this study should be followed by expression and enzymatic studies in order to assess whether the genetic potential translates to functional enzymatic activity, particularly with regards to aroma production under oenological conditions. In addition, the follow-up studies should also confirm if the high identity values observed among gene sequences of certain *O. oeni* strains translates to similarities in enzymatic activities.

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4.6 REFERENCES

Araque, I., Gil, J., Carreté, R., Bordons, A. and Reguant, C. (2009) Detection of *arc* genes related with the ethyl carbamate precursors in wine lactic acid bacteria. *J Agric Food Chem* **57**, 1841–1847.

Bartowsky, E.J. and Henschke, P.A. (2004) The 'buttery' attribute of wine–diacetyl–desirability, spoilage and beyond. Int *J Food Microbiol* **96**, 235–252.

Borneman, A.R., Bartowsky, E.J., McCarthy, J. and Chambers, P.J. (2010) Genotypic diversity in *Oenococcus oeni* by high-density microarray comparative genome hybridization and whole genome sequencing. *Appl Microbiol Biotechnol* **86**, 681–691.

De las Rivas, B., Rodríguez, H., Curiel, J.A., Landete, J.M. and Muñoz, R. (2009) Molecular screening of wine lactic acid bacteria degrading hydroxycinnamic acids. *J Agric Food Chem* **57**, 490–494.

Dicks, L.M.T., van Vuuren, H.J.J. and Dellaglio, F. (1990) Taxonomy of *Leuconostoc* species, particularly *Leuconostoc oenos*, as revealed by numerical analysis of total soluble cell protein patterns, DNA base compositions, and DNA-DNA hybridizations. *Int J Syst Bacteriol* **40**, 83–91.

Divol, B., Tonon, T., Morichon, S., Gindreau, E. and Lonvaud-Funel, A. (2003) Molecular characterization of *Oenococcus oeni* genes encoding proteins involved in arginine transport. *J Appl Microbiol* **94**, 738–746.

Dong, Y., Chen, Y.Y., Snyder, J.A. and Burne, R.A. (2002) Isolation and molecular analysis of the gene cluster for the arginine deiminase system from *Streptococcus gordonii* DL1. *Appl Environ Microbiol* **68**, 5549–5553.

Du Plessis, H.W., Dicks, L.M.T., Pretorius, I.S., Lambrechts, M.G. and Du Toit, M. (2004) Identification of lactic acid bacteria isolated from South African brandy base wines. *Int J Food Microbiol* **91**, 19–29.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Garmyn, D., Monnet, C., Martineau, B., Guzzo, J., Cavin, J.F. and Diviès, C. (1996) Cloning and sequencing of the gene encoding α-acetolactate decarboxylase from *Leuconostoc oenos*. *FEMS Microbiol Lett* **145**, 445–450.

Knoll, C., Du Toit, M., Schnell, S., Rauhut, D. and Irmler, S. (2011) Cloning and characterisation of a cystathionine β/γ-lyase from two *Oenococcus oeni* oenological strains. *Appl Microbiol Biotechnol* **89**, 1051–1060.

Krieling, S.J. (2003) An investigation into lactic acid bacteria as a possible cause of bitterness in wine. Masters Thesis. Stellenbosch University, Stellenbosch, South Africa.

Kumar, S., Nei, M., Dudley, J. and Tamura, K. (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* **9**, 299–306.

Kunkee, R.E. (1998) Some roles of L-malic acid in the malolactic fermentation. *FEMS Microbiol Rev* **8**, 55–72.

Lerm, E., Engelbrecht, L. and Du Toit, M. (2010) Malolactic Fermentation: The ABC's of MLF. *S Afr J Enol Vitic* **31**, 186–212.

Liu, S.-Q. (2002) Malolactic fermentation in wine – beyond deacidification. *J Appl Microbiol* **92**, 589–601.

Michlmayr, H., Schümann, C., Wurbs, P., Barreira Braz da Silva, N.M., Rogl, V., Kulbe, K.D. and del Hierro, A.M. (2010) A β-glucosidase from *Oenococcus oeni* ATCC BAA-1163 with potential for aroma release in wine: cloning and expression in *E. coli*. *World J Microbiol Biotechnol* **26**, 1281–1289.

Mills, D.A., Rawsthorne, H., Parker, C., Tamir, D. and Makarova, K. (2005) Genomic analysis of *Oenococcus oeni* PSU-1 and its relevance to winemaking. *FEMS Microbiol Rev* **29**, 465–475.

Mtshali, P.S., Divol, B., van Rensburg, P. and Du Toit M. (2010) Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines. *J Appl Microbiol* **108**, 1389–1397.

Ough, C.S., Crowell, E.A. and Gutlove, B.R. (1988) Carbamyl compound reactions with ethanol. *Am J Enol Vitic* **39**, 239–242.

Sarry, J.E. and Gunata, Z. (2004) Plant and microbial glycoside hydrolases: volatile release from glycosidic aroma precursors. *Food Chem* **87**, 509–521.

Spano, G., Rinaldi, A., Ugliano, M., Moio, L., Beneduce, L., Massa, S. (2005) A β-glucosidase gene isolated from wine *Lactobacillus plantarum* is regulated by abiotic stresses. *J Appl Microbiol* **98**, 855–861.

Swiegers, J.H., Bartowsky, E.J., Henschke, P.A. and Pretorius, I.S. (2005) Yeast and bacterial modulation of wine aroma and flavour. *Aust J Grape Wine Res* **11**, 139–173.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **24**, 4876–4882.

Tonon, T., Bourdineaud, J.P. and Lonvaud-Funel, A. (2001) The *arcABC* gene cluster encoding the arginine deaninase pathway of *Oenococcus oeni*, and arginine induction of a CRP-like gene. *Res Microbiol* **152**, 653–661.

Table 4.1 *Oenococcus oeni* strains investigated in this study

Strain names[a]	**Origin**
IWBT B021, IWBT B022, IWBT B030, IWBT B040, IWBT B041, IWBT B037, IWBT B035, IWBT B038, IWBT B036	Isolated from red grape and wine samples in wineries from the Western Cape region, South Africa (Krieling 2003)
IWBT B017, IWBT B018, IWBT B016, IWBT B013, IWBT B014, IWBT B011, IWBT B027, IWBT B023, IWBT B024, IWBT B026, IWBT B053, IWBT B055, IWBT B056, IWBT B054, IWBT B050	Isolated from brandy base wines (Du Plessis *et al*. 2004)
NCDO 1894	Isolated from Italian wine[b]
NCDO 2122	Isolated from Japanese wine[b]
DSMZ 20252^T	Isolated from French wine[b]

[a] Culture collections: IWBT, Institute for Wine Biotechnology, Stellenbosch University, South Africa; NCDO, National Collection of Dairy Organisms, UK; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany.

[b] Information was obtained from the relevant literature (Dicks *et al*. 1990).

Table 4.2 A list of primers used in this study

Gene	Description	Primer sequence (5′→3′)[a]	Reference
mleA	Malolactic enzyme	F-GTTGAATTYATGCCAATYGTKTATGA R-GCTTTWGCTTCKGCTAAYTTDGTTGG	This work
bgl	β-Glucosidase-related glycosidase	F-GAAAAAGCNGAWCTDGTWTCBGGNAAAGAT R-CCAGGCATTTCBAARTCYADACCDGCTTT	This work
estA	Predicted esterase	F-ATGGCATTTTTAGAAGTTAATTATTATTCACG R-CTATGACAAACGTTTTTCTGCTTGATAATT	This work
prtP	Serine protease	F-GTGACTGAAGAACAAGACCAAGGAAAAAC R-TTATTGTTTCAAAGTTTCAGTCATCTTAACCTT	This work
pad	Phenolic acid decarboxylase	F-AARAAYGAYCAYACYRTTGATTACC R-TTCTTCWACCCAYTTHGGGAAGAA	Mtshali *et al*. 2010
citD	Citrate lyase γ-subunit	F-ATGGAAATTAARAMAACKGCAKTMGC R-GCYGCYGTAATRGTYGKYGCYTTWAT	Mtshali *et al*. 2010
citE	Citrate lyase β-subunit	F-TTACGBCGSACRATGATGTTTGT R-TATTTTTCAATGTAATTDCCCTCC	Mtshali *et al*. 2010
citF	Citrate lyase α-subunit	F-ATGGYATGACRATTTCWTTYCAYCAYCA R-ATCAATVAHBSWRCCRTCRCGRTAYTC	Mtshali *et al*. 2010
maeP	Putative citrate transporter	F-ATGGGTGTTTTTTGGACATCG R-TCAAATAAAGTTGATGATACTCATTA	This work
alsD	α-Acetolactate decarboxylase	F-ATGAAAGATTTAACAAAAGCTTATC R-TTATTCTGTCTTTTCAATCGCTT	This work
alsS	α-Acetolactate synthase	F-ATGACAGAAAAGAAACGTTTTGGG R-TTAATCCATATCTCCTTCGATCAATTC	This work
metK	S-adenosylmethionine synthase	F-ATGAAAAAGTTTTTTACGAGTGAGTCGG R-TTAATTTGCCAAAAGAGCTTTAATTTTTTC	This work
metC	Cystathionine β-lyase	F-ATGACAGAATCCGATTGG R-TTAATCCTCCAATGC	This work
metB	Cystathionine γ-lyase	F-ATGAAATTCAATACAAAACTTATTCATG R-CTAAATCTTGCTGAATGAC	This work
gshR	Glutathione reductase	F-ATGAAAAACCAGCAATATG R-TTACAATTGGCCGGCTG	This work
arcA	Arginine deiminase	F-CAYGCNATGATGCAYYTNGAYACNGT R-GTRTTNSWNCCRTCRTTCCAYTGYTC	Araque *et al*. 2009
arcB	Ornithine transcarbamylase	F-ATGCAYTGYYTNCCNGCNTTYCAYGA R-CCNARNGTNGCNGCCATDATNGCYTT	Araque *et al*. 2009
arcC	Carbamate kinase	F-CAYGGNAAYGGNCCNCARGTNGGNAA R-CKNCKNYANCCNCKNCCNGCRTCYTC	Araque *et al*. 2009

[a] **F** – forward primer; **R** – reverse primer.

Table 4.3 PCR thermocycling conditions

Gene	Size (bp)	Initial denaturing	Cycles	Denaturing	Annealing	Extension	Final extension
mleA	989	94 °C, 3 min	35	94 °C, 30 s	54 °C, 1 min	72 °C, 1 min	72 °C, 10 min
bgl	704	94 °C, 5 min	30	94 °C, 1 min	53 °C, 45 s	72 °C, 1 min	72 °C, 10 min
estA	804	94 °C, 5 min	35	94 °C, 1 min	50 °C, 1 min	72 °C, 1 min	72 °C, 10 min
prtP	1278	94 °C, 5 min	35	94 °C, 1 min	52 °C, 1 min	72 °C, 1 min	72 °C, 10 min
pad	210	94 °C, 2 min	35	94 °C, 40 s	50 °C, 1 min	72 °C, 30 s	72 °C, 5 min
citD	245	94 °C, 3 min	35	94 °C, 45 s	54 °C, 30 s	72 °C, 1 min	72 °C, 5 min
citE	897	94 °C, 3 min	35	94 °C, 30 s	54 °C, 1 min	72 °C, 1 min	72 °C, 10 min
citF	1331	94 °C, 5 min	35	94 °C, 1 min	49 °C, 45 s	72 °C, 1 min	72 °C, 10 min
maeP	984	94 °C, 5 min	35	94 °C, 1 min	49 °C, 45 s	72 °C, 1 min	72 °C, 10 min
alsD	717	94 °C, 5 min	35	94 °C, 1 min	49 °C, 45 s	72 °C, 1 min	72 °C, 10 min
alsS	1683	94 °C, 5 min	35	94 °C, 1 min	49 °C, 45 s	72 °C, 1 min	72 °C, 10 min
metK	1167	94 °C, 2 min	30	94 °C, 1 min	48 °C, 1 min	72 °C, 1 min	72 °C, 5 min
metC	1134	94 °C, 2 min	30	94 °C, 1 min	45 °C, 1 min	72 °C, 1 min	72 °C, 5 min
metB	1137	94 °C, 2 min	30	94 °C, 1 min	46 °C, 1 min	72 °C, 1 min	72 °C, 5 min
gshR	1332	94 °C, 2 min	30	94 °C, 1 min	50 °C, 1 min	72 °C, 1 min	72 °C, 5 min
arcA	266	94 °C, 5 min	30	94 °C, 1 min	55 °C, 45 s	72 °C, 1 min	72 °C, 10 min
arcB	181	94 °C, 5 min	30	94 °C, 1 min	49 °C, 45 s	72 °C, 1 min	72 °C, 10 min
arcC	343	94 °C, 5 min	30	94 °C, 1 min	49 °C, 45 s	72 °C, 1 min	72 °C, 10 min

Table 4.4 Results of PCR detection of different enzyme-encoding genes in a population of *O. oeni* strains

	***Oenococcus oeni* strains**[a]			
Target gene	**IWBT (24 isolates)**	**NCDO 1894**	**NCDO 2122**	**DSMZ 20252**^{T}
mleA	24[b]	1	1	1
bgl	24	1	1	1
estA	24	1	1	1
prtP	24	1	1	1
pad	2	0	0	0
citD	24	1	1	1
citE	24	1	1	1
citF	24	1	1	1
maeP	24	1	1	1
alsD	24	1	1	1
alsS	24	1	1	1
metK	24	1	1	1
metC	24	1	1	1
metB	24	1	1	1
gshR	24	1	1	1
arcA	24	1	1	1
arcB	20	1	1	1
arcC	20	1	1	1

[a] Refer to Table 1 for full names of culture collections.
[b] The total number of positive strains.

Table 4.5 Identities found between DNA sequences of *O. oeni* IWBT B040 against those of other published *O. oeni* genomes

	% identity of *O. oeni* IWBT B040 gene sequences with those of		
Target gene	***O. oeni* PSU-1**	***O. oeni* ATCC BAA-1163**	***O. oeni* AWRI B429**
mleA	100%[a] (YP_811084[b])	100% (ZP_01544753)	99.9% (ZP_06554202)
bgl	99.7% (YP_811088)	99.4% (ZP_01543735)	99.8% (ZP_06554209)
estA	100% (YP_810319)	100% (ZP_01544534)	100% (ZP_06553286)
prtP	99.8% (YP_809767)	99.7% (ZP_01544672)	99.8% (ZP_06552695)
citD	100% (YP_810045)	98.5% (ZP_01544956)	100% (ZP_06552988)
citE	100% (YP_810046)	99.5% (ZP_01544955)	100% (ZP_06552989)
citF	99.9% (YP_810047)	99.8% (ZP_01544954)	100% (ZP_06552990)
maeP	100% (YP_810043)	98.1% (ZP_01544957)	100% (ZP_06552986)
alsD	100% (YP_811215)	99.9% (ZP_01544232)	100% (ZP_06554349)
alsS	99.9% (YP_811214)	99.8% (ZP_01544231)	99.9% (ZP_06554348)
metK	100% (YP_810424)	99.8% (ZP_01543877)	100% (ZP_06553415)
metB	99.6% (YP_811264)	99.6% (ZP_01544504)	99.5% (ZP_06554409)
metC	100% (YP_810358)	99.7% (ZP_01544503)	100% (ZP_06553338)
gshR	100% (YP_810750)	99.9% (ZP_01543773)	100% (ZP_06553795)
arcA	99.6% (YP_810682)	99.2% (ZP_01544331)	99.6% (ZP_06553717)
arcB	None[c]	None	100% (ZP_06554043)
arcC	None	None	100% (ZP_06554042)

[a] Percentage identity.
[b] GenBank accession number.
[c] (None), both *O. oeni* PSU-1 and ATCC BAA-1163 strains do not harbour *arcB* and *arcC* genes.

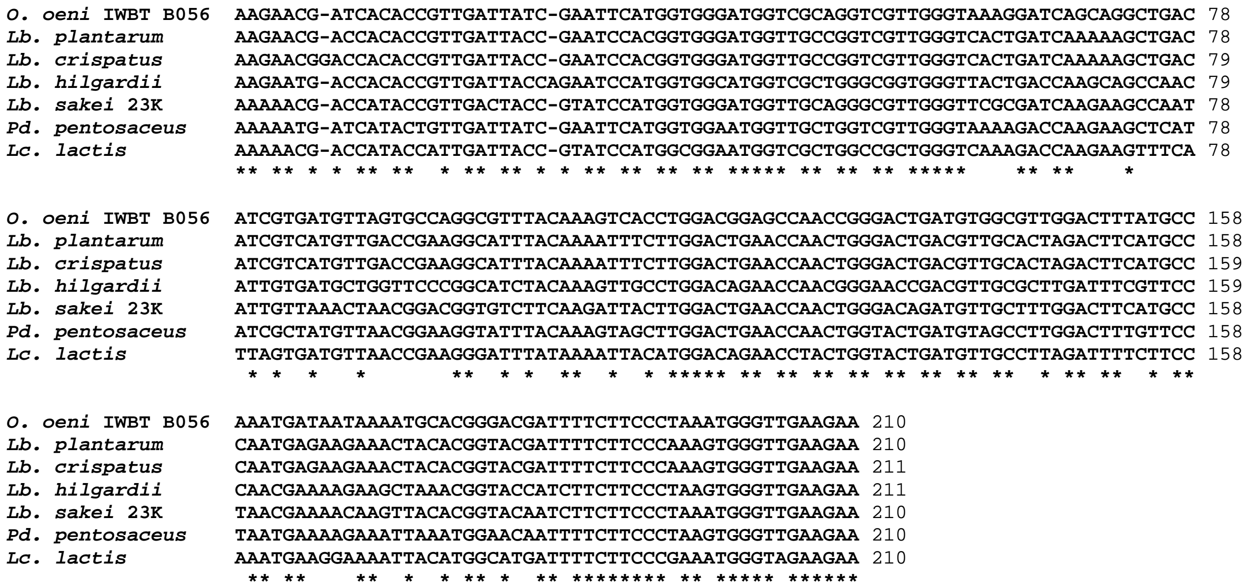


Figure 4.1. Multiple nucleotide sequence alignment of a partial phenolic acid decarboxylase (*pad*) gene of *Oenococcus oeni* IWBT B056 strain with related sequences of other bacterial strains extracted from GenBank databank: *Lactobacillus plantarum* (GenBank accession no. AF257163), *Lactobacillus crispatus* (AF257159), *Lactobacillus hilgardii* (AF257158), *Lactobacillus sakei* 23K (CR936503) *Pediococcus pentosaceus* ATCC 25745 (CP000422) and *Lactococcus lactis* IL1403 (AE005176). Due to high similarity (>99% identity) between the *pad* gene sequences of *O. oeni* IWBT B055 and IWBT B056, only one strain was considered in the alignment. The asterisks indicate highly conserved regions.

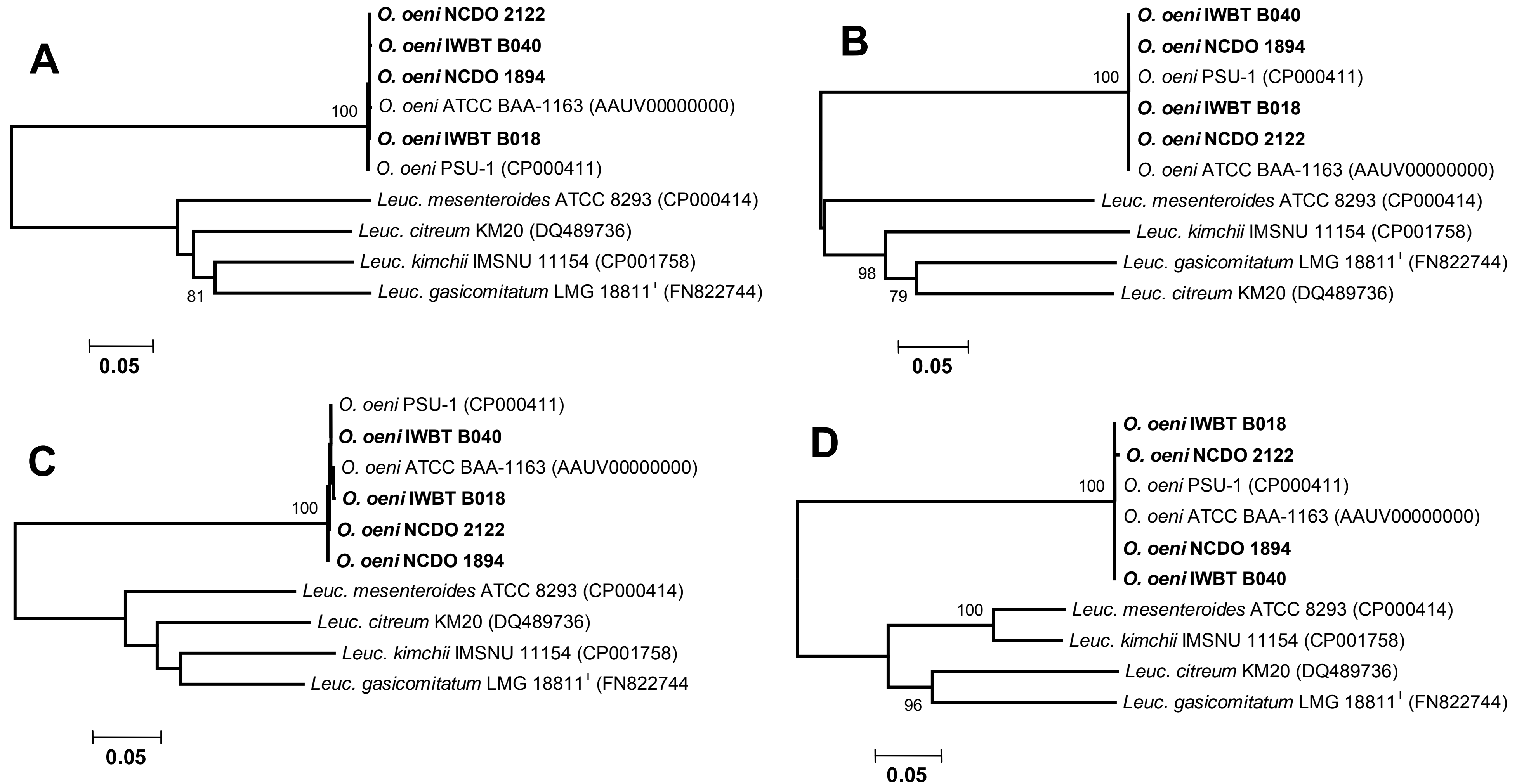
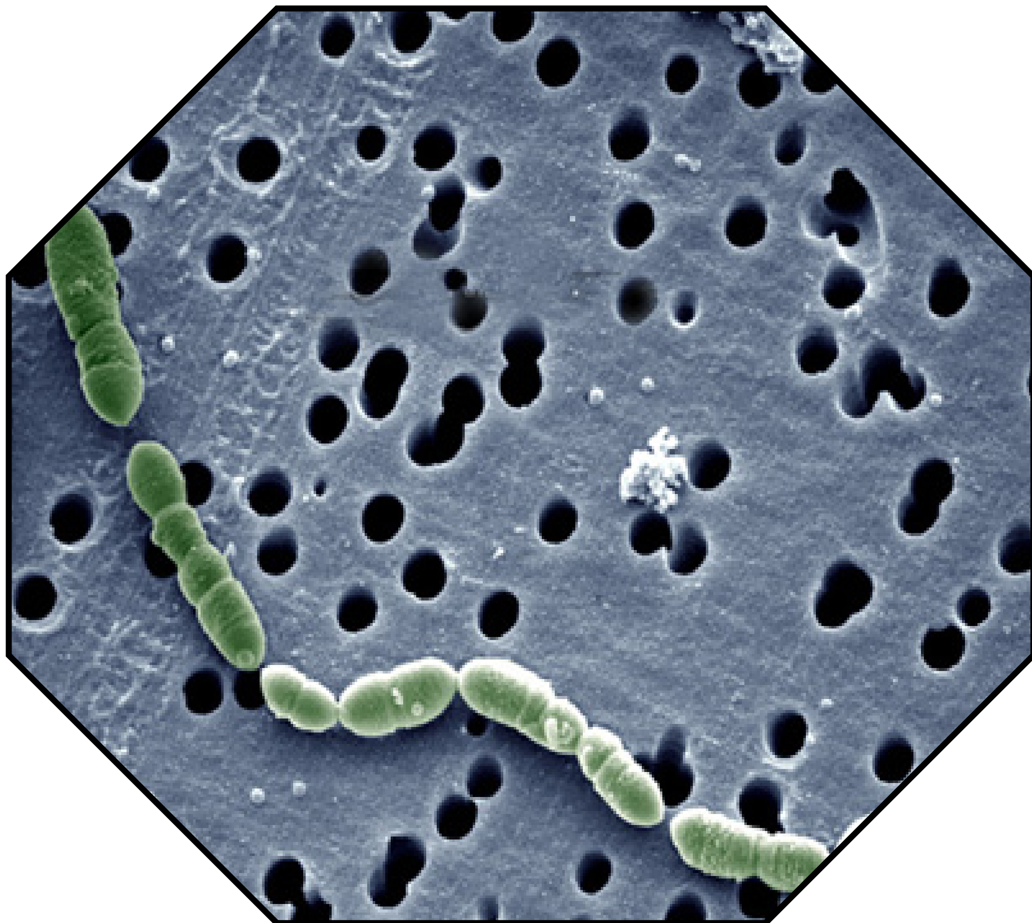


Figure 4.2. Phylogenetic trees showing the relationship between *Oenococcus oeni* strains and the closest relatives (GenBank accession numbers in parentheses) based on the nucleotide sequences of the (a) *alsS* (1508 nt), (b) *estA* (781 nt), (c) *metK* (1085 nt) and (d) *mleA* (914 nt) genes. Numbers at branching points represent the results of bootstrap analyses (expressed as percentages of 1000 replicates) using the neighbour-joining method. The scale bar represents the number of base substitutions per site.

CHAPTER 5

RESEARCH RESULTS III



Expression analysis of *Oenococcus oeni* malolactic enzyme gene under oenological conditions

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CHAPTER 5

Expression analysis of *Oenococcus oeni* malolactic enzyme gene under oenological conditions

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ABSTRACT

Aims: To investigate the effect of combined pH and ethanol on the expression of the malolactic enzyme-encoding gene of *Oenococcus oeni* under winemaking conditions using quantitative real-time PCR (qPCR).

Methods and Results: Three strains of *O. oeni* (IWBT B026, IWBT B053 and Lalvin VP41) were tested in a wine-like medium in order to evaluate the transcriptional response of the *mleA* gene under the conditions of combined pH and ethanol. In all the three strains, the *mleA* gene expression appeared to be negatively affected by high ethanol content in the medium, while low pH seemed to have an enhancing effect towards the expression of the *mleA* gene.

Conclusion: The data obtained in this study showed that, although low pH levels may inhibit the growth of wine LAB, however this condition has a positive impact towards the transcriptional response of the *mleA* gene.

Significance and Impact of the Study: Understanding the regulation of the *mleA* gene under different winemaking conditions will help select the best adapted strains capable of conducting malolactic fermentation to completion.

Keywords: *Oenococcus oeni*, malolactic fermentation, real-time PCR, *mleA* gene

5.1 INTRODUCTION

Oenococcus oeni is the main species of lactic acid bacteria (LAB) most commonly associated with wine during malolactic fermentation (MLF). This secondary fermentation process usually occurs after alcoholic fermentation by yeasts, although it may also occur during alcoholic fermentation. MLF brings about wine microbial stability and also enhances the organoleptic quality of the final product through the modification of aroma-active compounds (Kunkee 1991; Lonvaud-Funel 1999; Lerm *et al*. 2010). The other well-known effect of MLF is to reduce wine acidity via the conversion of a dicarboxylic acid (L-malate) into a monocarboxylic L-lactate and CO_2. The latter reaction is catalysed by the malate decaboxylase enzyme, encoded by the *mleA* gene, in the presence of NAD^+ and Mn^{+2} as co-factors (Bartowsky 2005).

The genetic locus involved in L-malic acid conversion (*mle*) has been identified in *O. oeni* and other LAB (Denayrolles *et al*. 1994; Labarre *et al*. 1996a). The three genes for the malolactic enzyme (*mleA*), malate permease (*mleP*) and regulatory protein (*mleR*) have been cloned and sequenced. These genes are present in a cluster with the *mleA* and *mleP* in a single operon, and the *mleR* transcribed in the opposite direction (Labarre *et al*. 1996a, b). MleR is a LysR-type regulatory protein and its involvement in the expression of *mleAP* remains unclear; no induction or repression of the malolactic enzyme was detected in the presence of L-malate (Labarre *et al*. 1996b).

During fermentation, the wine bacterium *O. oeni* has to cope with harsh physico-chemical conditions prevalent in wine, such as nutrient depletion, low pH, high ethanol content and the presence of SO_2 (Drici-Cachon *et al*. 1996; Davis *et al*. 1988). In such instances, *O. oeni* needs to develop various response mechanisms in order to adapt to these stress factors. One of the mechanisms involved in the adaptation of *O. oeni* to ethanol stress is by adjusting the membrane fluidity (Silveira *et al*. 2003). Previously, the effect of ethanol in *O. oeni* cells was investigated, and was shown to exert a disordering effect on the cytoplasmic membrane of *O. oeni* cells (Silveira *et al*. 2002) and also to trigger alterations in protein patterns of cells (Silveira *et al*. 2004). In addition, the low pH of the fermentation medium also impacts negatively towards the growth of *O. oeni*. The optimum pH reported for *O. oeni* growth is between 4.3 and 4.8 (Britz and Tracey 1990). Although the effect of ethanol on the cell membrane is well documented (Barry and Gawrisch 1994; Silveira *et al*. 2002), little work has been carried out to elucidate the combined effects of ethanol and pH on the viability of *O. oeni*.

The aim of this study was to evaluate the expression patterns of the *mleA* gene in three oenological strains of *O. oeni* using quantitative real-time PCR (qPCR). These strains were tested in a wine-like medium to evaluate the combined effect of ethanol and

pH on the expression of the *mleA* gene under the extreme conditions (i.e. high/low pH and ethanol) prevalent in wine.

5.2 MATERIALS AND METHODS

5.2.1 *Oenococcus oeni* strains

Three strains were chosen for the expression analysis of the *mleA* gene: one commercial strain (Lalvin VP41; Lallemand, Toulouse, France) and two natural *O. oeni* wine strains (IWBT B026 and IWBT B053; Du Plessis *et al.* 2004) from the culture collection of the Institute for Wine Biotechnology (Stellenbosch University, South Africa).

5.2.2 Culture conditions

Oenococcus oeni strains were cultured for four days at 30°C in MRS medium (BIOLAB Diagnostics, Wadeville, South Africa) supplemented with 20% (v/v) apple juice at pH 5.2. Cells were transferred into the adaptation medium (MRS 50 g l^{-1}, fructose 40 g l^{-1}, glucose 20 g l^{-1}, L-malate 4 g l^{-1} and Tween 80 1 g l^{-1}) at pH 4.6 and ethanol 6% (v/v) prior to inoculation into the modified FT80 medium, as described by Beltramo *et al.* (2006), to a final concentration of 10^7 CFU ml^{-1}. Fermentations were incubated at 20°C under the combination of pH and ethanol parameters. Fermentation conditions are shown in Figure 5.1. All fermentations were carried out in triplicate. To monitor the progress of MLF, samples were taken at regular intervals to measure the concentration of L-malic acid using an enzyme-based kit (Roche Diagnostics GmbH, Mannheim, Germany) and to determine the viable cell count by plating out serially-diluted samples on MRS agar plates added with 20% apple juice (pH adjusted to 5.2 with HCl).

5.2.3 RNA extraction

Cells were harvested by centrifugation and the total RNA extraction was performed using the method modified from Hoffman and Winston (1987). Briefly, after performing the chloroform extraction step, the nucleic acid was precipitated at –20°C for 30 min prior to centrifugation at 12,000 rpm for 10 min. The washed pellet was left to dry and then resuspended in DEPC-treated water. All RNA samples were treated with RNase-free DNAse (Roche Diagnostics). RNA concentrations were determined spectrophotometrically using a NanoDrop® ND-1000 (NanoDrop Technologies Inc., Wilmington, USA).

5.2.4 Real-time PCR experiments

cDNA was synthesised from 2 μg of RNA in a total volume of 25 μl using the Improm-II Reverse Transcriptase kit (Promega, Madison, USA) as recommended by the manufacturer. cDNA was amplified by real-time PCR with the *mleA* gene primers described previously (Beltramo *et al.* 2006). The primers 16S-qPCR1 (5′-CCT CGG GAT TTC ACA TCA GAC T-3′) and 16S-qPCR2 (5′-CCA GCA GCC GCG GTA AT-3′) targeting

O. oeni 16S rRNA gene, used as an internal control, were designed with the Primer Express 3.0 software (Applied Biosystems, Johannesburg, South Africa) by applying the default parameters. PCR efficiencies were determined by running a standard curve with serial dilutions of cDNA.

After 50X dilution, 1 μl of cDNA was added to a 20-μl real-time PCR mix containing 0.2 μl of each primer at 1 μM, 8.4 μl of RNAse-free water and 10.2 μl of KAPA SYBR® FAST Universal 2X qPCR Master Mix (Kapa Biosystems, Cape Town, South Africa). Amplifications were performed using a Real Time PCR System 7500 (Applied Biosystems) with the thermal cycling conditions designated as described by Beltramo *et al*. (2006). In each run, a negative control was included. For each measurement, the threshold value was automatically recorded by the instrument during each annealing step. The results were calculated using the comparative critical threshold ($2^{-\Delta\Delta C_T}$) method as described previously (Livak and Schmittgen 2001). The results were normalised with the 16S rRNA gene and are expressed as averages of three independent determinations.

5.2.5 Determination of the *mleA* gene sequences

The full *mleA* gene sequences of the two *O. oeni* strains (VP41 and IWBT B026) were determined by sequencing. DNA was subjected to PCR with the primers mleOE-F (5′-ATG ACA GAT CCA GTA AGT ATT TTA AAT GA-3′) and mleOE-R (5′-TTA GTA TTT CGG CTC CCA CCT-3′) deduced from the nucleotide sequences of the *mleA* gene from *O. oeni* PSU-1 (accession no. YP_811084) and ATCC BAA-1163 (accession no. ZP_01544753). PCR mixture (50 μl) contained 50 ng DNA, 0.6 μM of each primer, 250 μM of dNTP mix, 1X *Ex Taq* buffer and 1.25 units of *TaKaRa Ex Taq* DNA polymerase (Southern Cross Biotechnology, Cape Town, South Africa). The reaction conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 49°C for 45 s and 72°C for 1 min. The final extension step was carried out at 72°C for 10 min. PCR amplifications were analyzed by gel electrophoresis in 1% (w/v) agarose gel stained with ethidium bromide (0.5 μg ml^{-1}).

PCR-generated fragments were purified with QIAquick® PCR Purification kit (Southern Cross Biotechnology) and cloned into pGEM-T® Easy vector (Promega) following the instructions by the manufacturer. *Escherichia coli* DH5α cells grown in Luria-Bertani medium (LB; BIOLAB Diagnostics) supplemented with 100 μg ml^{-1} of ampicillin were used as the host for cloning procedures. Sequencing of the positive clones was performed by the Central DNA Sequencing Facility of Stellenbosch University, South Africa. The nucleotide sequences of the full *mleA* gene from VP41 and IWBT B026 will be submitted to GenBank database.

5.3 RESULTS AND DISCUSSION

5.3.1 Sequencing of *mleA* genes

The *mleA* genes of two *O. oeni* strains (IWBT B026 and VP41) tested in this study were amplified using the primers targeting the full *mleA* gene and subsequently cloned into the vector prior to sequencing. The obtained nucleotide gene sequences were aligned and compared to other DNA sequences of *O. oeni* strains published in GenBank database. The *mleA* sequence of IWBT B026 strain showed 99% identity to that of *O. oeni* PSU-1 (accession no. YP_811084), ATCC BAA-1163 (ZP_01544753) and AWRI B429 (ZP_06554202). On the other hand, the *mleA* nucleotide sequence of the commercial strain VP41 that we determined was 100% identical to that of AWRI B429, which is also VP41, and 99% identical to PSU-1 and ATCC BAA-1163.

5.3.2 Monitoring the evolution of MLF

The progress of MLF was monitored by measuring the concentration of L-malic acid from samples taken at regular intervals throughout the fermentation. MLF performance differed between the strains and under different fermentation conditions. In the absence of ethanol (at pH 3.2 and pH 3.8), all three strains (IWBT B026, IWBT B053 and VP41) finished MLF by day six (Figure 5.2). At 12% v/v ethanol, MLF was completed at day nine for IWBT B026 and VP41, and at day 13 for strain IWBT B053. At pH 3.8 and 15% v/v ethanol, the consumption of L-malic acid was poor for strains IWBT B053 and VP41 compared to IWBT B026; the latter strain completed MLF at day nine under this condition. However, at pH 3.2 in the presence of 15% v/v ethanol, IWBT B026 finished fermentation after day 20.

Apart from following the degradation patterns of L-malic acid, MLF was also monitored by performing the viable cell counts. It was observed from the cell numbers that the growth of bacterial cells was favoured in the absence of ethanol and at higher pH (i.e. pH 3.8). At 12% v/v ethanol, there was no significant loss of cell viability; the cell numbers remained above 10^6 CFU ml^{-1} until MLF was complete (data not shown). On the other hand, an increase in ethanol concentration retarded the proliferation of bacterial cells, and this was the case for the ethanol parameter of 15% v/v. Nevertheless, strain IWBT B026 seemed to be little affected by high ethanol content compared to IWBT B053 and VP41 (Figure 5.3).

5.3.3 Expression analysis of the *mleA* gene

The combined effect of pH and ethanol on *mleA* gene expression in *O. oeni* strains IWBT B026, IWBT B053 and VP41 was investigated using quantitative real-time PCR. The fermentation conditions included pH 3.2 and pH 3.8, as well as 0, 12 and 15% v/v ethanol. Comparisons of the relative expression of *mleA* were made between the strains under various fermentation conditions tested in this study.

Figure 5.4 shows the comparisons of the relative expression of the *mleA* gene of the two natural strains of *O. oeni* (IWBT B026 and IWBT B053) at day 2 and day 6 of MLF. Fifteen percent ethanol at pH 3.2 and pH 3.8 was used as the reference condition. At pH 3.2 (in both days), the *mleA* expression of IWBT B053 was significantly higher than that of IWBT B026 strain. At day 2 (pH 3.8), the expression of the *mleA* gene appeared to be lower for both strains in comparison to day 6. In addition, *mleA* gene expression was favoured at pH 3.2 than at pH 3.8 in day 2. The same trend was also seen in day 6, although the differences in relative expression between pH 3.2 and pH 3.8 were less pronounced.

Relative expression between the middle and the end of MLF was also compared using pH 3.2 and pH 3.8 with 15% v/v ethanol as the reference condition. At pH 3.2 in the middle of MLF (Figure 5.5A), the expression of *mleA* gene of IWBT B053 strain was higher than that of the other two strains (IWBT B026 and VP41). However, at pH 3.8, there were no differences observed in *mleA* gene expression between the strains (Figure 5.5B).

At the end of MLF, there was different *mleA* expression patterns observed between all the strains tested. The relative expression of the *mleA* gene of IWBT B053 remained higher under various fermentation conditions tested (Figure 5.6A, B). It was also noted that, at pH 3.2 with 12% v/v ethanol, *mleA* gene expression was higher for IWBT B053 compared to pH 3.8 with 12% v/v ethanol.

In summary, the results presented in this study have shown the differential expression patterns of the *mleA* gene of three *O. oeni* strains under various fermentation conditions simulating those of winemaking. From the results, it was demonstrated that the relative expression of the *mleA* gene is mostly favoured at low pH (i.e. pH 3.2) in comparison to pH 3.8. This is in accordance with the findings of Beltramo *et al.* (2006) who observed the enhancing effect of low pH towards the *mleA* gene expression when the tested strain was grown in a synthetic medium simulating wine-like conditions. In addition, the differences in the expression of the *mleA* gene were also reported previously (Beltramo *et al.*, 2006; Olguín *et al.*, 2010). On the other hand, high ethanol concentration of 15% v/v appeared to impact negatively on gene expression as the *mleA* gene relative expression was lower in this condition.

Generally, the presence of ethanol in combination with low pH are known to impact negatively towards the growth of malolactic bacteria, and the bacterial cells undergo a rapid death if exposed to these adverse conditions (G-Alegría *et al.* 2004). The optimum pH reported for the growth of *O. oeni* is above 4.0, and the acidic conditions result to the inhibition of bacterial growth (Britz and Tracey 1990). However, moderate levels of ethanol (10% v/v) may have a stimulatory effect on bacterial growth (G-Alegría *et al.* 2004) but not on the expression of the *mleA* gene. The findings from this study have clearly indicated

that the expression of the *mleA* gene is negatively affected by high ethanol content. In addition, the pH of 3.2 appeared to enhance gene expression in comparison to a less acidic pH condition of 3.8.

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5.5 REFERENCES

Barry, J.A. and Gawrisch, K. (1994) Direct NMR evidence for ethanol binding to the lipid–water interface of phospholipid bilayers. *Biochemistry* **33**, 8082–8808.

Bartowsky, E.J. (2005) *Oenococcus oeni* and malolactic fermentation – moving into the molecular arena. *Aust J Grape Wine Res* **11**, 174–187.

Beltramo, C., Desroche, N., Tourdot-Maréchal, R., Grandvalet, C. and Guzzo, J. (2006) Realtime PCR for characterizing the stress response of *Oenococcus oeni* in a wine-like medium. *Res Microbiol* **157**, 267–274.

Britz, T.J. and Tracey, R.P. (1990) The combination e!ect of pH, SO_2, ethanol and temperature on the growth of *Leuconostoc oenos*. *J Appl Bacteriol* **68**, 23–31.

Davis, C.R., Wibowo, D., Fleet, G.H. and Lee, T.H. (1988) Properties of wine lactic acid bacteria: their potential enological significance. *Am J Enol Vitic* **39**, 137–142.

Denayrolles, M., Aigle, M. and Lonvaud-Funel, A. (1994) Cloning and sequence analysis of the gene encoding *Lactococcus lactis* malolactic enzyme: relationships with malic enzymes. *FEMS Microbiol Lett* **116**, 79–86.

Drici-Cachon, Z., Guzzo, J., Cavin, F. and Diviès, C. (1996) Acid tolerance in *Leuconostoc oenos*. Isolation and characterisation of an acid-resistant mutant. *Appl Microbiol Biotechnol* **44**, 785–789.

Du Plessis, H.W., Dicks, L.M.T., Pretorius, I.S., Lambrechts, M.G. and Du Toit, M. (2004) Identification of lactic acid bacteria isolated from South African brandy base wines. *Int J Food Microbiol* **91**, 19–29.

G-Alegría, E., Lopez, I., Ruiz, J.I., Saenz, J., Fernandez, E., Zarazaga, M., Dizy, M., Torres, C. and Ruiz-Larrea, F. (2004) High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. *FEMS Microbiol Lett* **230**, 53–61.

Hoffman, C.S. and Winston, F. (1987) A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**, 267–272.

Kunkee, R.E. (1991) Some roles of malic acid in the malolactic fermentation in wine making. *FEMS Microbiol Rev* **88**, 55–72.

Labarre, C., Guzzo, J., Cavin, J.-F. and Divies, C. (1996a) Cloning and characterization of the genes encoding the malolactic enzyme and the malate permease of *Leuconostoc oenos*. *Appl Environ Microbiol* **62**, 1274–1282.

Labarre, C., Divies, C. and Guzzo, J. (1996b) Genetic organization of the mle locus and identification of a *mleR*-like gene from *Leuconostoc oenos*. *Appl Environ Microbiol* **62**, 4493–4498.

Lerm, E., Engelbrecht, L. and Du Toit, M. (2010). Malolactic Fermentation: The ABC's of MLF. *S Afr J Enol Vitic* **31**, 186–212.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* **25**, 402–408.

Lonvaud-Funel, A. (1999) Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie van Leeuwenhoek* **76**, 317–331.

Olguín, N., Bordons, A. and Reguant, C. (2010) Multigenic expression analysis as an approach to understanding the behaviour of *Oenococcus oeni* in wine-like conditions. *Int J Food Microbiol* **144**, 88–95.

Silveira, M.G., Baumgärtner, M., Rombouts, F.M. and Abee, T. (2004) Effect of adaptation to ethanol on cytoplasmic and membrane protein profiles of *Oenococcus oeni*. *Appl Environ Microbiol* **70**, 2748–2755.

Silveira, M.G., Golovina, E.A., Hoekstra, F.A., Rombouts, F.M. and Abee, T. (2003) Membrane fluidity adjustments in ethanol-stressed *Oenococcus oeni* cells. *Appl Environ Microbiol* **69**, 5826–5832.

Silveira, M. G., San Romao, M.V., Loureiro-Dias, M.C., Rombouts, F.M. and Abee, T. (2002) Flow cytometric assessment of membrane integrity of ethanol-stressed *Oenococcus oeni* cells. *Appl Environ Microbiol* **68**, 6087–6093.

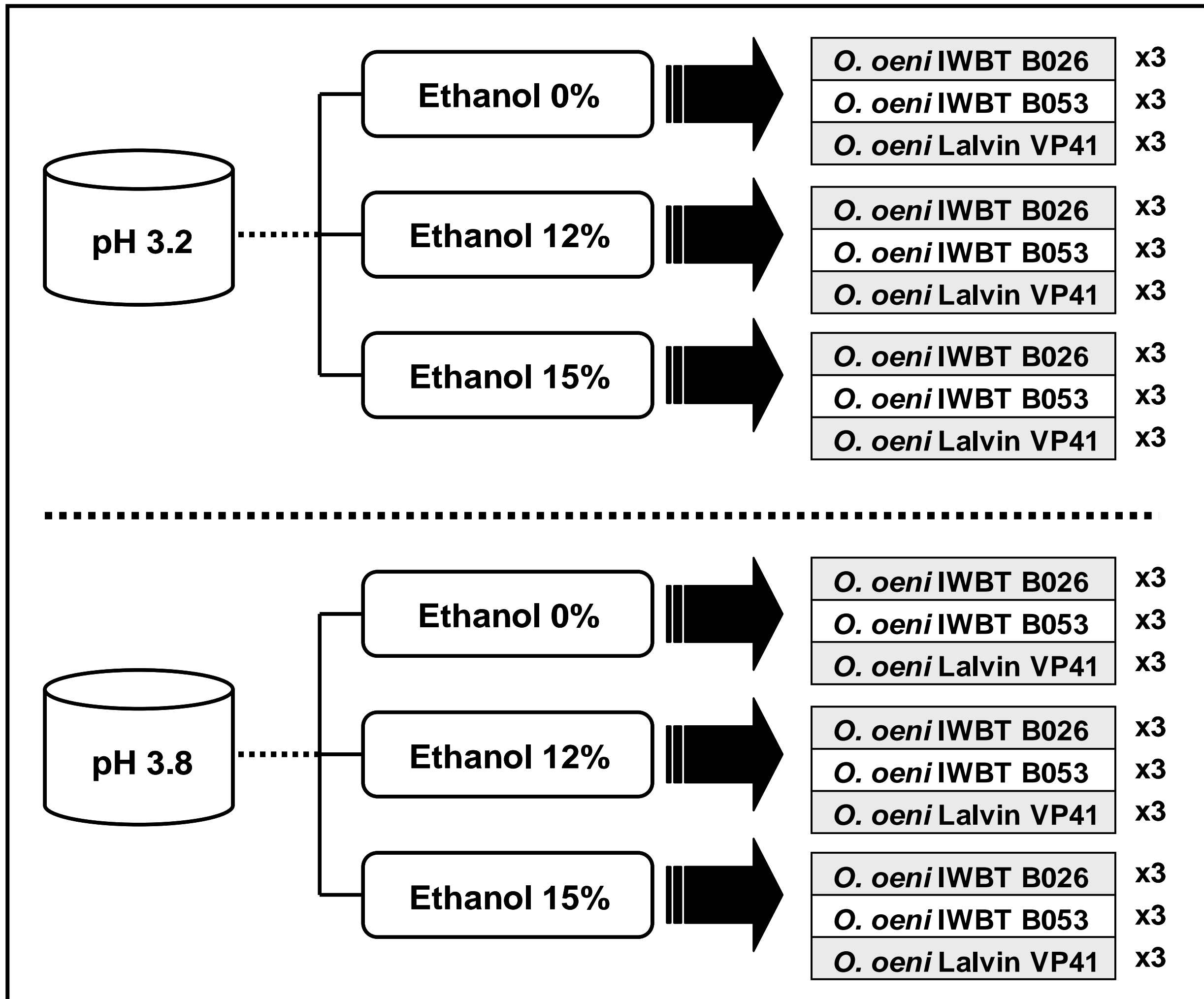

Figure 5.1. Experimental layout showing different conditions under which malolactic fermentation was performed. All fermentations, conducted with three *Oenococcus oeni* strains (IWBT B026, IWBT B053 and Lalvin VP41), were performed in triplicate for each parameter.

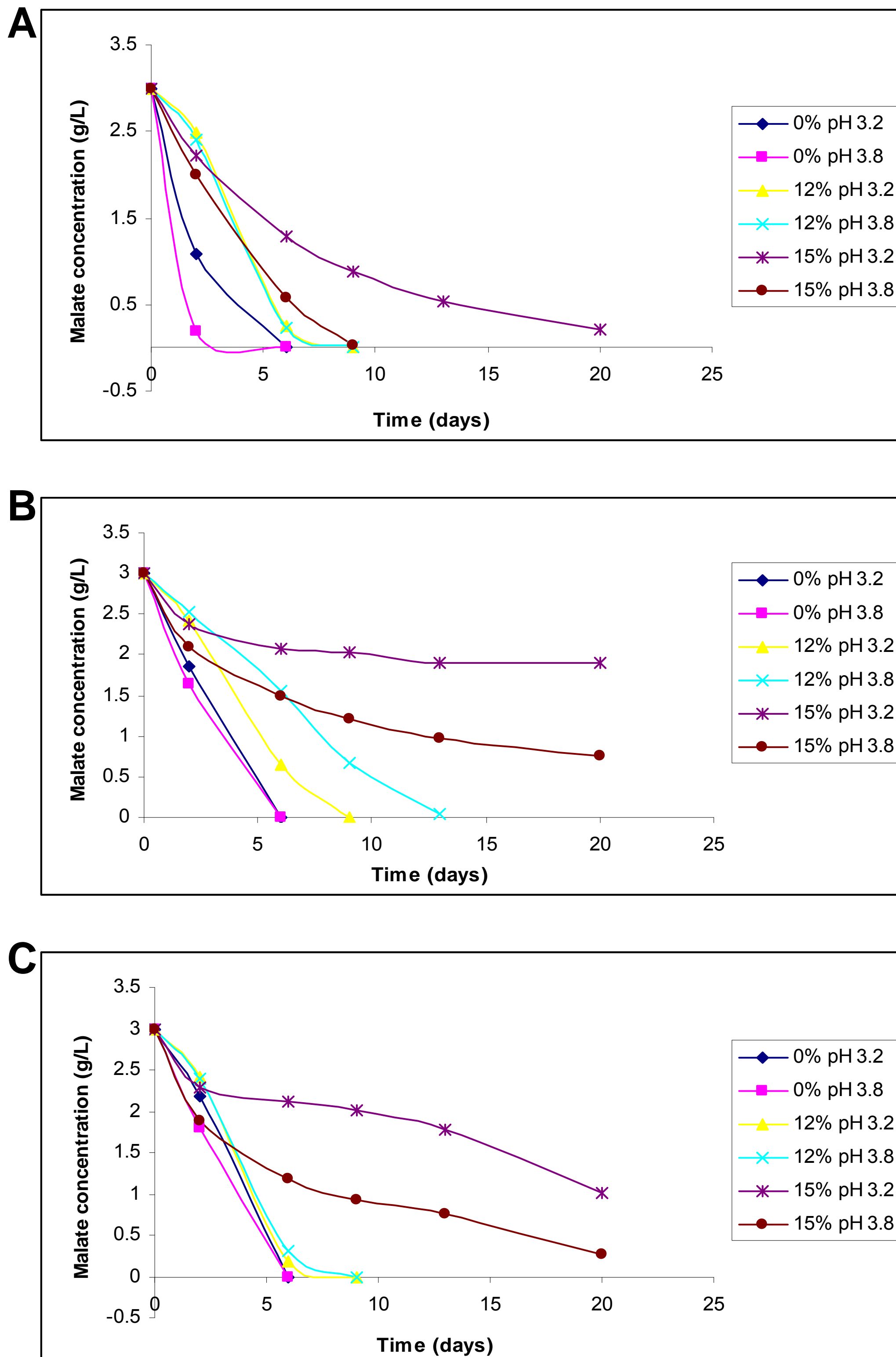

Figure 5.2. Evolution of L-malic acid consumption during MLF by *Oenococcus oeni* strains IWBT B026 (A), IWBT B053 (B) and Lalvin VP41 (C).

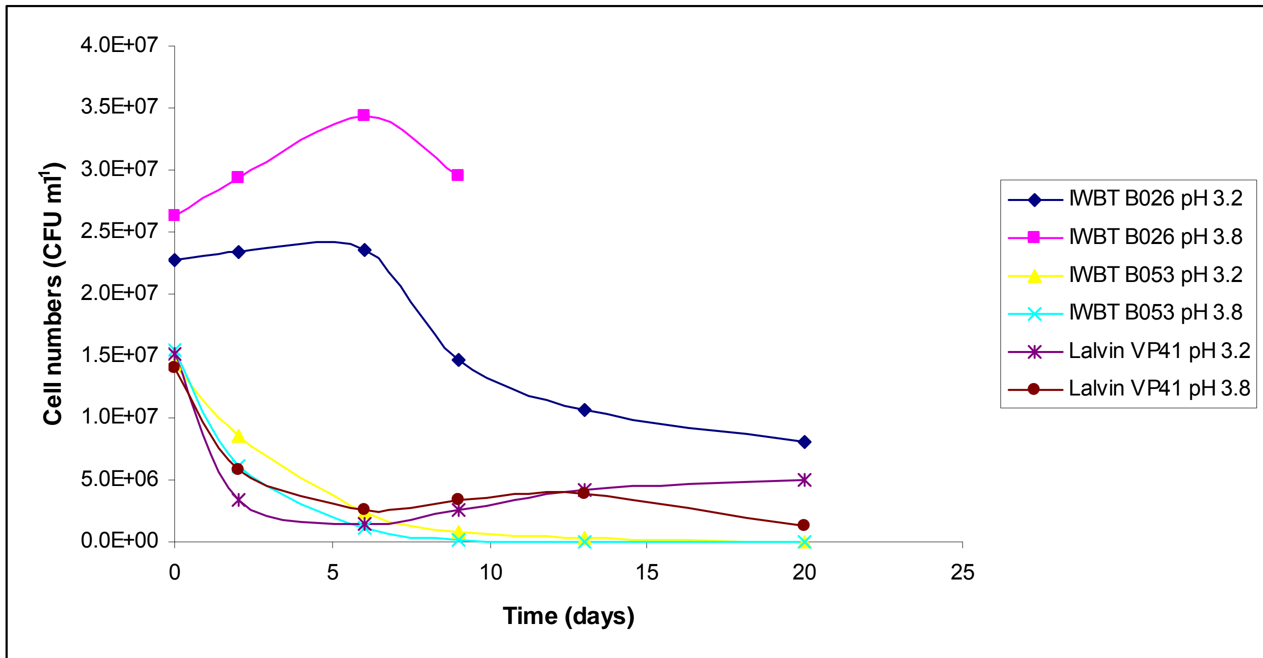

Figure 5.3. The bacterial cells numbers (CFU ml^{-1}) for *Oenococcus oeni* strains IWBT B026, IWBT B053 and Lalvin VP41 for the MLF with 15% v/v ethanol.

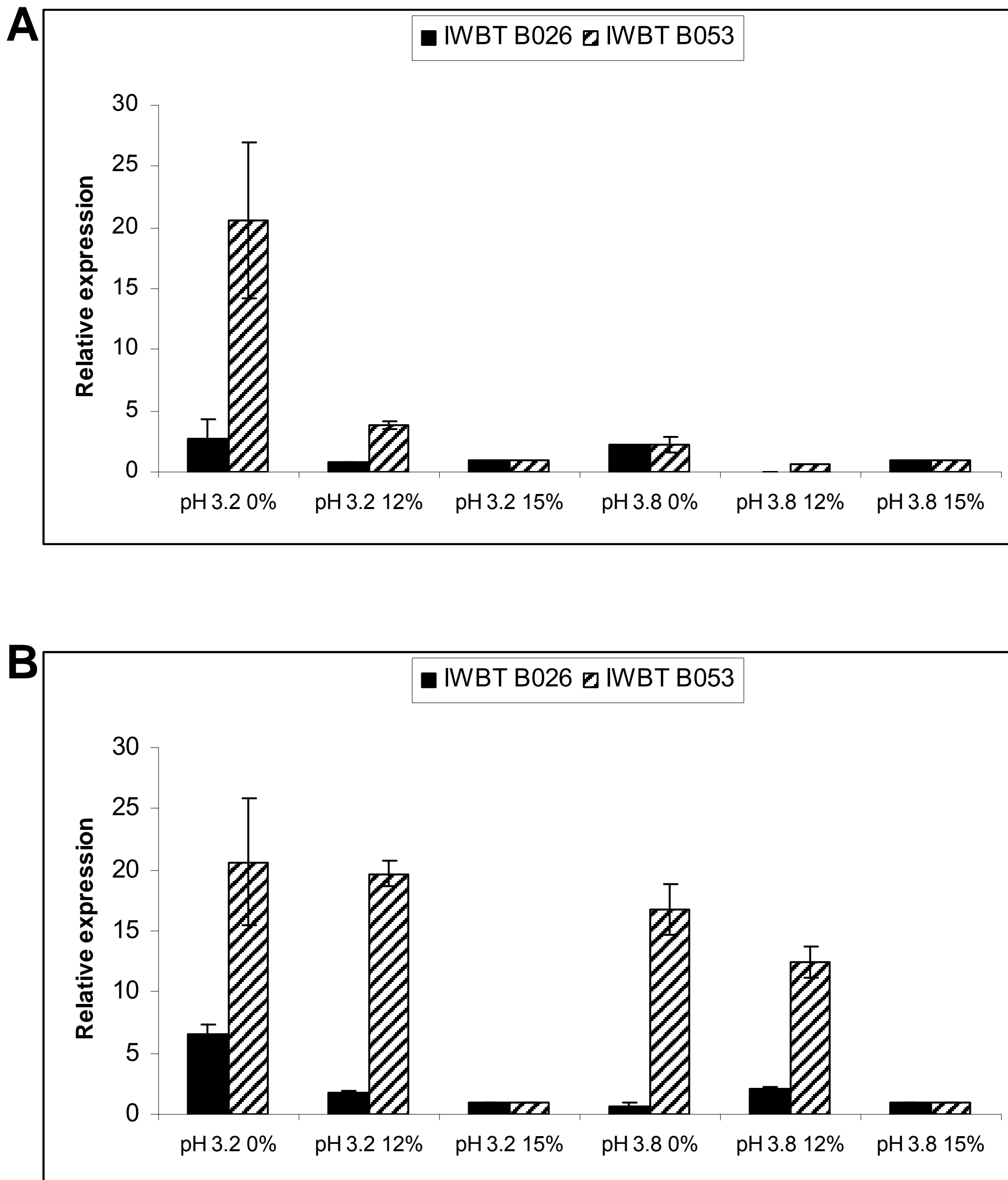

Figure 5.4. Comparison of the relative expression of the *mleA* gene at day 2 (A) and day 6 (B) for *O. oeni* strains IWBT B026 and IWBT B053. Ethanol of 15% v/v and pH 3.2 or pH 3.8 were used as the reference condition. Data shown are mean values with standard deviations (n = 3).

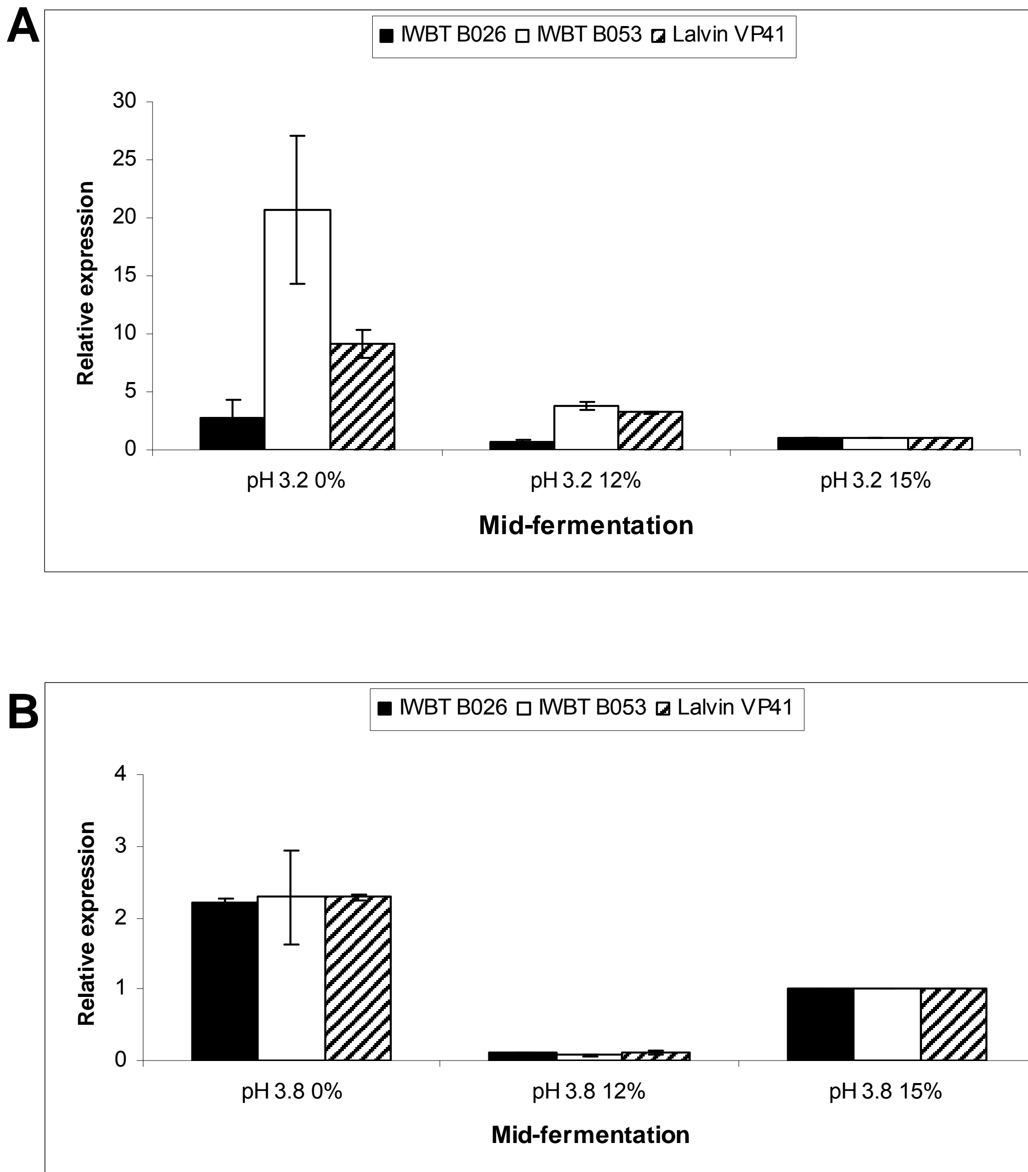

Figure 5.5. Relative expression of the *mleA* gene in a wine-like medium in the middle of MLF for pH 3.2 (A) and pH 3.8 (B) in the presence of different ethanol concentrations. The growth at 15% v/v ethanol and pH 3.2 or pH 3.8 was defined as the calibrator.

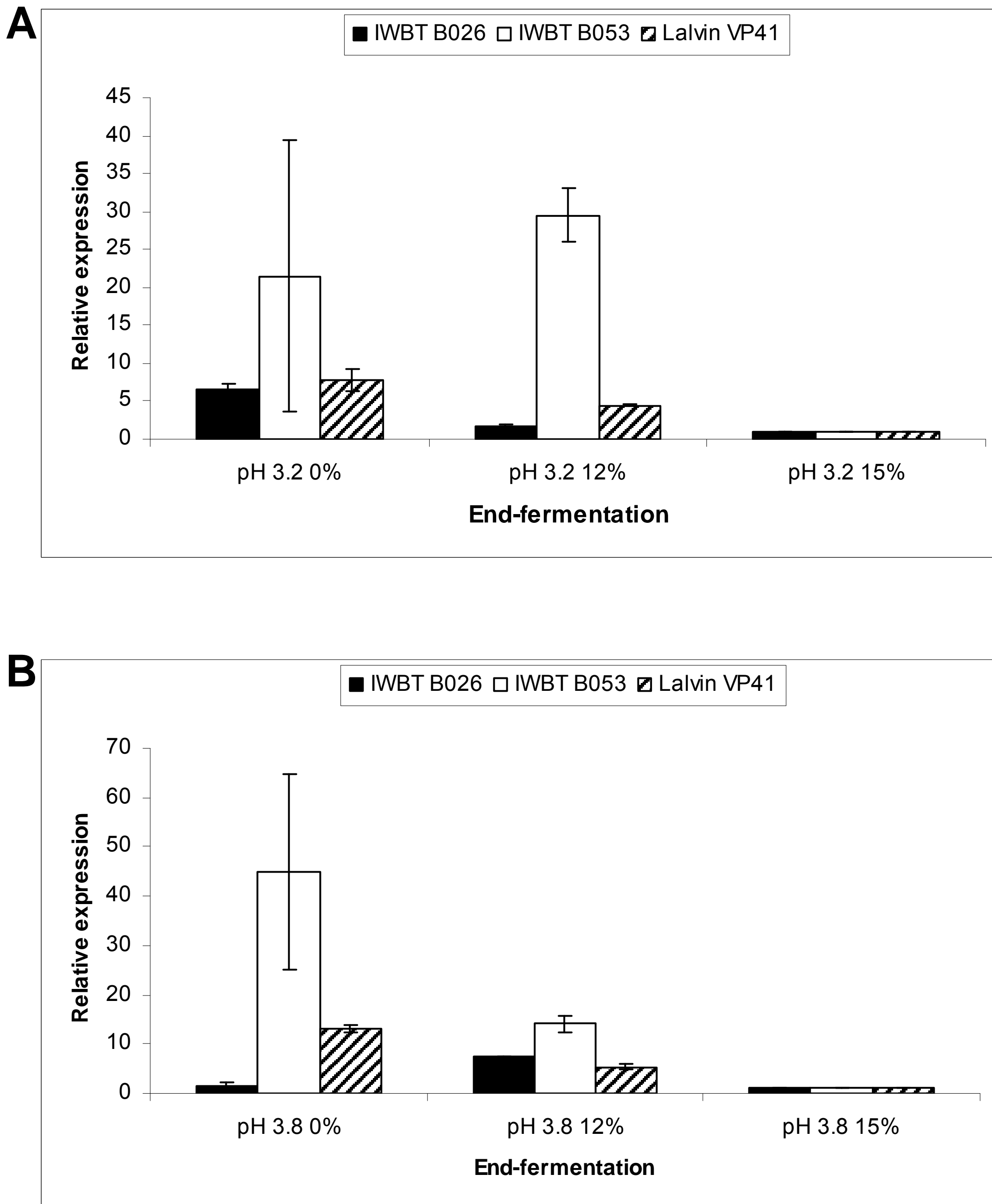

Figure 5.6. Relative expression of the *mleA* gene in a wine-like medium at the end of MLF for pH 3.2 (A) and pH 3.8 (B) in the presence of different ethanol concentrations. The growth at 15% v/v ethanol and pH 3.2 or pH 3.8 was defined as the calibrator.

CHAPTER 6

RESEARCH RESULTS IV

PCR detection of enzyme-encoding genes in *Leuconostoc mesenteroides* strains of wine origin

This manuscript will be submitted for publication to
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CHAPTER 6

PCR detection of enzyme-encoding genes in *Leuconostoc mesenteroides* strains of wine origin

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ABSTRACT

Fifteen isolates of lactic acid bacteria originating from South African grape and wine samples were identified as *Leuconostoc mesenteroides* subsp. *mesenteroides* through the taxonomic analysis of their 16S rDNA gene sequences. These isolates were further tested for the presence of genes coding for enzymes of oenological relevance using PCR detection technique. A type strain of *L. mesenteroides* (NCDO 529^T) was also incorporated for comparative analysis. From the PCR detection results, the *estA*, *prtP*, *alsD*, *alsS*, *metK*, *metC* and *metB* genes were present in all the strains tested. The *bgl* and *gshR* genes encoding β-glucosidase and glutathione reductase, respectively, were not detected in some strains. On the other hand, none of the tested strains possessed the genes encoding phenolic acid decarboxylase (*pad*), citrate permease (*citP*), citrate lyase (*citD*, *citE* and *citF*) and arginine deiminase pathway enzymes (*arcA*, *arcB* and *arcC*). The verification of PCR-generated fragments was performed by sequencing. GenBank database was used to search for homologous DNA sequences. Neighbour-joining trees based on nucleotide sequences of *alsS*, *estA*, *metK* and *mleA* genes were also constructed in order to study the phylogenetic relationship between *L. mesenteroides* strains and closely related species. Taken together, this study has improved our knowledge on the genetics of oenological strains of *L. mesenteroides*.

Keywords: *Leuconostoc mesenteroides*, PCR detection, genes, sequencing, wine

6.1 INTRODUCTION

Leuconostoc strains, including *Leuconostoc mesenteroides*, occupy a variety of niches including plants and fermented food products (Hemme and Foucaud-Scheunemann 2004). In dairy technology, for example, leuconostocs are linked to the production of flavour compounds (e.g. diacetyl) from citrate metabolism (Vedamuthu 1994). Although the genetics of *Leuconostoc* strains are poorly characterized, Bekal and others (1998) successfully cloned and characterized the *citCDEFG* gene cluster in a *L. mesenteroides* strain. Recently, sucrose phosphorylase gene from *L. mesenteroides* was cloned and expressed in *Escherichia coli* (Lee and others 2006, 2008). In addition, Zhang and others (2008) reported on the cloning, sequencing and expression of *dex*YG gene encoding dextransucrase, an enzyme that catalyzes the transfer of D-glucosyl units from sucrose to acceptor molecules, thus producing α-dextran as one of the by-products.

As such, *L. mesenteroides* is among a group of Gram-positive lactic acid bacteria commonly found in wine during malolactic fermentation. Despite the occurrence of *L. mesenteroides* in the wine matrix, however, there is still limited information on the role and impact of this bacterium in winemaking. In addition, the presence of a wide array of enzyme-encoding genes in wine-associated *L. mesenteroides* strains has not been well-characterized. The only genes that have been detected by a PCR approach are *arcA* and *arcB* genes encoding arginine deiminase and ornithine transcarbamylase enzymes, respectively (Araque and others 2009). The latter enzymes are involved in arginine metabolism via the arginine deiminase pathway. This is the extent of our knowledge regarding the presence of enzyme-encoding genes in *L. mesenteroides* strains associated with winemaking.

Therefore, this study was aimed at evaluating several isolates of *L. mesenteroides* for the presence of genes encoding enzymes of relevance in winemaking. The taxonomic identification of these isolates to the subspecies level was performed through the 16S rDNA gene sequence analysis. PCR-amplified fragments from randomly selected strains were also sequenced for verification and for studying the phylogenetic relationships between the strains.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial strains and growth conditions

A total of 15 strains were examined in this study. These strains were previously isolated during the 2001 and 2002 vintages from five different commercial wineries situated in the Western Cape region (South Africa) and identified as *L. mesenteroides* using species-specific primers (Krieling 2003). A type strain (*L. mesenteroides* NCDO 529^{T}) was also included in this study for comparative analysis. All strains were cultured on

MRS medium (BIOLAB Diagnostics, Wadeville, South Africa) and incubated at 30 °C until the colonies were clearly visible on the plates.

6.2.2 Identification of isolates to subspecies level

The subspecies of the *L. mesenteroides* isolates was identified through the taxonomic analysis of the 16S rDNA gene sequences. DNA templates (colony cells) were subjected to PCR in a Biometra Thermoblock (Biometra® GmbH, Göttingen, Germany) using the universal primers EubA (5′-AAG GAG GTG ATC CAN CCR CA-3′) and EubB (5′-AGA GTT TGA TCM TGG CTC AG-3′) targeting a 1500-bp region of the 16S rDNA (Cottrel and Kirchman 2000). PCR mixture (50 μL) contained template DNA, 0.7 μM each primer, 250 μM dNTP mix, 1X PCR buffer and 1.5 units of TaKaRa *Ex Taq*™ DNA polymerase (Southern Cross Biotechnology, Cape Town, South Africa). PCR amplification conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR-generated amplicons were checked on 1% (w/v) ethidium bromide-stained agarose gels, cleaned with Qiagen PCR Purification kit (Southern Cross Biotechnology) and then sequenced using the forward and reverse primers. Homology search of the closest relatives was performed with the NCBI database.

6.2.3 PCR assays with gene-specific primers

The primers used for PCR detection of genes are listed in Table 6.1. Colony PCR assays were performed in 25-μL reaction mixtures containing 0.6 μM of each primer, 1.5 mM of $MgCl_2$, 250 μM of dNTP mix, 1.25 units of Supertherm DNA polymerase (Southern Cross Biotechnology) and 1 X Supertherm buffer. PCR mixtures were cycled in a Biometra Thermoblock (Biometra® GmbH, Göttingen, Germany). PCR thermocycling conditions were as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 45-54 °C for 45 s and 72 °C for 1 min (annealing temperatures of the different reactions are reflected in Table 6.1). A final extension was performed at 72 °C for 10 min. PCR-amplified fragments were resolved on agarose gels containing ethidium bromide (0.5 μg/mL) and documented with UV transilluminator.

6.2.4 Sequence verification

To verify PCR-amplified fragments, one strain (IWBT B290) was selected for sequencing. The 50-μL PCR mix comprised template DNA, 0.6 μM of each primer, 250 μM of dNTPs, 1 X PCR buffer and 1.25 U of TaKaRa *Ex Taq*™ polymerase (Southern Cross Biotechnology). PCR mixtures were cycled and analysed as described above. PCR products were purified with QIAquick® PCR Purification Kit (Southern Cross Biotechnology), cloned into pGEM-T® Easy vector (Promega, Madison, WI, USA) and then sequenced by the Central DNA Sequencing Facility (Stellenbosch University, South Africa). The obtained nucleotide gene sequences were aligned and compared to those available in GenBank database.

6.2.5 Phylogenetic analyses

To study phylogenetic relationship between *L. mesenteroides* subsp. *mesenteroides* strains (IWBT B288, IWBT B290, IWBT B296, IWBT B298 and IWBT B301) of wine origin and closely related species, the nucleotide sequences of four randomly selected genes (*mleA*, *alsS*, *metK* and *estA*) were used to construct the phylogenetic trees. DNA sequences of the closest relatives were retrieved from GenBank database. Multiple sequence alignments were created with the CLUSTAL_X software (Thompson and others 1997). Phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei 1987) of the MEGA program version 4.1 (Kumar and others 2008) with Kimura's two-parameter distance correction model (Kimura 1980). Bootstrapping analysis was used to test the reliability of the topologies using 1000 replications (Felsenstein 1985).

6.2.6 Nucleotide sequences

The nucleotide sequences of the *mleA*, *bgl*, *estA*, *prtP*, *alsD*, *alsS*, *metK*, *metB*, *metC* and *gshR* genes of *L. mesenteroides* IWBT B290 strain will be submitted to DDBJ/EMBL/GenBank databases.

6.3 RESULTS AND DISCUSSION

6.3.1 Species identification

The taxonomic identification of *L. mesenteroides* isolates to the subspecies level was performed by determining their 16S rDNA gene sequences. These sequences were aligned and compared to other DNA sequences of the closest relatives published in GenBank database. The homology search of the obtained nucleotide sequences against database sequences revealed that our strains are highly similar (>99% identity) to other strains of *L. mesenteroides* subsp. *mesenteroides*: ATCC 8293 (accession no. CP000414, locus tag LEUM_r0019), MGB93-1 (HM218010), NM183-5 (HM218762), NM28-7 (HM218148) and NM27-4 (HM218137).

The phylogenetic relationship between the studied isolates and their closest relatives was also studied by constructing a neighbour-joining tree based on nucleotide gene sequences of the 16S rDNA (1440 nt). As shown in Figure 6.1, *L. mesenteroides* isolates tested in this study (indicated in bold) clustered together with other *L. mesenteroides* subsp. *mesenteroides* strains published in GenBank database, suggesting that our isolates belong to the subspecies *mesenteroides*.

6.3.2 PCR detection of genes

A total of 16 strains were tested for the presence of genes encoding enzymes of oenological relevance using a PCR detection method. PCR assays generated single PCR

products of the expected sizes reflected in Table 6.2. Results of PCR amplifications are also shown in Table 6.2.

From the PCR detection results, it is worth noting that the *estA*, *prtP*, *alsD*, *alsS*, *metK*, *metC* and *metB* genes were present in all *L. mesenteroides* strains tested. In contrast, none of the *arcA*, *arcB*, *arcC*, *citD*, *citE*, *citF*, *citP* and *pad* genes were detected. In addition, a type strain NCDO 529^T strain did not yield a positive signal for the *bgl* and *mleA* genes encoding β-glucosidase and malolactic enzyme, respectively.

Of all the strains tested for the presence of the *gshR* gene, only 12 yielded a positive signal. A type strain NCDO 529^T also did not present the *gshR* gene. The latter gene encodes glutathione reductase involved in cell defense against oxygen stress by maintaining a high intracellular glutathione (GSH) (Jänsch and others 2007). Although GSH is absent in most Gram-positive bacteria lacking the *gshA* or *gshAB* genes required for GSH synthesis (Lee and others 2010), certain other lactic acid bacteria are known for their ability to accumulate glutathione (Wiederholt and Steele 1994). The major physiological role of this compound in bacteria is to protect the cells against acid stress (Riccillo and others 2000), osmotic stress (Smirnova and others 2001), toxic electrophiles (Ferguson 1999) and oxidative stress (Carmel-Harel and Storz 2000; Smirnova and others 1999).

In relation to the presence of the *arcABC* gene cluster encoding proteins involved in arginine metabolism, all the *L. mesenteroides* strains tested in this study did not possess any of these genes. From the literature, all species in the genus *Leuconostoc* are described as non-arginine-degrading (Hemme and Foucaud-Scheunemann 2004). Surprisingly, Araque and others (2009) demonstrated the presence of either *arcA* or *arcB* in *L. mesenteroides* strains originating from olives and wine. These authors also found a correlation between the presence of *arcA* gene and the formation of citrulline from arginine metabolism. These results therefore suggest that arginine-degrading ability in *L. mesenteroides* species is rather strain-dependent.

To detect the presence of a *bgl* gene coding for β-glucosidase protein, degenerated primers were designed to amplify an internal 704-bp region (partial gene). Table 6.2 shows that only 44% (7/16 strains) of the tested strains possessed this gene. Although this study has demonstrated the presence of the *bgl* gene in *L. mesenteroides* strains, it remains unknown if this gene yields an active enzyme under oenological conditions given that most β-glucosidases in wine are regulated by winemaking parameters (Spano and others 2005). This aspect therefore merits further investigation.

Of the entire *L. mesenteroides* strains tested for the presence of the *pad* gene, none possessed this gene. These results are in agreement with the findings of De las Rivas and

others (2009) who developed a PCR assay for the detection of the *pad* gene from several wine LAB species including *L. mesenteroides*. In their study, De las Rivas and others (2009) also found a correlation between the presence of the *pad* gene encoding a phenolic acid decarboxylase and volatile phenol production.

The three genes (*citD*, *citE* and *citF*) encoding enzymes involved in citrate metabolism pathway were also investigated in this study. However, none of the *L. mesenteroides* strains possessed these genes. All the strains were either tested with degenerated primers described by Mtshali and others (2010) or by using the oligonucleotides (designed in this study) based on the *citDEF* gene cluster of *L. mesenteroides* subsp. *cremoris* strain described by Bekal and others (1998) (data not shown). In both instances, none of the strains tested positive. An *in silico* analysis of *L. mesenteroides* subsp. *mesenteroides* ATCC 8293 genome (accession no. CP000414) also revealed that this strain does not harbour the citrate lyase gene cluster. Nevertheless, Bekal and others (1998) successfully cloned and characterized the *citCDEFG* gene cluster in a *L. mesenteroides* subsp. *cremoris* strain originating from the dairy industry. In addition, no *citP* gene was detected in all strains tested in this study. The *citP* gene encodes citrate permease enzyme, which mediates the uptake of citrate into the cell.

6.3.3 Sequence and phylogenetic analyses

The verification of PCR-generated fragments was performed by sequencing the corresponding genes from the *L. mesenteroides* IWBT B290 strain selected. The obtained nucleotide sequence data were aligned and compared to other DNA sequences available in GenBank database. The BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed that we amplified and sequenced the correct genes. Table 6.3 shows percentage identities of nucleotide sequences from the IWBT B290 strain and related species of the two closely related genera: *Leuconostoc* and *Oenococcus*. The highest sequence homology (91-99% identity) was observed with *L. mesenteroides* subsp. *mesenteroides* ATCC 8293 strain (CP000414).

Based on the percentage identities shown in Table 6.3, it appears that strain IWBT B288 is quite distant from other closely related species of *Leuconostoc*, and even a bit distant from *L. mesenteroides* subsp. *mesenteroides* ATCC 8293 (CP000414). This trend is also evidenced in the phylogenetic trees constructed based on DNA sequences of the *alsS*, *estA*, *metK* and *mleA* genes (Figure 6.2). In the phylogenies of the *alsS*, *estA* and *metK* genes, ATCC 8293 clustered separately from the wine isolates whereas in the *mleA* phylogeny, the ATCC 8293 strain appeared to be intermixed with the tested strains. These findings may clearly suggest that there is a genetic heterogeneity between strains of the same species.

6.4 CONCLUSION

In this study, we have demonstrated, with a molecular approach, the existence of certain genes encoding enzymes of oenological relevance in *L. mesenteroides* strains originating from wine. Notably, certain other genes are absent in *L. mesenteroides* strains and this might suggest that they are not essential for the survival of these strains. However, it still remains to be further determined if the enzymes encoded by PCR-detected genes are active and functional under winemaking conditions. This aspect therefore warrants further studies. The phylogenetic analysis of *L. mesenteroides* isolates also indicated that there is a genetic variability amongst different strains of the same species.

6.5 ACKNOWLEDGEMENTS

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6.6 REFERENCES

Araque I, Gil J, Carreté R, Bordons A, Reguant C. 2009. Detection of *arc* genes related with the ethyl carbamate precursors in wine lactic acid bacteria. J Agric Food Chem 57:1841–1847.

Bekal S, van Beeumen J, Samyn B, Garmyn D, Henini S, Diviès C, Prévost H. 1998. Purification of *Leuconostoc mesenteroides* citrate lyase and cloning and characterization of the *citCDEFG* gene cluster. J Bacteriol 180:647–654.

Carmel-Harel O, Storz G. 2000. Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. Annu Rev Microbiol 54:439–461.

Cottrell MT, Kirchman DL. 2000. Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. Appl Environ Microbiol 66:5116–5122.

De las Rivas B, Rodríguez H, Curiel JA, Landete JM, Muñoz R. 2009. Molecular screening of wine lactic acid bacteria degrading hydroxycinnamic acids. J Agric Food Chem 57:490–494.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Ferguson GP. 1999. Protective mechanisms against toxic electrophiles in *Escherichia coli*. Trends Microbiol 7:242–247.

Hemme D, Foucaud-Scheunemann C. 2004. *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. Int Dairy J 14:467–494.

Jänsch A, Korakli M, Vogel RF, Gänzle MG. 2007. Glutathione reductase from *Lactobacillus sanfranciscensis* DSM20451^T: contribution to oxygen tolerance and thiol exchange reactions in wheat sourdoughs. Appl Environ Microbiol 73, 4469–4476.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120.

Krieling SJ. 2003. An investigation into lactic acid bacteria as a possible cause of bitterness in wine. Masters Thesis. Stellenbosch University, Stellenbosch, South Africa.

Kumar S, Nei M, Dudley J, Tamura K. 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9:299–306.

Lee JH, Moon YH, Kim N, Kim YM, Kang HK, Jung JY, Abada E, Kang SS, Kim D. 2008. Cloning and expression of the sucrose phosphorylase gene from *Leuconostoc mesenteroides* in *Escherichia coli*. Biotechnol Lett 30:749–754.

Lee KB, Pi KB, Kim EB, Rho BS, Kang SK, Lee HG, Choi YJ. 2010. Glutathione-mediated response to acid stress in the probiotic bacterium, *Lactobacillus salivarius*. Biotechnol Lett 32:969–972.

Lee JH, Yoon SH, Nam SH, Moon YH, Moon YY, Kim D. 2006. Molecular cloning of a gene encoding the sucrose phosphorylase from *Leuconostoc mesenteroides* B-1149 and the expression in *Escherichia coli*. Enzyme Microbial Technol 39:612–620.

Mtshali PS, Divol B, van Rensburg P, du Toit M. 2010. Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines. J Appl Microbiol 108:1389–1397.

Riccillo PM, Muglia CI, de Bruijn FJ, Roe AJ, Booth IR, Aguilar OM. 2000. Glutathione is involved in environmental stress responses in Rhizobium tropici, including acid tolerance. J Bacteriol 182:1748–1753.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Smirnova GV, Krasnykh TA, Oktyabrsky ON. 2001. Role of glutathione in the response of *Escherichia coli* to osmotic stress. Biochemistry (Moscow) 66:973–978.

Smirnova GV, Muzyka NG, Glukhovchenko MN, Krasnykh TA, Oktyabrsky ON. 1999. Oxidative stress resistance of *Escherichia coli* strains deficient in glutathione biosynthesis. Biochemistry (Moscow) 64:1111–1116.

Spano G, Rinaldi A, Ugliano M, Moio L, Beneduce L, Massa S. 2005. A β-glucosidase gene isolated from wine *Lactobacillus plantarum* is regulated by abiotic stresses. J Appl Microbiol 98:855–861.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882.

Vedamuthu ER. 1994. The dairy *Leuconostoc*: use in dairy products. J Dairy Sci 77:2725–2737.

Wiederholt KM, Steele JL. 1994. Glutathione accumulation in lactococci. J Dairy Sci 77:1183–1188.

Zhang H, Hu Y, Zhu C, Zhu B, Wang Y. 2008. Cloning, sequencing and expression of a dextransucrase gene (*dex*YG) from *Leuconostoc mesenteroides*. Biotechnol Lett 30:1441–1446.

Table 6.1–A list of primers designed in this study

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Annealing temp.
mleA	GTTGAATTYATGCCAATYGTKTATGA	GCTTTWGCTTCKGCTAAYTTDGTTGG	54 °C
bgl	GAAAAAGCNGAWCTDGTWTCBGGNAAAGAT	CCAGGCATTTCBAARTCYADACCDGCTTT	53 °C
estA	ATGGCTTTTTTAGAAGTTAATTATTATTC	TTAACTCAATCTCTCTTCCTGAACATAG	45 °C
prtP	ATGAAAAAGCATTCTTTAATTATCG	CTACTTTTCAGTATTCTTAATAAATGTC	45 °C
citP	ATGGAGATAGAATTATGATGAATCA	AAATATCTAAATTACTTCATGAATGTG	49 °C
alsD	ATGACAACAATATATCAACATGGTA	CTAATTTTTTCCGCCTTCACT	49 °C
alsS	ATGGCAAATAAAAAATATGGTGCAG	TTATCCTTCAGAACCAATCAATTGTG	49 °C
metK	ATGGCAAAGTATTTCACATCGG	TTAAAGTAAGTTTTTGATTTCTTTCACCTT	49 °C
metC	ATGAGTGATTGGACAAATATTATTGATG	CTAAATTAAGGCTAATGCACTATCCAA	49 °C
metB	ATGAAATTTGATACACAACTTATTCATG	TTATTTCAATTGATTAAAACCTTGTTCTAG	49 °C
gshR	ATGGCGGAACAGTACGATG	TTAATACAAATATTGTAAGTCACTAGCCG	49 °C

The primers for amplifying *arcA*, *arcB*, *arcB*, *citD*, *citE*, *citF* and *pad* genes are similar to those reported in literature (Araque and others 2009; Mtshali and others 2010)

Table 6.2–Results of PCR detection of enzyme-encoding genes with gene-specific primers

Target gene	Function	Size (bp)	*Leuconostoc mesenteroides* strains	
			IWBT (15 isolates)	NCDO (529^T)
mleA	Malolactic enzyme	989	15[a]	0
bgl	β-Glucosidase	704	7	0
estA	Predicted esterase	792	15	1
prtP	Serine protease	858	15	1
pad	Phenolic acid dercaboxylase	210	0	0
citD	Citrate lyase γ-subunit	245	0	0
citE	Citrate lyase β-subunit	897	0	0
citF	Citrate lyase α-subunit	1331	0	0
citP	Citrate permease	1357	0	0
alsD	α-Acetolactate decarboxylase	720	15	1
alsS	α-Acetolactate synthase	1686	15	1
metK	S-adenosylmethionine synthase	1158	15	1
metC	Cystathionine β-lyase	1140	15	1
metB	Cystathionine γ-lyase	1140	15	1
gshR	Glutathione reductase	1332	12	0
arcA	Arginine deiminase	266	0	0
arcB	Ornithine transcarbamylase	181	0	0
arcC	Carbamate kinase	343	0	0

Culture collections: IWBT, Institute for Wine Biotechnology, Stellenbosch University, South Africa; NCDO, National Collection of Dairy Organisms, UK.
[a] The total number of strains positive.

Table 6.3–Percentage identity of DNA sequences from *L. mesenteroides* IWBT B290 with other published closest relatives

	% identity of *Leuconostoc mesenteroides* subsp. *mesenteroides* IWBT B290 with				
Target gene	**OE-OE**[a]	**Leuc-M**	**Leuc-C**	**Leuc-G**	**Leuc-K**
mleA	68[b] (OEOE_1563[c])	98 (LEUM_1005)	75 (LCK_00751)	76 (LEGAS_0941)	92 (LKI_10035)
bgl	69 (OEOE_1569)	99 (LEUM_0875)	none[d]	none	none
estA	70 (OEOE_0720)	97 (LEUM_1482)	70 (LCK_00616)	70 (LEGAS_1200)	69 (LKI_01580)
prtP	none	92 (LEUM_1808)	70 (LCK_01484)	70 (LEGAS_0381)	68 (LKI_07085)
metK	69 (OEOE_0838)	97 (LEUM_1697)	79 (LCK_01370)	79 (LEGAS_0489)	80 (LKI_07665)
metB	65 (OEOE_1758)	97 (LEUM_1288)	none	74 (LEGAS_0159)	72 (LKI_03395)
metC	65 (OEOE_0765)	97 (LEUM_1804)	74 (LCK_01478)	68 (LEGAS_0159)	74 (LKI_07100)
gshR	none	99 (LEUM_1019)	none	89 (LEGAS_1861)	99 (LKI_00285)
alsD	none	98 (LEUM_0549)	68 (LCK_00459)	68 (LEGAS_1345)	68 (LKI_02730)
alsS	72 (OEOE_1703)	96 (LEUM_0522)	77 (LCK_01334)	77 (LEGAS_0526)	78 (LKI_07840)

[a] Abbreviations: **OE-OE**, *Oenococcus oeni* PSU-1 (accession no. CP000411); **Leuc-M**, *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 (CP000414); **Leuc-C**, *Leuconostoc citreum* KM20 (DQ489736); **Leuc-G**, *Leuconostoc gasicomitatum* LMG 18811[T] (FN822744); **Leuc-K**, *Leuconostoc kimchii* IMSNU 11154 (CP001758).
[b] Percentage identity.
[c] Gene locus tag.
[d] (none), no hits found during the BLAST search.

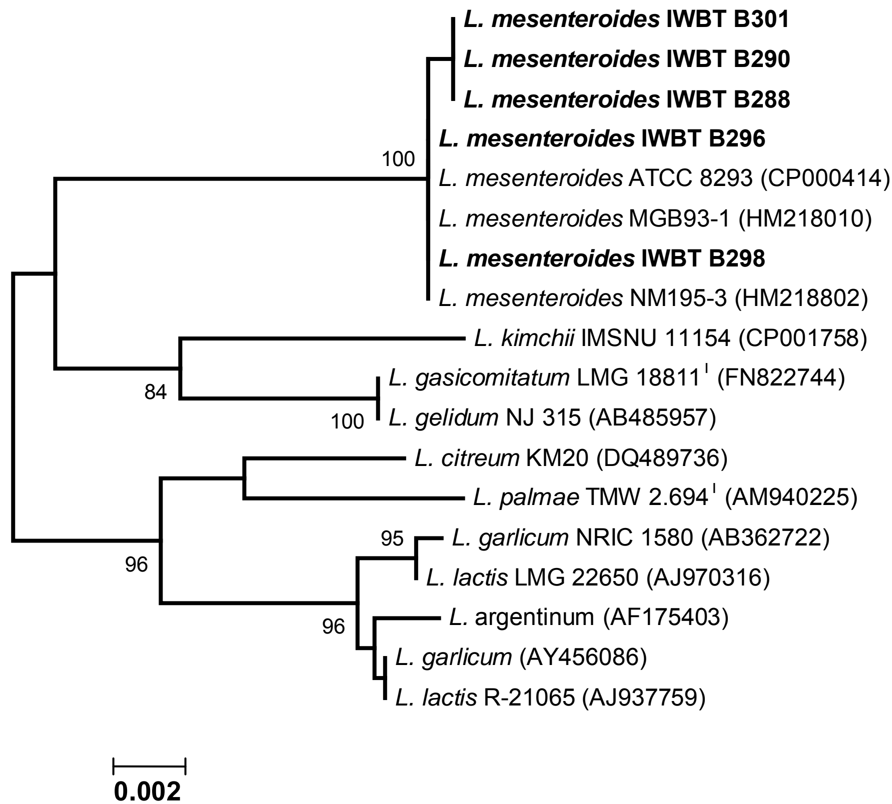

Figure 6.1. Phylogenetic tree constructed with the neighbour-joining method based on 16S rDNA gene sequences to show the relationship between *L. mesenteroides* isolates tested in this study (in bold) and the closest relatives published in GenBank database (accession numbers in parentheses). Bootstrap values >80% are shown at branching points. The horizontal scale bar indicates the numbers of base substitutions per site.

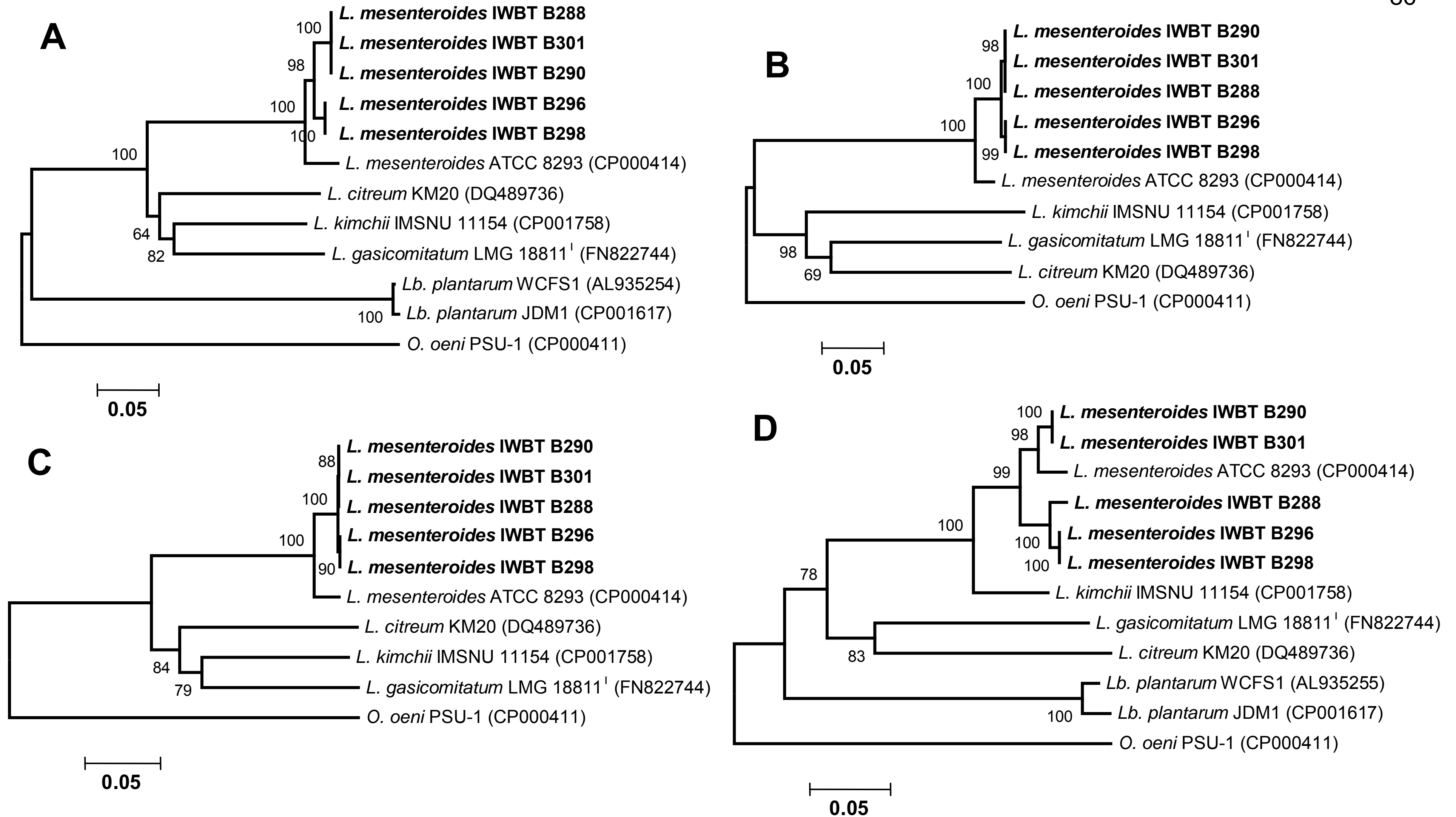

Figure 6.2. Neighbour-joining trees created with nucleotide sequences of the (a) *alsS* (1535 nt), (b) *estA* (769 nt), (c) *metK* (1121 nt) and (d) *mleA* (946 nt) genes, showing the relationship of five *L. mesenteroides* subsp. *mesenteroides* strains of wine origin (in bold) to other closely related taxa (GenBank accession numbers in parentheses). Numbers at branching points represent the results of bootstrap analyses (expressed as percentages of 1000 replicates). The horizontal scale bar indicates the number of base substitutions per site.

CHAPTER 7

RESEARCH RESULTS V

Identification and characterization of *Lactobacillus florum* strains isolated from South African grape and wine samples

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CHAPTER 7

Identification and characterization of *Lactobacillus florum* strains isolated from South African grape and wine samples

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ABSTRACT

A total of 210 strains of lactic acid bacteria were examined in this study. Among these, 30 strains previously isolated from South African grape and wine samples remained unidentified. The identification of these isolates was performed by BLAST and phylogenetic analyses of 16S rDNA gene sequences, which indicated that the isolates belonged to *Lactobacillus florum*. In this work, we also designed a discriminative species-specific primer FLOR targeting the 16S rDNA gene of *Lb. florum*. The validity and specificity of this primer was confirmed. Of particular interest in this study was to further evaluate the identified strains for the presence of genes encoding enzymes of oenological relevance. Reference strains included three flower-associated *Lb. florum* (F9-1^T, F9-2 and F17) and two *Lactobacillus lindneri* (AWRI B530 and DSM 20691) strains. *Lb. lindneri* strains were incorporated as being the closest relatives of *Lb. florum*. PCR detection results revealed that all *Lb. florum* strains and *Lb. lindneri* AWRI B530 (grape isolate) possessed the majority of the tested genes relative to DSM 20691 (beer isolate); these enzyme-encoding genes included malolactic enzyme, peptidases (PepC, PepI, PepN), citrate lyase (α- and β-subunits), phenolic acid decarboxylase and arginine deiminase pathway enzymes (arginine deiminase and ornithine transcarbamylase). Sequence verification of PCR-generated fragments was performed. The sequence data were used to construct the phylogenetic trees, which indicated that our *Lb. florum* isolates cluster with other *Lb. florum* strains but rather distinct from other LAB species, with *Lb. lindneri* being the next closest species.

Keywords: *Lactobacillus florum*, 16S rDNA, species-specific primer, PCR detection, phylogenetic trees

7.1 INTRODUCTION

Lactic acid bacteria (LAB) are a group of Gram-positive microorganisms occurring naturally in a variety of niches, including the gastrointestinal tract, plants and fermented foods such as dairy products, meat and alcoholic beverages (Hammes and Hertel, 2006; Mohania et al., 2008). On the other hand, fructophilic LAB represent a new group of bacteria having been described recently (Endo and Okada, 2008; Endo et al., 2009). Based on their characteristics, these bacteria grow well on D-fructose or D-glucose in the presence of electron acceptors such as pyruvic acid, but poorly on D-glucose without electron acceptors (Endo and Okada, 2008).

Endo et al. (2009) isolated three novel strains of fructophilic LAB from South African flowers of peony and bietou. These strains were closely related to *Lactobacillus lindneri* and *Lactobacillus sanfranciscensis* (95.4% and 93.7% similarity, respectively) based on the phylogenetic analysis of 16S rDNA gene sequences. Because the sequence similarities between the new isolates and closely related species were lower than the recommended value (98.7-99%) for species differentiation as proposed by Stackebrandt and Ebers (2006), the new isolates therefore represented a novel species for which the name *Lactobacillus florum* was adopted (Endo et al., 2010). To date, reports on the occurrence of *Lb. florum* in nature are very scarce. This may be attributed to the fact that only few conventional isolation media contain D-fructose as a substrate, thus making it possible that fructophilic LAB are not selected for (Endo et al., 2009). In light of this, rapid and reliable methods are required for a quick detection and differentiation of *Lb. florum* strains from a variety of habitats.

From an oenological perspective, LAB play a pivotal role in conducting malolactic fermentation (MLF). Among the LAB species associated with MLF, *Oenococcus oeni* is usually the main bacterium involved in carrying out this secondary fermentation to completion. Of all the lactobacilli, *Lactobacillus plantarum* is considered a new potential candidate for MLF (Du Toit et al., 2010). Other species such as *Lactobacillus hilgardii*, *Lactobacillus brevis* and *Lactobacillus buchneri* are rather associated with spoilage. Another species typically implicated in beer spoilage is *Lactobacillus lindneri*; it causes an increase in acidity, turbidity and, in some instances, can result to the production of off-flavours (Back, 2005). The latter bacterium has recently been detected on Australian grapes, and its occurrence in wine causes a clear inhibition of the growth of *O. oeni* in a mixed culture (Bae et al., 2006).

Quite recently, several species of yeasts and bacteria were tested for their ability to form indole during wine fermentation (Arevalo-Villena et al., 2010). Among the bacterial strains tested, *Lb. lindneri* was found to be the highest producer of indole. This compound, derived from the microbial catabolism of tryptophan, imparts a ‘plastic-like’ off-flavour in

wine (Capone et al., 2010). These preliminary results are therefore indicative that *Lb. lindneri* may impact negatively in wine when present during fermentation. This area therefore merits further research in order to elucidate the metabolic activity of *Lb. lindneri*, and hence its impact on wine production.

In this work, we examined several isolates of LAB originating from South African grape and wine samples. The identification of these isolates was performed by phylogenetic analysis of their 16S rDNA gene sequences. In addition, a discriminative species-specific primer for *Lb. florum* targeting the 16S rDNA gene was designed. We further investigated the isolates for the presence of genes coding for enzymes of relevance in winemaking, such as malolactic enzyme, β-glucosidase, protease, peptidases (PepC, PepI, PepN and PepM), citrate lyase (α-, β- and γ-subunits), α-acetolactate synthase, phenolic acid decarboxylase, amino acid decarboxylases (histidine decarboxylase, ornithine decarboxylase and tyrosine decarboxylase), as well as enzymes associated with sulphur and arginine metabolic pathways.

7.2 MATERIALS AND METHODS

7.2.1 Microorganisms and growth conditions

Thirty isolates only identified as *Lactobacillus* sp. were investigated in this study. They were previously isolated from grape and wine samples obtained from five different wineries situated in the Western Cape region, South Africa, during the 2001 and 2002 vintages (Krieling, 2003). An additional number of strains representing 24 different species were used as PCR controls to validate the specificity of *Lb. florum* species-specific primers. The type or reference strains used in this study are listed in Table 7.1. Three *Lb. florum* strains (F9-1^T, F9-2 and F17) were kindly provided by Dr. Akihito Endo (Department of Microbiology, Stellenbosch University, South Africa).

Oenococcus oeni strains were grown anaerobically at 30°C on MRS agar medium (BIOLAB Diagnostics, Wadeville, Gauteng, South Africa) supplemented with 20% apple juice (pH adjusted to 5.2 with HCl); anaerobic conditions were created with Anaerocult® A (Merck, Darmstadt, Germany). All other LAB strains were cultured at 30°C on normal MRS agar medium (BIOLAB Diagnostics).

7.2.2. Preparation of template DNA

Chromosomal DNA or bacterial colony cells were used as template DNA for PCR assays. The total chromosomal DNA from overnight MRS cultures of individual strains was extracted as described previously (Mtshali et al., 2010).

7.2.3. Determination of 16S rDNA sequences

Amplification and sequencing of the 16S rDNA gene from the group of our isolated strains were performed using universal eubacterial primers EubA and EubB (Cottrel and Kirchman, 2000). All primer sets used for PCR assays are listed in Table 7.2, and were synthesized by the Integrated DNA Technologies, Inc. (Coralville, IA, USA). PCR was performed in a 50-μL amplification reaction mixture containing template DNA, 0.7 μM of each primer, 250 μM of dNTPs, 1X *Ex Taq*™ Buffer (TaKaRa Bio Inc., Southern Cross Biotechnology, Cape Town, South Africa) and 1.5 U of *TaKaRa Ex Taq*™ polymerase (TaKaRa Bio Inc.). PCR amplifications were carried out using TRIO-Thermoblock (Biometra® GmbH, Göttingen, Germany), and cycled through the temperature profiles indicated in Table 7.3. Amplified products were resolved on a 1% agarose gel (Whitehead Scientific, Cape Town, South Africa), purified with QIAquick® PCR Purification Kit (Qiagen, Southern Cross Biotechnology, Cape Town, South Africa) and subsequently sequenced by the Central DNA Sequencing Facility (Stellenbosch University, South Africa). For identification of the closest relatives, the obtained sequences were assembled, aligned and compared to those in GenBank database.

7.2.4. PCR with species-specific primers

To design the primers specific for *Lb. florum*, 16S rDNA gene sequences of *Lb. florum* and other closely related species (Table 7.2) were aligned. While the 16S rDNA gene sequence data of *Lb. florum* strains of grape and wine origin were generated in this study, all other gene sequences were extracted from GenBank database. The forward primer FLOR (5′-GCT GCC CAG TTG CTA GTC-3′) specific for *Lb. florum* was designed from the V1 variable region of the 16S rDNA gene located near the 5’ terminus of the 16S rDNA sequence. The universal reverse primer 907r (5′-CCG TCA ATT CCT TTG AGT TT-3′) was taken from the work published previously (Yasui et al., 1997). PCR samples were cycled and analysed as described above. For verification of PCR-amplified fragments, two strains (F9-1^T of flower origin and IWBT B322 of wine origin) were selected for sequencing using the forward and reverse primers. Sequence similarity searches were performed with GenBank databank.

7.2.5. PCR detection of enzyme-encoding genes

For PCR amplifications to detect the presence of different enzyme-encoding genes, template DNA (*ca.* 50 ng DNA or bacterial colony cells), 1.5 mM $MgCl_2$, 0.6 μM of each primer, 250 μM dNTP mix, 1X PCR buffer and 1.25 U Supertherm polymerase (Hoffman-La Roche, Southern Cross Biotechnology, Cape Town, South Africa) were used in a total reaction mix of 25 μL. When required for sequencing, the volume of the total PCR mix was increased to 50 μL in the presence of template DNA, 0.6 μM of each primer, 250 μM dNTP mix, 1X PCR buffer and 1.25 U *Ex Taq*™ DNA polymerase (TaKaRa Bio Inc.). The reactions were cycled through the temperature profiles indicated in Table 7.3. PCR products were checked by agarose gel electrophoresis and documented with UV

transilluminator. The verification of PCR-generated amplicons was performed as described above.

A multiplex PCR assay was also applied for simultaneous detection of amino acid decarboxylase genes (*hdc*, *tdc* and *odc*) using three sets of primers described by Marcobal et al. (2005). Multiplex PCR mixture was performed in a 25-μL amplification reaction mixture containing 50 ng template DNA, 1.75 mM $MgCl_2$, 200 μM dNTP mix, 0.3 μM *hdc* primer set, 2 μM *tdc* primer set, 1 μM *odc* primer set, 1X PCR Buffer and 1.5 U Supertherm polymerase (Hoffman-La Roche). After PCR amplification, amplicons were analyzed as described above.

7.2.6. Phylogenetic analysis

Multiple alignments of nucleotide gene sequences were created using the program CLUSTAL X (Thompson et al., 1997) and MEGA 4.1 software (Kumar et al., 2008). The neighbour-joining method (Saitou and Nei, 1987) with Kimura's two-parameter distance correction model was used to construct phylogenetic trees. The robustness of individual branches was estimated by bootstrapping with 1000 replications (Felsenstein, 1985).

7.2.7. Nucleotide sequences

The nucleotides sequences of the 16S rDNA, *mleA*, *pepC*, *pepI*, *pepN*, *citE* and *citF* genes of *Lb. florum* IWBT B322 will be deposited in GenBank/EMBL/DDBJ databases.

7.3 RESULTS

7.3.1 Species identification

The identification of 30 isolated strains of *Lactobacillus* was performed by analyzing the 16S rDNA gene sequences obtained after subjecting DNA to PCR with universal eubacterial primers EubA and EubB. The alignment of DNA sequences revealed that the determined nucleotide sequences were >99% similar between all the tested strains. These sequences were also compared to those available in GenBank database and the highest identity (99.9%) was recorded with *Lb. florum* F9-1^T (GenBank accession no. AB498045), *Lb. florum* F9-2 (AB498046) and *Lb. florum* F17 (AB498047) strains.

The newly determined 16S rDNA sequences were also compared to other closely related lactobacilli and the level of sequence identity was 95% with *Lb. lindneri* AWRI B530 (grape isolate), 95% with *Lb. lindneri* DSM 20690 (X95421), 94% with *Lb. sanfranciscensis* LMG 16002 (EU350220) and 93% with *Lactobacillus homohiochii* LMG 9478^T (AJ621552). A phylogenetic tree was also constructed using the neighbour-joining method to show the phylogenetic relationship of our strains with those retrieved from GenBank database (Fig. 7.1). Due to high sequence similarity (>99%) between our strains,

only one strain was included in the phylogenetic tree. Phylogenetic analysis based on 16S rRNA gene sequences indicated that our strain clustered together with *Lb. florum* strains (F9-1, F9-2 and F17), and separately from other closely related species. It can also been seen in Fig. 7.2 that *Lb. lindneri* AWRI B530 clusters separately from both *Lb. florum* strains and the beer isolates of *Lb. lindneri*.

7.3.2. Design of a species-specific primer

To develop a species-specific primer for a rapid and accurate detection of *Lb. florum*, 16S rDNA gene sequences of *Lb. florum* and closely related species were aligned (Fig. 7.2). The specific primer FLOR was then designed from the variable region located near the 5′ terminus of the 16S rDNA sequence. When applied to all *Lb. florum* strains tested, this primer together with a reverse universal primer 907r yielded PCR amplicons corresponding to an expected size of 907 bp. The specificity of the FLOR primer was also verified by testing different strains representing 23 different species. As expected, these strains produced no signal as an indication that this primer is only specific for *Lb. florum* (Table 7.5).

To verify that the *Lb. florum* primer set amplified the target gene (16S rDNA), PCR amplifications of two selected *Lb. florum* strains (F9-1^T and IWBT B322) were sequenced. After the obtained sequence data were assembled and aligned, a comparative analysis of DNA sequences was performed. When the sequences of the two strains were compared to one another, they displayed 100% identity. A homology search using the newly determined 16S rDNA gene sequences was also performed by comparing them to other DNA sequences published in the NCBI database. The BLAST results confirmed that the sequenced fragments corresponded to the 16S rDNA genes of *Lb. florum* strains F9-1^T (AB498045), F9-2 (AB498046) and F17 (AB498047).

7.3.3. Genetic screening for enzymes of oenological relevance

The 30 isolates were also genetically screened for the presence of genes encoding enzymes of oenological relevance. The reference strains of *Lb. florum* (F9-1^T, F9-2 and F17) and *Lb. lindneri* (AWRI B530 and DSM 20691) were also tested. Positive PCR amplifications yielded DNA fragments with expected sizes reflected in Table 2. From the PCR detection results shown in Table 7.6, it is worth noting that our isolates together with *Lb. florum* strains of flower origin tested positive for the presence of genes coding for the malolactic enzyme (encoded by *mleA*), peptidases (*pepC* and *pepI*), phenolic acid decarboxylase (*pad*) and arginine deiminisase enzymes (*arcA* and *arcB*). The rest of other genes, such as *bgl*, *prtP*, *pepM*, *citD*, *alsS*, *metK*, *metB*/*metC*, *arcC* and amino acid decarboxylase genes (*hdc*, *odc* and *tdc*), were not detected.

The PCR assay aiming to amplify the gene coding for glutathione reductase (*gshR*) yielded amplicons for four isolates. The primers used for *gshR* gene amplification were

designed after aligning nucleotide gene sequences of *Lactobacillus plantarum* WCFS1 (AL935252), *Lb. plantarum* JDM1 (CP001617) and *Leuconostoc mesenteroides* ATCC 8293 (CP000414). PCR-amplified fragments from these strains were gel-isolated, purified and finally sequenced. Multiple sequence alignments of the obtained DNA sequences indicated that the nucleotide sequences from these strains were >99% similar to each other. The homology search with other bacterial DNA sequences was performed in the NCBI database (http://www.ncbi.nlm.nih.gov). However, no similarity was found.

On the other hand, the two *Lb. lindneri* strains (AWRI B530 and DSM 20691) were also subjected to PCR with the same primer sets. While *Lb. lindneri* DSM 20691 (beer isolate) only exhibited the presence of malolactic enzyme (*mleA*) gene, the wine-originated strain *Lb. lindneri* AWRI B530 also possessed genes encoding citrate lyase α-subunit (*citF*), citrate lyase β-subunit (*citE*), MetK, arginine deiminase (*arcA*) and peptidases (*pepC*, *pepI*, *pepN* and *pepM*).

The suitability of citD-deg1/citD-deg2, alsS-deg1/alsS-deg2, Prot-deg1/Prot-deg2 and CBGL-deg1/CBGL-deg2 gene-specific primers was confirmed by identification of the corresponding genes from LAB strains belonging to *Lb. plantarum*, *Oenococcus oeni* and *Leuc. mesenteroides*. As expected, the latter strains yielded PCR fragments of expected sizes (data not shown) in comparison to the tested strains, which showed no presence of *citD*, *alsS*, *prtP* and *metB*/*metC* genes, respectively. The primer sets used for PCR assays were designed from nucleotide gene sequences of LAB species originating not only from wine but also from other fermented foods such as dairy products.

7.3.4. Phylogenetic analysis

In this study, three genes (*mleA*, *citE* and *pepN*) from randomly selected strains were sequenced for phylogenetic analysis. These strains comprised two references (F9-1^T and F17) and six isolates from our collection. Phylogenetic relationships between the tested isolates and closely related species were studied by constructing phylogenetic trees based on nucleotide sequences of the three randomly selected genes: *mleA*, *citE* and *pepN*. From the phylogeny analysis, it was noted that our isolates and reference strains of *Lb. florum* formed their own cluster in comparison to other closely related species whose gene sequences were retrieved from GenBank database. Among the isolates examined, none of them clustered with AWRI B530 or DSM 20691. Surprisingly, AWRI B530 appeared to be intermediate between DSM 20691 and the *Lb. florum* strains (Fig. 7.3A). In the phylogenetic trees based on *mleA* and *citE* gene sequences (Figs. 7.3B), *Lb. florum* and *Lb. lindneri* strains were distinct from other bacterial species. However, an exception was observed for the phylogeny based on *pepN* gene (Fig. 7.3C) in which *Lb. lindneri* AWRI B530 formed a cluster with *Lactobacillus fermentum* IFO 3956 (AP008937).

7.4 DISCUSSION

Traditionally, identification of *Lactobacillus* strains relied on the determination of biochemical properties. However, phenotypical methods clearly have inherent limitations in discriminating bacterial strains possessing similar physiological traits (Mohania et al., 2008). Many recent studies have therefore focused their attention towards developing molecular biology techniques as an alternative method for a rapid and accurate identification of *Lactobacillus* species. The determination of 16S rRNA gene sequence provides an accurate basis for phylogenetic analysis and identification of *Lactobacillus* (Vandamme et al., 1996), and this gene is the most universal gene targeted in bacterial diversity studies (Mohania et al., 2008). In addition, the use of species-specific primers offers a rapid, accurate and sensitive tool for species identification in comparison to phenotype-based methods. The species-specific oligonucleotides are usually designed from the variable regions of the 16S rDNA gene sequence. These variations therefore allow for the selection of species-specific targets for primer design.

In this study, the isolates of *Lactobacillus* originating from South African grape and wine samples were investigated. The identification of these new isolates was performed by sequencing the 16S rDNA gene. In search of the closest relatives, these isolates exhibited 99.9% identity with *Lb. florum* strains described recently by Endo et al. (2010). We therefore identified the new isolates as *Lb. florum*. From the phylogenetic tree analysis based on the 16S rDNA sequences (Fig. 7.1), it was also interesting to note that our isolates clustered together with F9-1^T (AB498045), F9-2 (AB498046) and F17 (AB498047) strains. Surprisingly, *Lb. lindneri* AWRI B530 clustered separately from other beer strains of *Lb. lindneri* published in the NCBI database. The percentage identity of AWRI B530 strain relative to beer isolates was also slightly lower than the recommended value for species identification using the 16S rDNA sequence analysis.

Besides using 16S rDNA sequence analysis for species identification, we also developed a novel PCR assay that is specific for *Lb. florum* and can suitably be employed for the direct detection and identification of any *Lb. florum* isolate. The variations in the V1 region of *Lb. florum* 16S rDNA gene sequence allowed us to design species-specific PCR primer (FLOR) for the identification of *Lb. florum*. The reverse primer 907r, taken from Yasui et al. (1997), was universal across bacterial species. Both primers (FLOR and 907r) were then tested against all *Lb. florum* strains, which gave positive amplicons of expected size. The validation of FLOR/907r primer specificity was performed by testing other strains representing 23 species and belonging to *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus*. As expected, none of these strains tested positive.

Apart from identifying the new isolates as *Lb. florum*, our interest was to further investigate these isolates for the presence of genes encoding enzymes of oenological

relevance in order to evaluate the potential impact – positive or negative – of the presence of this bacterium in wine. The genes were detected by PCR with gene-specific primers. The corresponding PCR-amplified fragments from randomly selected strains were sequenced for confirmation analysis. The obtained nucleotide sequences were assembled, aligned and subsequently used to study their homology patterns in comparison to those published in GenBank database.

Among the enzyme-encoding genes investigated in this study, it was demonstrated that all *Lb. florum* strains together with *Lb. lindneri* AWRI B530 possessed several genes encoding peptidases (*pepC*, *pepN* and *pepI*). The *pepM* gene was only detected in *Lb. lindneri* AWRI B530 strain. Of all the strains tested for *prtP* gene, none of them yielded a signal during PCR detection with gene-specific primers. The presence of the *prtP* gene in various species of *Lactobacillus* was reported previously (Mtshali et al., 2010). The genes investigated in this work encode enzymes related to proteolysis and peptidolysis in which proteins are degraded into smaller peptides and amino acids necessary for the growth of LAB (Juillard et al., 1995). Although the distribution of peptidase enzyme-encoding genes in wine-associated LAB is poorly understood, the presence of several peptidase genes in AWRI B530 and *Lb. florum* strains suggests that they might play a role in peptidolysis.

In relation to the PCR assay aimed at detecting the presence of the *arcABC* gene cluster, *Lb. florum* and *Lb. lindneri* strains were tested using the primer sets reported previously (Araque et al., 2009). The *arcABC* gene cluster encodes three enzymes (arginine deiminase, ornithine transcarbamylase and carbamate kinase, respectively) involved in arginine catabolism via the arginine deiminase pathway (Liu et al., 1996). From the PCR detection results, all *Lb. florum* strains exhibited the presence of *arcA* and *arcB* genes whereas *Lb. lindneri* AWRI B530 strain only presented the *arcA* gene. The *arcC* gene was not detected in any of the strains tested. Taken together, the presence of genes encoding an incomplete arginine deiminase pathway might suggest that these strains have the genetic potential to induce wine spoilage by forming ethyl carbamate (also known as urethane) from arginine metabolism. Urethane is a carcinogenic compound with negative health implications, and is formed in a reaction between ethanol and *N*-carbamyl compounds such as citrulline and carbamyl phosphate (i.e. the intermediates of arginine metabolism) (Ough et al., 1988).

The *Lb. florum* and *Lb. lindneri* strains were also tested for the presence of genes coding for enzymes involved in the sulphur metabolic pathway. These genes (*metK* and *metB*/*metC*) encode S-adenosylmethionine synthase and cystathionine γ-lyase/β-lyase involved in the conversion of methionine to diverse volatile sulphur compounds, respectively. Of all the strains tested, none of them possessed the *metB* or *metC* gene. Only *Lb. lindneri* AWRI B530 possessed the methionine biosynthetic gene (*metK*). These

results therefore suggest the possibility that the AWRI B530 strain has the potential to produce beneficial aroma-active components to influence wine sensory properties.

The absence of amino acid decarboxylase genes from all *Lb. florum* and *Lb. lindneri* strains was revealed by multiplex PCR as described previously (Marcobal et al., 2005). The plate assay method was also employed to corroborate the PCR detection results. These findings are indicative that none of the tested strains is able to produce biogenic amines. In principle, biogenic amines are mainly formed by the microbial decarboxylation of amino acids present in fermented foods and beverages. The formation of these amines in fermented products is undesirable because if absorbed by consumers at an excessively high content, they may induce headaches, respiratory distress, heart palpitation, hyper-hypotension, and several allergenic disorders (Smit et al., 2008).

In this work, we have also demonstrated the presence of the *pad* gene coding for a phenolic acid decarboxylase in all *Lb. florum* strains originating from flowers and wine. The presence of this gene in several wine LAB isolates was also reported previously (de las Rivas et al., 2009; Mtshali et al., 2010). However, we could not obtain an amplicon when applying the *pad* primers in both *Lb. lindneri* strains (AWRI B530 and DSM 20691). The presence of phenolic acid decarboxylase enzyme in phenolic acid-degrading strains is directly linked to the formation of volatile phenols (Liu, 2002). As such, the PCR detection of the genetic determinant of this enzyme can therefore be used as a preliminary tool for identifying LAB strains with potential to produce volatile phenols in wine (de las Rivas et al., 2009).

Finally, our study has also demonstrated the existence of citrate lyase genes (*citE* and *citF*) in *Lb. lindneri* AWRI B530 and all *Lb. florum* strains tested, albeit the *citD* gene was not detected in any of these strains. These genes are related to citrate metabolism by citrate-utilising LAB. The metabolism of citrate in wine relates to the production of diacetyl, which imparts a pleasant buttery attribute in wine (Liu, 2002). However, this compound can be regarded as an off-flavour when present in wine at higher concentrations (Fornachon and Lloyd, 1965; Rankine et al., 1969). The presence of citrate lyase genes in AWRI B530 and *Lb. florum* strains therefore suggests that these strains have the genetic potential to utilize citrate. However, it remains to be further investigated if the citrate pathway in these strains is still active as not all the genes involved in the pathway are present. On the other hand, the degenerate primers used for PCR assays could not yield any signal in a beer isolate (*Lb. lindneri* DSM 20691).

In conclusion, we have genetically identified *Lb. florum* strains isolated from South African grape and wine samples using 16S rDNA sequence analysis. To our knowledge, this is the first study to report on the occurrence of *Lb. florum* on the grapes and in wine. We also established a novel PCR assay using a species-specific PCR primer for the rapid

detection of *Lb. florum* species, which can also be exploited to confirm the identity of *Lb. florum* strains originating from various sources. From the phylogenetic analysis of the tested strains with closest relatives published in GenBank database, it was noted that *Lb. florum* strains of wine origin clustered together with those of flower origin (99.9% identity). On the other hand, the wine-originated AWRI B530 strain of *Lb. lindneri* did not cluster with other *Lb. lindneri* strains of beer origin.

For further inquisition, we identified several enzyme-encoding genes from all the strains tested, with the exception of the beer strain (DSM 20691) that only possessed the *mleA* gene. From the enzyme profiling perspective, it was noteworthy that *Lb. lindneri* AWRI B530 and all *Lb. florum* strains possessed the majority of enzyme-encoding genes tested relative to the DSM 20691 strain. In addition, there was a high degree of genetic diversity between *Lb. florum* and *Lb. lindneri* strains tested. Of particular interest to note is the fact that *Lb. lindneri* AWRI B530 appears to be intermediate between *Lb. florum* strains and beer isolates of *Lb. lindneri* in relation to its phylogenetic relatedness to beer isolates and its similarity to *Lb. florum* regarding the gene pool. These findings therefore warrant a speculation that AWRI B530 might represent a new novel species rather than *Lb. lindneri*. Altogether, this work has extended our knowledge on the occurrence of *Lb. florum* strains in various niches as well as on the presence of various enzyme-encoding genes of oenological relevance.

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7.6 REFERENCES

Araque, I., Gil, J., Carreté, R., Bordons, A., Reguant, C., 2009. Detection of *arc* genes related with the ethyl carbamate precursors in wine lactic acid bacteria. Journal of Agricultural and Food Chemistry 57, 1841–1847.

Arevalo-Villena, M., Bartowsky, E.J., Capone, D., Sefton, M.A., 2010. Production of indole by wine-associated microorganisms under oenological conditions. Food Microbiology 27, 685–690.

Back, W., 2005. Brewery. In: Back, W. (Ed.), Colour Atlas and Handbook of Beverage Biology. Verlag Hans Carl, Nürnberg, pp. 10–112.

Bae, S., Fleet, G.H., Heard, G.M., 2006. Lactic acid bacteria associated with wine grapes from several Australian vineyards. Journal of Applied Microbiology 100, 712–727.

Capone, D.L., van Leeuwen, K., Pardon, K.H., Daniel, M.A., Elsey, G.A., Coulter, A.D., Sefton, M.A., 2010. Idenfitication and analysis of 2-chloro-6-methylph2,6-dichlorophenol and indole – causes of taints and off-flavours in wines. Australian Journal of Grape and Wine Research 16, 210–217.

Cottrell, M. T., Kirchman, D.L., 2000. Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. Applied and Environmental Microbiology 66, 5116–5122.

de las Rivas, B., Rodríguez, H., Curiel, J.A., Landete, J.M., Munoz, R., 2009. Molecular screening of wine lactic acid bacteria degrading hydroxycinnamic acids. Journal of Agricultural and Food Chemistry 57, 490–494.

Du Toit, M., Engelbrecht, L., Lerm, E., Krieger-Weber, S., 2010. *Lactobacillus*: the next generation of malolactic fermentation starter cultures–an overview. Food Bioprocess Technol. DOI 10.1007/s11947-010-0448-8 (In press).

Endo, A., Futagawa-Endo, Y., Dicks, L.M.T., 2009. Isolation and characterization of fructophilic lactic acid bacteria from fructose-rich niches. Systematic and Applied Microbiology 32, 593–600.

Endo, A., Futagawa-Endo, Y., Sakamoto, M., Kitahara, M., Dicks, L.M.T., 2010. *Lactobacillus florum* sp. nov., a fructophilic species isolated from flowers. International Journal of Systematic Evolutionary Microbiology 60, 2478–2482.

Endo, A., Okada, S., 2008. Reclassification of the genus *Leuconostoc* and proposals of *Fructobacillus fructosus* gen. nov., comb. nov., *Fructobacillus durionis* comb. nov., *Fructobacillus ficulneus* comb. nov. and *Fructobacillus pseudoficulneus* comb. nov. International Journal of Systematic Evolutionary Microbiology 58, 2195–2205.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Fornachon, J.C.M., Lloyd, B., 1965. Bacterial production of diacetyl and acetoin in wine. Journal of the Science of Food and Agriculture 19, 374–378.

Hammes, W.P., Hertel, C., 2006. The genera *Lactobacillus* and *Carnobacterium*. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), The Prokaryote, third ed., vol.4, Springer, NewYork, NY, pp.320–403.

Juillard, V., Le Bars, D., Kunji, E.R., Konings, W.N., Gripon, J.C., Richard, J., 1995. Oligopeptides are the main source of nitrogen for *Lactococcus lactis* during growth in milk. Applied and Environmental Microbiology 61, 3024–3030.

Krieling, S.J., 2003. An investigation into lactic acid bacteria as a possible cause of bitterness in wine. Masters Thesis. Stellenbosch University, Stellenbosch, South Africa.

Kumar, S., Nei, M., Dudley, J., Tamura, K., 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Briefings in Bioinformatics 9, 299–306.

Le Jeune, C., Lonvaud-Funel, A., Ten Brink, B., Holstra, H., van der Vossen, J.M.B.M., 1995. Development of a detection system for histidine decarboxylating lactic acid bacteria based on DNA probes, PCR and activity test. Journal of Applied Bacteriology 78, 316–326.

Liu, S.-Q., 2002. Malolactic fermentation in wine – beyond deacidification. Journal of Applied Microbiology 92, 589–601.

Liu, S.-Q., Pritchard, G.G., Hardman, M.J., Pilone, G.J., 1996. Arginine metabolism in wine lactic acid bacteria: is it via the arginine deiminase pathway or the arginase-urease pathway. Journal of Applied Bacteriology 81, 486–492.

Lucas, P., Lonvaud-Funel, A., 2002. Purification and partial gene sequence of the tyrosine decarboxylase of *Lactobacillus brevis* IOEB 9809. FEMS Microbiology Letters 211, 85–89.

Marcobal, Á, de las Rivas, B., Moreno-Arribas, M.V., Muñoz, R., 2005. Multiplex PCR method for the simultaneous detection of histamine-, tyramine-, and putrescine-producing lactic acid bacteria in foods. Journal of Food Protection 68, 874–878.

Mohania, D., Nagpal, R., Kumar, M., Bhardwaj, A., Yadav, M., Jain, S., Marotta, F., Singh, V., Parkash, O., Yadav, H., 2008. Molecular approaches for identification and characterization of lactic acid bacteria. Journal of Digestive Diseases 9, 190–198.

Mtshali, P.S., Divol, B., du Toit, M., 2010. Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines. Journal of Applied Microbiology 108, 1389–1397.

Ough, C.S., Crowell, E.A., Gutlove, B.R., 1988. Carbamyl compound reactions with ethanol. American Journal of Enology and Viticulture 39, 239–242.

Rankine, B.C., Fornachon, J.C.M., Bridson, D.A., 1969. Diacetyl in Australian dry red wines and its significance in wine quality. Vitis 8, 129–134.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4, 406–425.

Smit, A.Y., du Toit, W.J., du Toit, M., 2008. Biogenic amines in wine: understanding the headache. South African Journal of Enology and Viticulture 29, 109–127.

Spano, G., Rinaldi, A., Ugliano, M., Moio, L., Beneduce, L., Massa, S., 2005. A β-glucosidase gene isolated from wine *Lactobacillus plantarum* is regulated by abiotic stresses. Journal of Applied Microbiology 98, 855–861.

Stackebrandt, E., Ebers, J., 2006. Taxonomic parameters revisited: tarnished gold standards. Microbiology Today 33, 152–155.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24, 4876–4882.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K., Swings, J., 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiology Reviews 60, 407–438.

Yasui, T., Okamoto, T., Taguchi, H., 1997. A specific oligonucleotide primer for the rapid detection of *Lactobacillus lindneri* by polymerase chain reaction. Canadian Journal of Microbiology 43, 157–163.

Table 7.1
Reference or type strains used as PCR controls to test the specificity of FLOR primer.

Bacterial species	Strain number	Culture collection
Lactobacillus spp.		
Lb. brevis	ATCC 14869	American Type Culture Collection
Lb. buchneri	NCIB 8007	National Collection of Industrial Bacteria
Lb. casei	LMG 13552	Laboratorium voor Microbiologie
Lb. curvatus	LMG 13553	Laboratorium voor Microbiologie
Lb. delbrueckii	DSM 20074	Deutsche Sammlung von Mikroorganismen und Zellkulturen
Lb. fermentum	DSM 20052	Deutsche Sammlung von Mikroorganismen und Zellkulturen
Lb. florum	F9-1, F9-2, F17	Department of Microbiology (MICRO; Stellenbosch)
Lb. hilgardii	DSM 20176	Deutsche Sammlung von Mikroorganismen und Zellkulturen
Lb. jensenii	NCFB 2165	National Collection of Food Bacteria
Lb. kunkeei	DSM 12361	Deutsche Sammlung von Mikroorganismen und Zellkulturen
Lb. lindneri	AWRI B530	Australian Wine Research Institute
	DSM 20691	Deutsche Sammlung von Mikroorganismen und Zellkulturen
Lb. malefermentans	ATCC 11305	American Type Culture Collection
Lb. paraplantarum	DSM 10667	Deutsche Sammlung von Mikroorganismen und Zellkulturen
Lb. pentosus	DSM 20314	Deutsche Sammlung von Mikroorganismen und Zellkulturen
Lb. plantarum	DSM 20174	Deutsche Sammlung von Mikroorganismen und Zellkulturen
Lb. reuteri	LMG 13557	Laboratorium voor Microbiologie
Lb. sakei	NCFB 2714	National Collection of Food Bacteria
Leuconostoc spp.		
Leuc. citreum	LMG 13562	Laboratorium voor Microbiologie
Leuc. mesenteroides	NCDO 529	National Collection of Dairy Organisms
Pediococcus sp.		
Pd. pentosaceus	LMG 13561	Laboratorium voor Microbiologie
Oenococcus sp.		
O. oeni	NCDO 2122	National Collection of Dairy Organisms

Table 7.2

Primers used for PCR amplifications.

Primer name	Sequence (5' to 3')	Target	Size (bp)	Source
EubA EubB	F-AAGGAGGTGATCCANCCRCA R-AGAGTTTGATCMTGGCTCAG	Eubacterial 16S rDNA (universal)	1540	Cottrell and Kirchman, 2000
MLE-3 MLE-4	F-GTTGAATTYATGCCAATYGTKTATGA R-GCTTTWGCTTCKGCTAAYTTDGTTGG	Malolactic enzyme	989	This study
BGL-1 BGL-2	F-GTGACTATGGTAGAGTTTCC R-TCAAAACCCATTCCGTTCCCCA	β-Glucosidase	1392	Spano et al., 2005
Prot-deg1 Prot-deg2	F-TTRGCNATYGGSTCDCCKHTSGGHWCTRATT R-GGAATWGCAAADCCCATBCCYTCRAC	Serine protease	270	This study
pepC-for pepC-rev	F-GGNCGTTGYTGGATGTTYGCBGCHTTRAAYAC R-TTADGCHAAWGHWCCCATTGGRTCCCAHGG	Cysteine aminopeptidase	1142	This study
pepI-for pepI-rev	F-CCYGGTGGHAABCAYGARTAYTGGGAARAC R-GCRTTRTCDATCATGTGRTGRTGRCCRCCDT	Proline iminopeptidase	737	This study
pepN-for pepN-rev	F-ATGGAAAACTGGGGNYTDGTNACHTAYCG R-ACNRCNGGRTADCCNGGYTGTTCVARCCANG	Membrane alanine aminopeptidase	569	This study
pepM-for pepM-rev	F-GGHTTTGAAGGHTAYAARTATKCNACBTGTGT R-AYCATBGGTTCRATNGTAATBGTCATVCC	Methionine aminopeptidase	443	This study
citD-deg1 citD-deg2	F-ATGGAAATTAARAMAACDGCMDTHGCVGGAAC R-GCYTTDATRACVMWRTCSARYGCSCCYTTRTCRACA	Citrate lyase γ-subunit	227	This study
citE-1 citE-2	F-TTACGBCGSACRATGATGTTTGT R-TATTTTTCAATGTAATTDCCCTCC	Citrate lyase β-subunit	894	Mtshali et al., 2010
citF-a citF-b	F-ATGGYATGACRATTTCWTTYCAYCAYCA R-ATCAATVAHBSWRCCRTCRCGRTAYTC	Citrate lyase α-subunit	1331	Mtshali et al., 2010
alsS-deg1 alsS-deg2	F-GGTTAYGAYSCSRTYGAATATGARCCNCG R-ATTTCYTCTTGRAAYTTRACCATRTCGTA	α-Acetolactate synthase	620	This study
PAD-1 PAD-3	F-AARAAYGAYCAYACYRTTGATTACC R-TTCTTCWACCCAYTTHGGGAAGAA	Phenolic acid decarboxylase	210	Mtshali et al., 2010
Sams-1 Sams-2	F-GAAMGMCAYTTATTTACDTCDGA R-AATBCCAGCWGGBCGYAARTCAAA	S-adenosylmethionine synthase	1050	This study
CBGL-deg1 CBGL-deg2	F-ATGAAATTYRAWACMMAAYTWATTCAYGGYGG R-ACCVACHGAKARRCGRATYAGYTCGTCTT	Cystathionine β-lyase/γ-lyase	1080	This study
gshR-1 gshR-2	F-ATGGCGGAACAGTACGATG R-TTAATACAAATATTGTAAGTCACTAGCCG	Glutathione reductase	1332	This study
ADI-for ADI-rev	F-CAYGCNATGATGCAYYTNGAYACNGT R-GTRTTNSWNCCRTCRTTCCAYTGYTC	Arginine deiminase	266	Araque et al., 2009

OTC-for OTC-rev	F-ATGCAYTGYYTNCCNGCNTTYCAYGA R-CCNARNGTNGCNGCCATDATNGCYTT	Ornithine transcarbamylase	181	Araque et al., 2009
CK-for CK-rev	F-CAYGGNAAYGGNCCNCARGTNGGNAA R-CKNCKNYANCCNCKNCCNGCRTCYTC	Carbamate kinase	343	Araque et al., 2009
JV16 JV17	F-AGATGGTATTGTTTCTTATG R-AGACCATACACCATAACCTT	Histidine decarboxylase	367	Le Jeune et al., 1995
ALF-P1 ALF-P2	R-CCRTARTCNGGNATAGCRAARTCNGTRTG F-GAYATNATNGGNATNGGNYTNGAYCARG	Tyrosine decarboxylase	924	Lucas and Lonvaud-Funel, 2002
ODC3 ODC16	F-GTNTTYAAYGCNGAYAARACNTAYTTYGT R-TACRCARAATACTCCNGGNGGRTANGG	Ornithine decarboxylase	1446	Marcobal et al., 2005

Abbreviations: F, forward primer; R, reverse primer.
N: G, A, T or C; **R**: G or A; **M**: A or C; **Y**: T or C; **K**: G or T; **W**: A or T; **D**: G, A or T; **S**: G or C; **H**: A, C or T; **B**: G, T or C; **V**: G, C or A.

Table 7.3
PCR amplification profiles.

Primer pair	Initial denaturing	Cycles	Denaturing	Annealing	Extension	Final extension
EubA/EubB	95°C, 2 min	35	95°C, 1 min	55°C, 1 min	72°C, 1 min	72°C, 10 min
FLOR/907r	94°C, 5 min	35	94°C, 1 min	53°C, 45 s	72°C, 1 min	72°C, 10 min
MLE-3/MLE-4	94°C, 3 min	35	94°C, 30 s	54°C, 1 min	72°C, 1 min	72°C, 10 min
BGL-1/BGL-2	94°C, 5 min	30	94°C, 1 min	50°C, 40 s	72°C, 1 min	72°C, 10 min
Prot-deg1/Prot-deg2	94°C, 5 min	30	94°C, 1 min	55°C, 45 s	72°C, 1 min	72°C, 10 min
pepC-for/pepC-rev	94°C, 5 min	30	94°C, 1 min	54°C, 45 s	72°C, 1 min	72°C, 10 min
pepI-for/pepI-rev	94°C, 5 min	30	94°C, 1 min	53°C, 45 s	72°C, 1 min	72°C, 10 min
pepN-for/pepN-rev	94°C, 5 min	30	94°C, 1 min	49°C, 45 s	72°C, 1 min	72°C, 10 min
pepM-for/pepM-rev	94°C, 5 min	30	94°C, 1 min	49°C, 45 s	72°C, 1 min	72°C, 10 min
citD-deg1/citD-deg2	94°C, 5 min	30	94°C, 1 min	49°C, 45 s	72°C, 1 min	72°C, 10 min
citE-1/citE-2	94°C, 3 min	35	94°C, 30 s	54°C, 1 min	72°C, 1 min	72°C, 10 min
citF-a/citF-b	94°C, 5 min	35	94°C, 1 min	49°C, 45 s	72°C, 1 min	72°C, 10 min
alsS-deg1/alsS-deg2	94°C, 5 min	30	94°C, 1 min	55°C, 45 s	72°C, 1 min	72°C, 10 min
PAD-1/PAD-3	94°C, 2 min	35	94°C, 40 s	50°C, 1 min	72°C, 1 min	72°C, 10 min
Sams-1/Sams-2	94°C, 3 min	35	94°C, 30 s	54°C, 1 min	72°C, 1 min	72°C, 10 min
CBGL-deg1/CBGL-deg2	94°C, 5 min	30	94°C, 1 min	49°C, 45 s	72°C, 1 min	72°C, 10 min
gshR-1/gshR-2	94°C, 5 min	35	94°C, 1 min	49°C, 45 s	72°C, 1 min	72°C, 10 min
ADI-for/ADI-rev	94°C, 5 min	30	94°C, 1 min	55°C, 45 s	72°C, 1 min	72°C, 10 min
OTC-for/OTC-rev	94°C, 5 min	30	94°C, 1 min	49°C, 45 s	72°C, 1 min	72°C, 10 min
CK-for/CK-rev	94°C, 5 min	30	94°C, 1 min	49°C,45 s	72°C, 1 min	72°C, 10 min
JV16/ JV17	95°C, 10 min	30	95°C, 30 s	52°C, 30 s	72°C, 2 min	72°C, 10 min
ALF-P1/ALF-P2	95°C, 10 min	30	95°C, 30 s	52°C, 30 s	72°C, 2 min	72°C, 10 min
ODC3/ODC16	95°C, 10 min	30	95°C, 30 s	52°C, 30 s	72°C, 2 min	72°C, 10 min

Table 7.4

LAB species used for the design of FLOR primer specific for *Lactobacillus florum*.

Bacterial species	Strain number [a]	Origin (source/accession no.)
Lactobacillus florum	F9-1	Flower (AB498045)
	F9-2	Flower (AB498046)
	F17	Flower (AB498047)
	IWBT B322	Grape (IWBT) [b]
Lactobacillus lindneri	DSM 20690	Beer (X95421)
	L2	Beer (X95422)
	LTH 2505	Beer (X95423)
Lactobacillus sanfranciscensis	LMG 16002	Unknown (EU350220)
Lactobacillus homohiochii	DSM 20571	Unknown (AM113780)
Lactobacillus fructivorans	DSM 20203	Unknown (X76330)

[a] Refer to Table 1 for full names of culture collections.
[b] IWBT, Institute for Wine Biotechnology collection, Stellenbosch University, South Africa.

Table 7.5

PCR detection results of reference strains and isolates by specific primers for *Lactobacillus florum*.

Bacterial species	Number of strains tested	Positive strains	Negative strains
Lactobacillus spp.	158		
Lb. brevis	8	0	8
Lb. buchneri	1	0	1
Lb. casei	1	0	1
Lb. curvatus	1	0	1
Lb. delbrueckii	1	0	1
Lb. fermentum	1	0	1
Lb. florum	33	33	0
Lb. hilgardii	3	0	3
Lb. jensenii	1	0	1
Lb. kunkeei	1	0	1
Lb. lindneri	2	0	2
Lb. malefermentans	1	0	1
Lb. paracasei	9	0	9
Lb. paraplantarum	3	0	3
Lb. pentosus	11	0	11
Lb. plantarum	78	0	78
Lb. reuteri	1	0	1
Lb. sakei	2	0	2
Leuconostoc spp.	17		
Leuc. citreum	2	0	2
Leuc. mesenteroides	15	0	15
Pediococcus spp.	10		
Pd. acidilactici	1	0	1
Pd. parvulus	6	0	6
Pd. pentosaceus	3	0	3
Oenococcus sp.	25		
O. oeni	25	0	25
Total	**210**	**33**	**177**

Table 7.6

PCR detection results with gene-specific primers.

Target gene	Gene description	*Lactobacillus florum*		*Lactobacillus lindneri*	
		IWBT	MICRO	AWRI	DSM
mleA	Malolactic enzyme	30[a] (30[b])	03 (03)	01 (01)	01 (01)
bgl	β-Glucosidase	30 (00)	03 (00)	01 (00)	01 (00)
prtP	Serine protease	30 (00)	03 (00)	01 (00)	01 (00)
pepC	Cysteine aminopeptidase	30 (29)	03 (03)	01 (01)	01 (00)
pepI	Proline iminopeptidase	30 (28)	03 (03)	01 (01)	01 (00)
pepN	Membrane alanine aminopeptidase	30 (30)	03 (03)	01 (01)	01 (00)
pepM	Methionine aminopeptidase	30 (00)	03 (00)	01 (01)	01 (00)
citD	Citrate lyase γ-subunit	30 (00)	03 (00)	01 (00)	01 (00)
citE	Citrate lyase β-subunit	30 (30)	03 (03)	01 (01)	01 (00)
citF	Citrate lyase α-subunit	30 (30)	03 (03)	01 (01)	01 (00)
alsS	α-Acetolactate synthase	30 (00)	03 (00)	01 (00)	01 (00)
pad	Phenolic acid decarboxylase	30 (30)	03 (03)	01 (00)	01 (00)
metK	S-adenosylmethionine synthase	30 (00)	03 (00)	01 (01)	01 (00)
metB/metC	Cystathionine γ-lyase/β-lyase	30 (00)	03 (00)	01 (00)	01 (00)
gshR	Glutathione reductase	30 (04[c])	03 (00)	01 (00)	01 (00)
arcA	Arginine deiminase	30 (30)	03 (03)	01 (01)	01 (00)
arcB	Ornithine transcarbamylase	30 (30)	03 (03)	01 (00)	01 (00)
arcC	Carbamate kinase	30 (00)	03 (00)	01 (00)	01 (00)
hdc	Histidine dercarboxylase	30 (00)	03 (00)	01 (00)	01 (00)
tdc	Tyrosine decarboxylase	30 (00)	03 (00)	01 (00)	01 (00)
odc	Ornithine decarboxylase	30 (00)	03 (00)	01 (00)	01 (00)

Refer to Table 1 for the descriptions of culture collections.
[a] The total number of strains tested.
[b] The total number of positive strains.
[c] False positive.

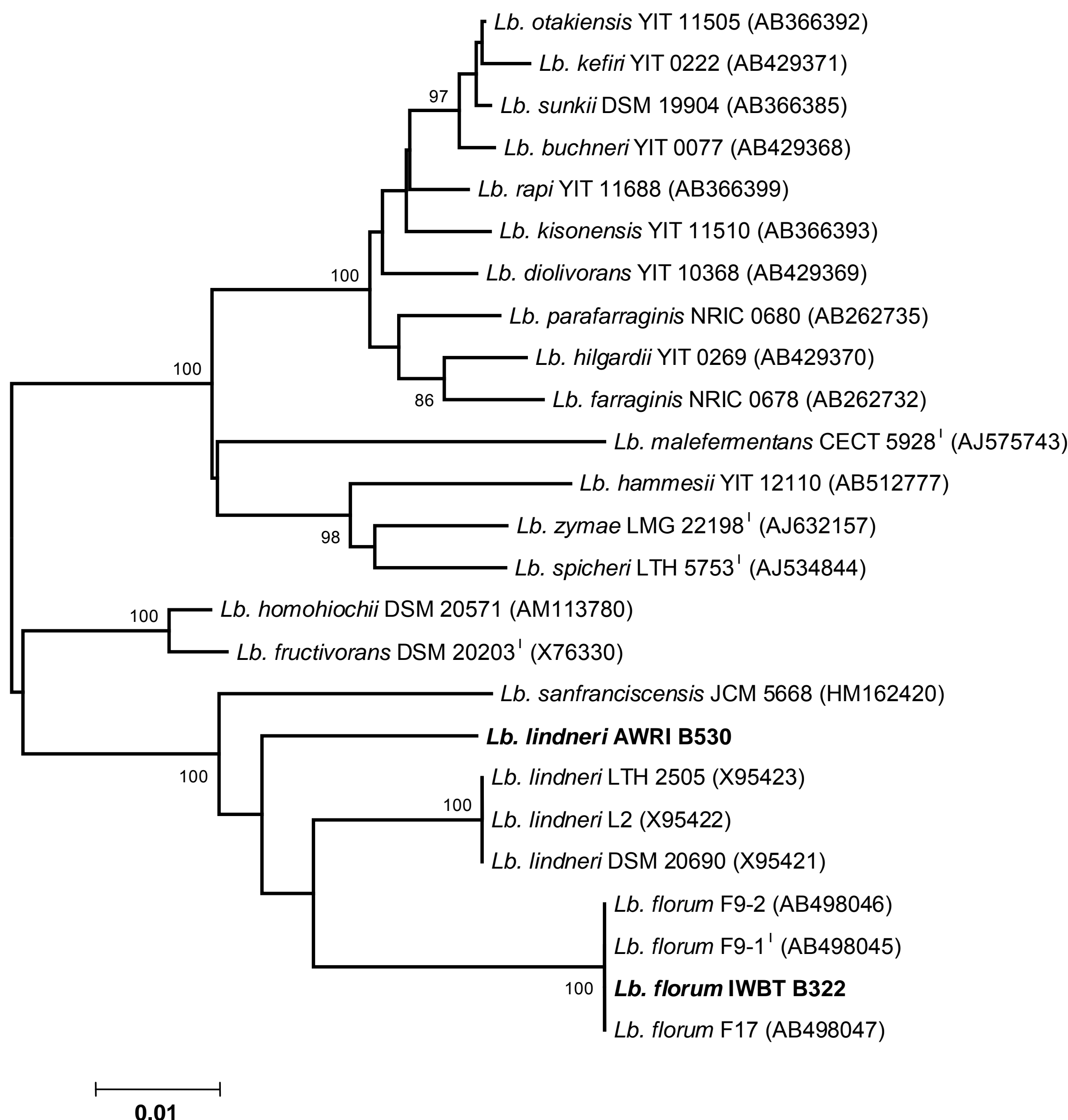

Figure 7.1. Phylogenetic tree based on 16S ribosomal DNA gene sequences showing the relationship of *Lactobacillus florum* IWBT B322 strain with closely related species (accession numbers in parenthesis). Only one strain from our culture collection was included in the phylogenetic tree due to high sequence similarity (>99%) between all our strains. The tree was constructed using the neighbour-joining method, and the numbers at branching points are bootstrap values (expressed as percentages of 1000 replicates). The horizontal scale bar represents the number of base substitutions per site. Abbreviations: IWBT, Institute for Wine Biotechnology; AWRI, Australian Wine Research Institute.

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FLOR                 ---------------------GCTGCCCAGTTGCTAGTC------------------------------------------------ 18
florum IWBT B322     ---ACATGCAAGTCGAACGAGGCTGCCCAGTTGCTAGTCGGTGCTTGCACTGACGAACAATTGGATCCAGCCGAGTGGCGAACTGGT 84
florum F9-1          ---ACATGCAAGTCGAACGAGGCTGCCCAGTTGCTAGTCGGTGCTTGCACTGACGAACAATTGGATCCAGCCGAGTGGCGAACTGGT 84
lindneri AWRI B530   ---ACATGCAAGTCGAACGCGGTCTCCTAATTGAAAAACCGTGCAAGCACGGGTTGGATTTAGATCGGACCGAGTGGCGAACTGGT 84
lindneri DSM 20690   ---ACATGCAAGTCGAACGAGGTCTCCTAACTGATAGCTGGTGCTTGCATCAGCTTGACGATAGATCTGACCGAGTGGCGAACTGGT 84
homohiochii          ---ACATGCAAGTCGAACGAGCTGCGCCTAATGATAGTTGATGCTTGCATTAGCTTGACTTAAGTTAGCAGCGAGTGGCGAACTGGT 84
fructivorans         ---ACATGCAAGTCGAACGAGCTGCGCCTAATGATAGTTGATGCTTGCATTAGCTTGACTTAAGTTAGCAGCGAGTGGCGAACTGGT 84
```

Figure 7.2. Multiple sequence alignments showing the V1 variable region of the 16S rDNA sequence from where the species-specific primer (FLOR) for *Lb. florum* was designed. LAB strains: *Lb. florum* IWBT B322, *Lb. florum* F9-1^T (AB498045), *Lb. lindneri* AWRI B530, *Lb. lindneri* DSM 20690 (X95421), *Lb. homohiochii* DSM 20571 (AM113780) and *Lb. fructivorans* (X76330).

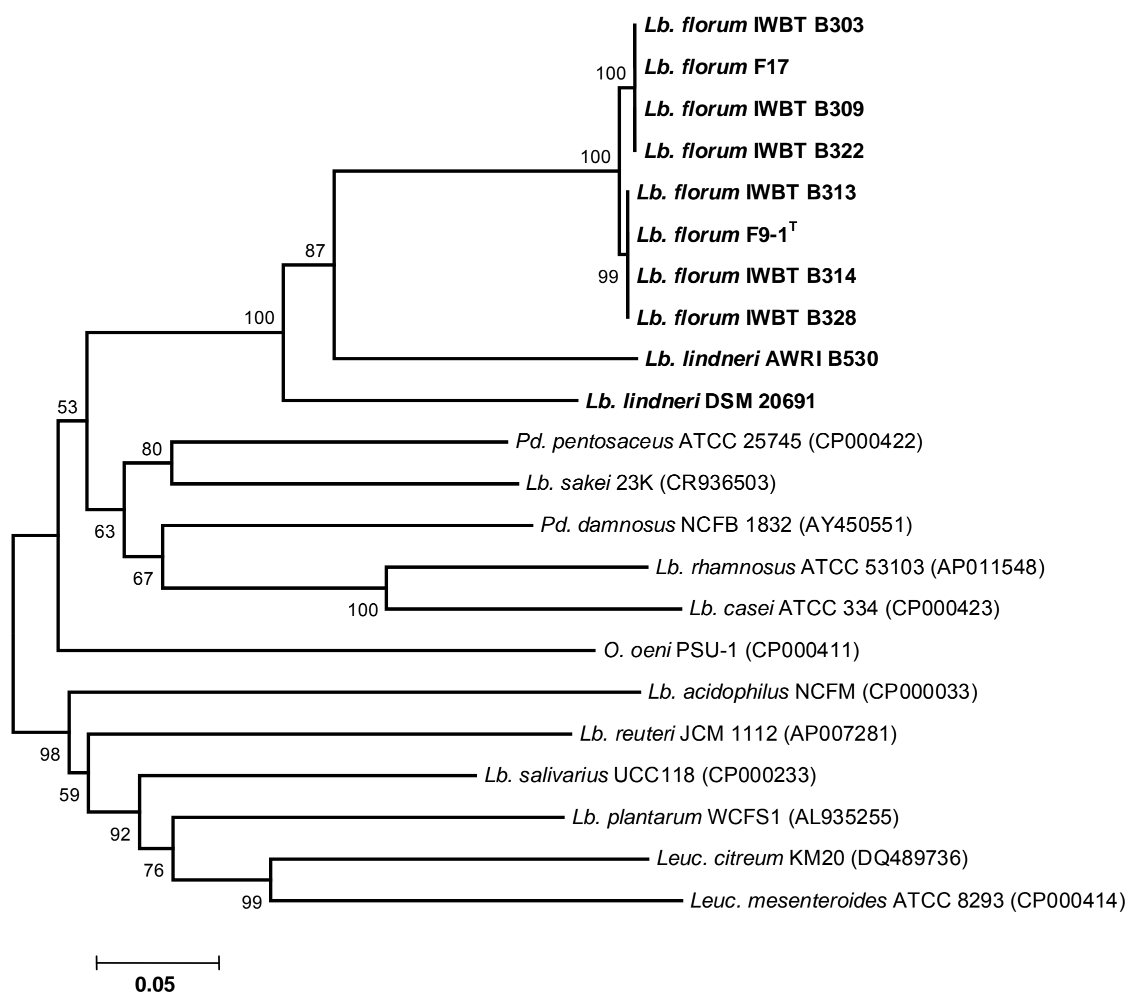


Figure 7.3A. Phylogenetic tree based on *mleA* gene sequences of the representative strains of *Lactobacillus florum* (IWBT B303, IWBT B309, IWBT B313, IWBT B314, IWBT B322, IWBT B328, F17 and F9-1^T), together with closely related species (accession numbers in parenthesis). The tree was constructed using the neighbour-joining method, and the numbers at branching points are bootstrap values (expressed as percentages of 1000 replicates). The horizontal scale bar represents the number of base substitutions per site. Refer to Figure 7.1 for abbreviations.

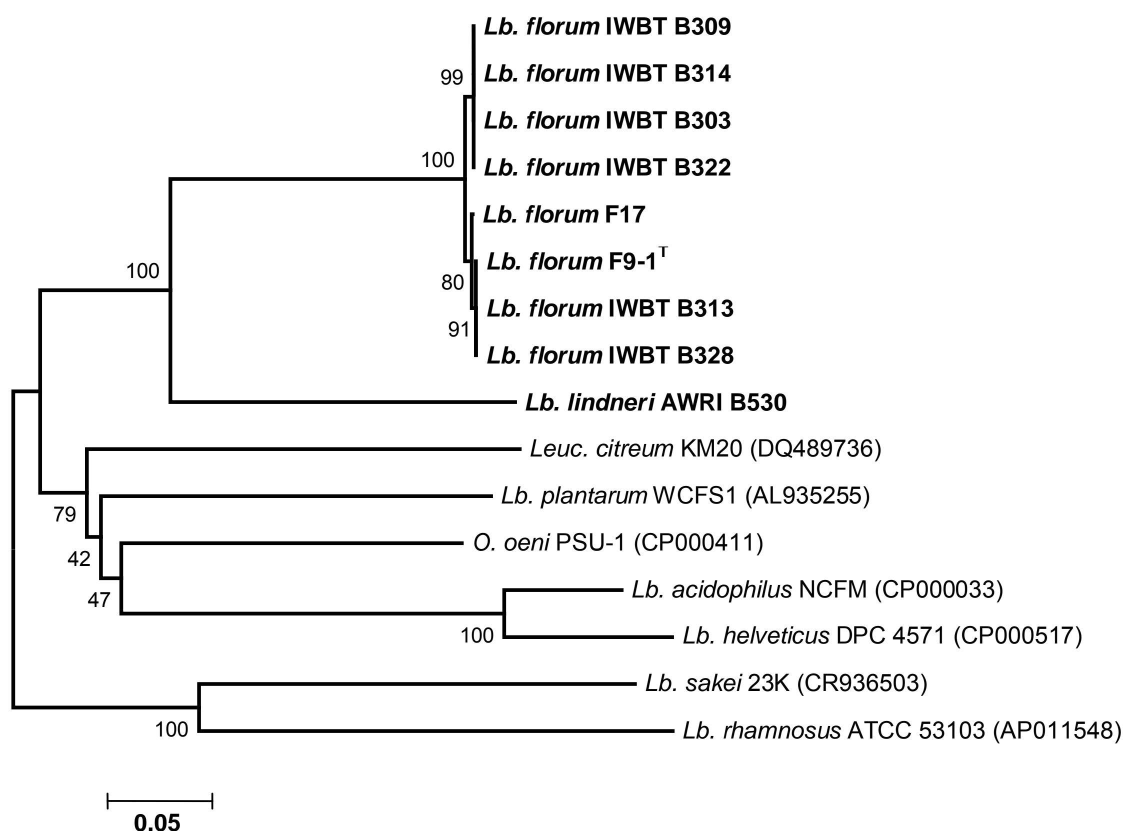


Figure 7.3B. Phylogenetic tree based on *citE* gene sequences of the representative strains of *Lactobacillus florum* (IWBT B303, IWBT B309, IWBT B313, IWBT B314, IWBT B322, IWBT B328, F17 and F9-1^T), together with closely related species (accession numbers in parenthesis). The tree was constructed using the neighbour-joining method, and the numbers at branching points are bootstrap values (expressed as percentages of 1000 replicates). The horizontal scale bar represents the number of base substitutions per site. Refer to Figure 7.1 for abbreviations.

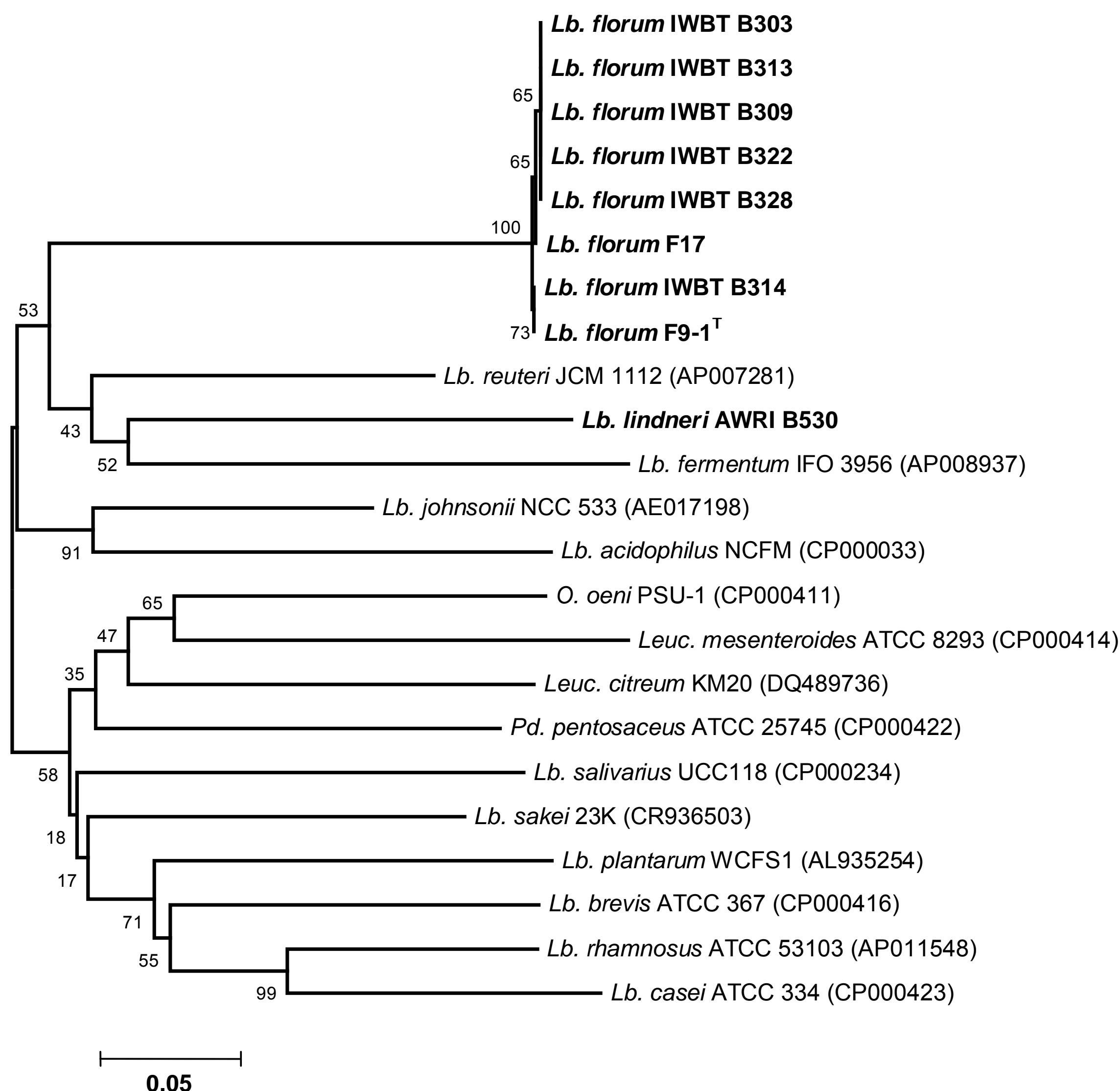
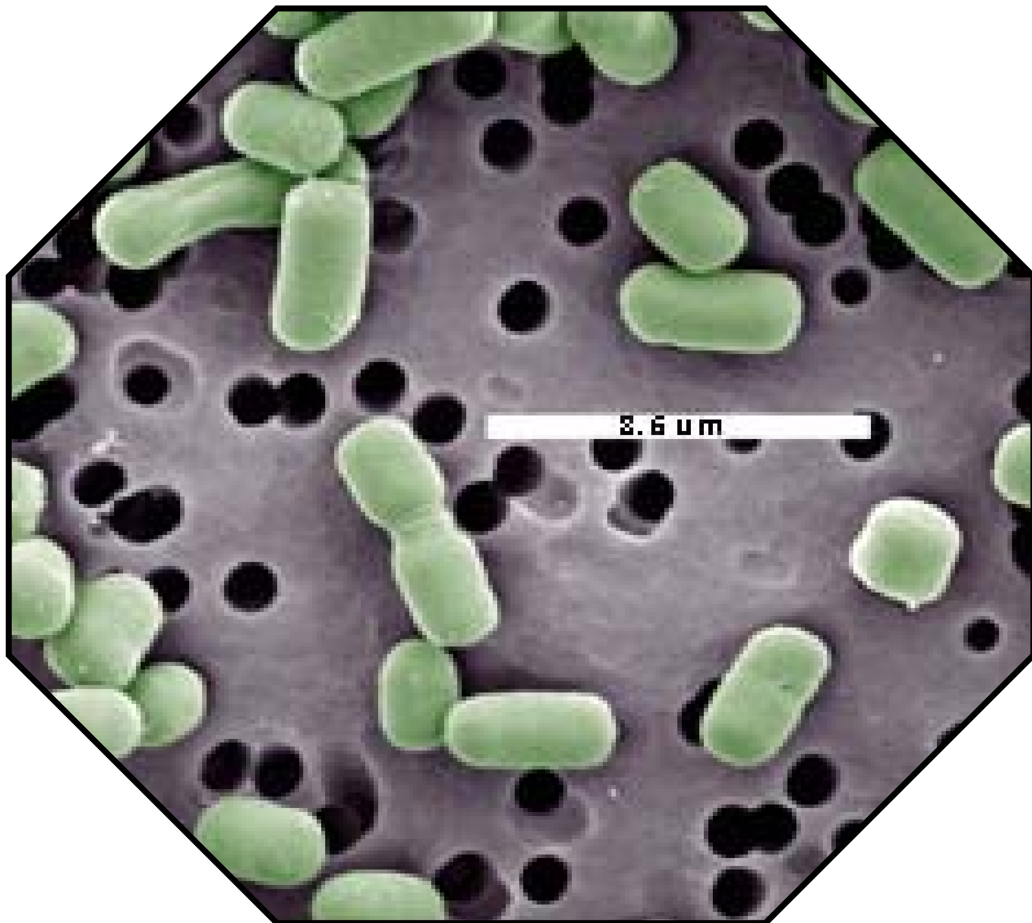


Figure 7.3C. Phylogenetic tree based on *pepN* gene sequences of the representative strains of *Lactobacillus florum* (IWBT B303, IWBT B309, IWBT B313, IWBT B314, IWBT B322, IWBT B328, F17 and F9-1^T), together with closely related species (accession numbers in parenthesis). The tree was constructed using the neighbour-joining method, and the numbers at branching points are bootstrap values (expressed as percentages of 1000 replicates). The horizontal scale bar represents the number of base substitutions per site. Refer to Figure 7.1 for abbreviations.

CHAPTER 8

RESEARCH RESULTS VI



Lactobacillus and *Pediococcus* genes related to peptide and amino acid utilisation in wine

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CHAPTER 8

Lactobacillus and *Pediococcus* genes related to peptide and amino acid utilisation in wine

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ABSTRACT

In this study, a total of 104 strains of lactic acid bacteria were tested for the presence of genes encoding enzymes related to peptide and amino acid utilization in wine. Primers for PCR amplifications were designed from conserved regions of the genes from various LAB species belonging to *Lactobacillus, Leuconostoc, Pediococcus* and *Oenococcus*. As expected, PCR assays generated single DNA fragments of correct sizes. From the PCR detection results, it was observed that the genes tested for were distributed across different species of lactobacilli and pediococci investigated. However, some strains of *Pediococcus* did not present certain enzyme-encoding genes, such as *pepO*, *pepT*, *metK* and *gshR*. In addition, *pepX* and *metB*/*metC* genes were not detected in any of the *Pediococcus* strains tested. *Lactobacillus plantarum* IWBT B349 strain was selected for gene sequence verification. From the comparative sequence analysis, it was observed that nucleotide gene sequences of this strain are highly identical to those of other *L. plantarum* strains (WCFS1, JDM1 and ATCC 14917) published in GenBank database. Altogether, the results presented in this study provide an indication that lactobacilli and pediococci strains of wine origin have the genetic potential to degrade peptides and amino acids in wine.

Keywords: *Lactobacillus*, pediococci, peptidases, amino acids, PCR detection, sequencing

8.1 INTRODUCTION

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria occurring on grapes and in wine during malolactic fermentation (MLF). The latter process, carried out mainly by *Oenococcus oeni* and *Lactobacillus plantarum*, involves the conversion of L-malic acid to L-lactic acid and CO_2. This reaction contributes to wine acidity reduction, increase microbial stability and changes in the sensory attributes of wine (Bartowsky, 2005; Lerm *et al*., 2010). Generally, LAB are fastidious microorganisms with multiple amino acid requirements, thus requiring the proteolytic system necessary to degrade proteins (present in the environment in which they occur) into smaller peptides and amino acids crucial for their growth (Juillard *et al*., 1995). In the dairy industry, for example, the proteolytic activity of bacterial species also favours the development of texture and flavour of fermented milk products (El-Soda *et al*., 2000; Hynes *et al*., 2003; Lynch *et al*., 1999).

While the proteolytic system of *Lactococcus lactis*, a model microorganism used as the starter culture in the dairy industry, has been extensively investigated (Tan *et al*., 1993; Tynkkynen *et al*., 1993; Hagting *et al*., 1994; Foucaud *et al*., 1995; Kunji *et al*., 1996), the proteolytic enzymes of bacterial species of wine origin remain poorly understood. Nevertheless, the production of exocellular proteinases by *O. oeni* strains was detected previously (Rollan *et al*., 1993; Manca de Nadra *et al*., 1997, 1999; Remize *et al*., 2005), albeit Davis *et al*. (1988) observed no protease production by several wine LAB strains of *Oenococcus*, *Lactobacillus* and *Pediococcus*. More recently, Mtshali *et al*. (2010) demonstrated the presence of protease enzyme-encoding gene (*prtP*) in several wine lactobacilli and the PCR detection results indicated that this gene is distributed across the LAB species. The presence of *prtP* proteinase gene in a natural isolate of *L. plantarum* originating from home-made cheese has also been reported (Strahinic *et al*., 2010). This gene encodes an enzyme that participates in protein degradation to form small peptides and amino acids. The resulting peptides will further be translocated into the cell via the specific transport system where they will be acted upon by a variety of peptidases (Kunji et al., 1996).

Following the primary and secondary proteolysis, some peptidases are able to release sulphur-containing amino acids from peptide degradation (Kunji *et al*., 1996). In the dairy industry, the catabolism of sulphur amino acids by microbial enzymes plays a key role in the development of a typical cheese flavour (Dias & Weimer, 1999). The LAB originating from cheese are also known to metabolise methionine into methanethiol, a volatile sulphur compound contributing to cheese flavour (Weimer *et al*., 1999). In wine, Pripis-Nicolau *et al*. (2004) showed that the LAB isolated from wine could degrade methionine to form volatile sulphur compounds. From our knowledge, this aspect has not been investigated at a molecular level with intent to ascertain the potential of wine LAB to possess genes associated with the metabolism of sulphur-containing compounds. In addition, the

distribution of genes encoding a variety of peptidases in wine-associated LAB species is still devoid of thorough investigation.

Therefore, this study was aimed at investigating the presence of genes encoding enzymes involved in the proteolytic and amino acid catabolic pathways in lactobacilli and pediococci of oenological origin. These enzyme-encoding genes tested in this study included peptide transporter (DtpT), aminopeptidases (PepC, PepN, PepM), endopeptidase (PepO), tripeptidase (PepT), proline peptidase (PepX, PepI), as well as those in the sulphur metabolic pathway.

8.2 MATERIALS AND METHODS

8.2.1 Bacterial strains and growth conditions

The bacterial strains used in this work were previously isolated from grape and wine samples obtained from five different commercial wineries situated in the Western Cape region, South Africa, during the 2001 and 2002 harvest seasons (Krieling, 2003). Taxonomic identification of these isolates was performed by PCR with species-specific primers and by 16S rDNA sequence analysis. The type and reference strains included in this work are presented in Table 8.1. All the strains were cultured at 30°C in MRS medium (BIOLAB Diagnostics, Wadeville, South Africa).

8.2.2 Primer design

To design the primers for PCR assays, we aligned nucleotide sequences of the target genes from several LAB species belonging to four LAB genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus*. Two conserved domains of each gene were then selected to design the primers for PCR amplifications. All PCR primers used in this study (Table 8.2) were synthesised by the Integrated DNA Technologies, Inc. (Coralville, IA, USA).

8.2.3 Colony PCR assays

PCR amplifications were performed in a Biometra Thermocycler machine (Biometra® GmbH, Göttingen, Germany). The 25-μL PCR mixture comprised a template DNA, 0.6 μM of each primer, 1.5 mM $MgCl_2$, 250 μM of dNTP mix, 1X PCR buffer and 1.25 units of Supertherm DNA polymerase (Southern Cross Biotechnology, Cape Town, South Africa). The reaction conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 49–54°C for 45 s and 72°C for 1 min (specific annealing temperatures are indicated in Table 8.2). The final extension step was carried out at 72°C for 10 min. PCR fragments were checked by gel electrophoresis in 1% w/v agarose gels containing ethidium bromide (0.5 μg mL^{-1}) and documented with Alpha Imager.

8.2.4 Sequence verification

To verify if the PCR-generated amplicons corresponded to the target genes, *L. plantarum* IWBT B349 was chosen for sequencing. DNA fragments from this strain were amplified with the same primers in a 50-μL reaction containing TaKaRa *Ex Taq*™ polymerase (Southern Cross Biotechnology) instead of Supertherm polymerase. Amplicons were cleaned with QIAquick® PCR Purification Kit (Qiagen, Southern Cross Biotechnology, Cape Town, South Africa) and subsequently sequenced using the corresponding forward and reverse primers. GenBank database was used to search for homologous DNA sequences.

8.2.5 Phylogenetic analysis

In an attempt to study the phylogenetic relationship between the LAB strains tested in this study with closely related taxa, five strains were randomly selected from which the *pepC* gene was amplified and sequenced. The obtained nucleotide gene sequence data were assembled and compared to the database sequences. The phylogenetic trees were inferred using the neighbour-joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) from the MEGA 4.1 software (Kumar et al., 2008) and are in the units of the number of base substitutions per site. Bootstrapping analysis was performed to evaluate the reliability of the topologies of constructed phylogenetic trees using 1000 bootstrap replications (Felsenstein, 1985).

8.3 RESULTS

8.3.1 PCR detection of genes

A total of 104 strains of lactobacilli and pediococci were tested in this study. These strains were screened for the presence of peptidase and sulphur metabolic pathway genes using a PCR detection approach with primers specific for different enzymes. PCR assays generated amplicons of expected sizes as shown in Table 8.2. The PCR detection results of different enzyme-encoding genes are reflected in Table 8.3. Among the lactobacilli screened, more than 80% possessed all the genes tested for. It was also worth noting that the *pepC* and *pepM* genes were present in all the *Lactobacillus* species. Although *L. plantarum* strains seemed to possess different combinations of the tested genes, other bacterial species possessed all these genes, including *Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus delbrueckii* and *Lactobacillus reuteri*. Among the *Lactobacillus fermentum* strains tested, only the reference strain (DSM 20052) did not present the *pepO* and *pepX* genes. Amongst the 10 strains of pediococci, all possessed the *dtpT*, *pepC*, *pepI*, *pepN* and *pepM* genes. On the other hand, the *pepX* and *metB*/*metC* genes were not detected in any of the *Pediococcus* strains tested.

8.3.2 Sequence verification

PCR amplifications of IWBT B349 strain of *L. plantarum* were verified by sequencing using the same sets of primers for PCR amplifications. After the newly determined sequence data were assembled and aligned, a comparative analysis of DNA sequences was performed. A homology search was performed by comparing these sequences to other DNA sequences published in GenBank database. The BLAST results confirmed that the sequenced fragments corresponded to the genes tested, and the highest homology of nucleotide sequences was recorded with three *L. plantarum* strains (JDM1, WCFS1 and ATCC 14917) published in the NCBI databank (Table 8.4). From the comparative analysis of sequences, it was observed that IWBT B349 displayed the highest homology to the JDM1 strain compared to WCFS1 and ATCC 14917. However, an exception was only observed for the glutathione reductase-encoding gene (*gshR*) of IWBT B349 whose identity to JDM1 was only 99.8%.

8.3.3 Phylogenetic analysis

The phylogenetic relationship between bacterial strains was also studied by constructing neighbour-joining trees based on nucleotide sequences of the *pepC* gene as an example. Five strains belonging to *L. plantarum*, *Lactobacillus paracasei*, *Lactobacillus paraplantarum*, *Pediococcus parvulus* and *Pediococcus pentosaceus* were randomly selected from which the *pepC* genes were PCR-amplified and subsequently sequenced. As shown in Figure 8.1, *L. plantarum* IWBT B349 clustered with other *L. plantarum* strains (WCFS1 and JDM1) whose *pepC* gene sequences were retrieved from GenBank database. Similarly, *P. pentosaceus* LMG 13561 also clustered with ATCC 25745 strain of the same species.

8.4 DISCUSSION

The proteolytic system has been studied extensively in lactococci. This system is composed of the protein breakdown, peptide transport and hydrolysis (Kunji *et al.*, 1996). The degradation of proteins is initiated by an extracellular cell wall-associated proteinase (PrtP) enzyme, which releases small peptides and amino acids essential for bacterial growth. The degradation products of proteins are then transported across the cell membrane into the cell via the peptide transporters (e.g. DtpT, DtpP, Opp). Once inside the cell, these peptides are further hydrolysed into amino acids by a variety of intracellular peptidases (Kunji *et al.*, 1996; Christensen *et al.*, 1999).

Previously, we demonstrated the existence of serine protease (*prtP*) gene in several species of *Lactobacillus* originating from South African wines (Mtshali *et al.*, 2010). PCR detection results indicated that the *prtP* gene was distributed across different species. Another recent study also reported the presence of *prtP* proteinase gene in a natural strain

of *L. plantarum* isolated from home-made cheese (Strahinic *et al*., 2010). In this work, different oenological species of lactobacilli and pediococci were screened for the presence of genes encoding various peptidases. These peptidases can be classified into aminopeptidases (PepC, PepN, PepM, PepA), endopeptidases (PepO, PepF), dipeptidases (PepD, PepV), tripeptidase (PepT) and proline-specific peptidases (PepX, PepI, PepR, PepP, PepQ) (Liu *et al*., 2010). Among the bacterial strains tested in this study, it was shown that peptidase genes are present in the majority of different species. The presence of the *dtpT* gene encoding a di-/tripeptide transporter was also detected in all pediococci strains as well as in the majority of lactobacilli (>90%).

In order to complement the amino acid auxotrophy, LAB require a range of proteolytic and peptidolytic enzymes to enable them to garner amino acids from the proteins and peptides. As such, wine also encompasses proteins which can ultimately be degraded by protein-utilising LAB to produce smaller peptides and amino acids. Other studies have indicated that wine peptides act as carbon and nitrogen substrates for bacterial growth (Aredes Fernandez *et al*., 2004). The presence of peptidolytic enzyme-encoding genes in wine lactobacilli and pediococci strains tested in this study has implications on the genetic capability of these strains to release amino acids from peptides. This can also favour the development of wine flavour, as it has been reported for dairy LAB (El-Soda *et al*., 2000; Hynes *et al*., 2003).

Following the breakdown of peptides by various peptidases, sulphur-containing amino acids can also be released (Kunji *et al*., 1996). These sulphurous amino acids have been shown to enhance flavour formation in dairy products such as cheese (Dias & Weimer, 1999). In this study, we have also tested the LAB strains for the presence of enzyme-encoding genes involved in the sulphur metabolic pathway. Different *Lactobacillus* species displayed the presence of these genes (i.e. *metK*, *gshR* and *metB*/*metC*), albeit not all the strains possessed these genes. Some strains of pediococci only possessed the *metK* and *gshR* genes whereas *metB*/*metC* gene was not detected in any of the strains tested.

In conclusion, the results presented in this study provide an indication that *Lactobacillus* and *Pediococcus* strains of wine origin possess the genetic potential to degrade peptides and amino acids during winemaking. However, it remains to be further confirmed if these peptidase enzyme-encoding genes are not repressed by winemaking parameters such as pH, temperature and ethanol.

8.5 ACKNOWLEDGEMENTS

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8.6 REFERENCES

Aredes Fernandez PA, Saguir FM, Manca de Nadra MC (2004) Effect of dipeptides on the growth of *Oenococcus oeni* in synthetic medium deprived of amino acids. *Curr Microbiol* **49:** 361–365.

Bartowsky EJ (2005) *Oenococcus oeni* and malolactic fermentation – moving into the molecular arena. *Aust J Grape Wine Res* **11:** 174–187.

Christensen JE, Dudley EG, Pederson JA, Steele JL (1999) Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* **76:** 217–246.

Davis CR, Wibowo D, Fleet GH & Lee TH (1988) Properties of wine lactic acid bacteria: their potential enological significance. *Am J Enol Vitic* **39:** 137–142.

Dias B & Weimer B (1999) Production of volatile sulphur compounds in Cheddar cheese slurries. *Int Dairy J* **9:** 605–611.

El-Soda M, Madkor SA & Tong PS (2000) Adjunct cultures: recent development and potential significance to the cheese industry. *J Dairy Sci* **83:** 609–619.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783–791.

Foucaud C, Kunji ER, Hagting A, Richard J, Konings WN, Desmazeaud M & Poolman, B (1995) Specificity of peptide transport systems in *Lactococcus lactis*: evidence for a third system which transports hydrophobic di- and tripeptides. *J Bacteriol* **177:** 4652–4657.

Hagting A, Kunji E, Leenhouts K, Poolman B & Konings W (1994) The di- and tripeptide transport protein of *Lactococcus lactis*. A new type of bacterial peptide transporter. *J Biol Chem* **269:** 11391–11399.

Hynes E, Bach C, Lamberet G, Ogier JC, Son O & Delacroix-Buchet A (2003) Contribution of starter lactococci and adjunct lactobacilli to proteolysis, volatile profile and sensory characteristics of washed-curd cheese. *Lait* **83:** 31–43.

Juillard V, Le Bars D, Kunji ER, Konings WN, Gripon JC & Richard J (1995) Oligopeptides are the main source of nitrogen for *Lactococcus lactis* during growth in milk. *Appl Environ Microbiol* **61:** 3024–3030.

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16:** 111–120.

Krieling SJ (2003) An investigation into lactic acid bacteria as a possible cause of bitterness in wine. Masters Thesis. Stellenbosch University, Stellenbosch, South Africa.

Kumar S, Nei M, Dudley J & Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* **9:** 299–306.

Kunji ERS, Mierau I, Hagting A, Poolman B & Konings WN (1996) The proteolytic systems of lactic acid bacteria. *Antonie van Leeuwenhoek* **70:** 187–221.

Lerm E, Engelbrecht L & Du Toit M (2010) Malolactic Fermentation: The ABC's of MLF. *S Afr J Enol Vitic* **31:** 186–212.

Liu M, Bayjanov JR, Renckens B, Nauta A & Siezen RJ (2010) The proteolytic system of lactic acid bacteria revisited: a genomic comparison. *BMC Genomics* **11:** 36–50.

Lynch CM, Muir DD, Banks JM, McSweeney PLH & Fox PF (1999) Influence of adjunct cultures of *Lactobacillus paracasei* ssp. *paracasei* or *Lactobacillus plantarum* on Cheddar cheese ripening. *J Dairy Sci* **82:** 1618–1628.

Manca de Nadra MC, Farias ME, Moreno-Arribas MV, Pueyo E & Polo MC (1997) Proteolytic activity of *Leuconostoc oenos*: effect on proteins and polypeptides from white wine. *FEMS Microbiol Lett* **150:** 135–139.

Manca de Nadra MC, Farias ME, Moreno-Arribas V, Pueyo E & Polo MC (1999) A proteolytic effect of *Oenococcus oeni* on the nitrogenous macromolecular fraction of red wine. *FEMS Microbiol Lett* **174:** 41–47.

Mtshali PS, Divol B & Du Toit M (2010) Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines. *J Appl Microbiol* **108:** 1389–1397.

Pripis-Nicolau L, de Revel G, Bertrand A & Lonvaud-Funel A (2004) Methionine catabolism and production of volatile sulphur compounds by *Oenococcus oeni*. *J Appl Microbiol* **96:** 1176–1184.

Remize F, Augagneur Y, Guilloux-Benatier M & Guzzo J (2005) Effect of nitrogen limitation and nature of the feed upon *Oenococcus oeni* metabolism and extracellular protein production. *J Appl Microbiol* **98:** 652–661.

Rollan GC, Farias ME & Manca de Nadra MC (1993) Protease production by *Leuconostoc oenos* strains isolated from wine. *World J Microbiol Biotechnol* **9:** 587–589.

Saitou N & Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4:** 406–425.

Strahinic I, Kojic M, Tolinacki M, Fira D & Topisirovic L (2010) The presence of *prtP* proteinase gene in natural isolate *Lactobacillus plantarum* BGSJ3–18. *Lett Appl Microbiol* **50:** 43–49.

Tan PS, Poolman B, Konings WN (1993) Proteolytic enzymes of *Lactococcus lactis*. *J Dairy Res* **60:** 269–286.

Tynkkynen S, Buist G, Kunji E, Kok J, Poolman B & Venema G (1993) Genetic and biochemical characterization of the oligopeptide transport system of *Lactococcus lactis*: demonstration of the essential role of oligopeptide transport in the utilization of casein. *J Bacteriol* **175:** 7523–7532.

Weimer B, Seefeldt K & Dias B (1999) Sulfur metabolism in bacteria associated with cheese. *Antonie van Leeuwenhoek* **76:** 247–261.

Table 8.1. Reference or type strains included in this work

Bacterial species	Strain number	Culture collection
Lactobacillus spp.		
Lb. casei	LMG 13552	Laboratorium voor Microbiologie
Lb. curvatus	LMG 13553	Laboratorium voor Microbiologie
Lb. delbrueckii	DSM 20074	German Collection of Microorganisms and Cell Cultures
Lb. fermentum	DSM 20052	German Collection of Microorganisms and Cell Cultures
Lb. hilgardii	DSM 20176	German Collection of Microorganisms and Cell Cultures
Lb. malefermentans	ATCC 11305	American Type Culture Collection
Lb. paracasei	DSM 5622	German Collection of Microorganisms and Cell Cultures
Lb. paraplantarum	DSM 10667	German Collection of Microorganisms and Cell Cultures
Lb. pentosus	DSM 20314	German Collection of Microorganisms and Cell Cultures
Lb. plantarum	DSM 20174	German Collection of Microorganisms and Cell Cultures
Lb. reuteri	LMG 13557	Laboratorium voor Microbiologie
Lb. sakei	NCFB 2714	National Collection of Food Bacteria
Pediococcus sp.		
Pd. pentosaceus	LMG 13561	Laboratorium voor Microbiologie
	NCDO 813	National Collection of Dairy Organisms
	NCDO 1859	National Collection of Dairy Organisms

Table 8.2. PCR primers designed in this study, annealing temperatures and amplicon sizes

Target gene	Application	Primer sequence (5′→3′)	Annealing temp.	Amplicon size [a] (bp)
dtpT	Di-tripeptide transporter	F-GAAATGTGGGARCGDTTYAGTTAYTAYGG R-ATCNGYYARGAACCACAKACWCATCATYTG	50°C	1242-1269
pepC	Cysteine aminopeptidase	F-GGNCGTTGYTGGATGTTYGCBGCHTTRAAYAC R-TTADGCHAAWGHWCCCATTGGRTCCCAHGG	54°C	1131-1143
pepI	Proline iminopeptidase	F-CCYGGTGGHAABCAYGARTAYTGGGAARAC R-GCRTTRTCDATCATGTGRTGRTGRCCRCCDT	53°C	734-743
pepN	Membrane alanine aminopeptidase	F-ATGGAAAACTGGGGNYTDGTNACHTAYCG R-ACNRCNGGRTADCCNGGYTGTTCVARCCANG	49°C	569
pepM	Methionine aminopeptidase	F-GGHTTTGAAGGHTAYAARTATKCNACBTGTGT R-AYCATBGGTTCRATNGTAATBGTCATVCC	49°C	443
pepO	Endopeptidase O	F-ATYTTVCCDGAYAMDACNTACTAYGMHGA R-CCACCARTTATBCADVTTNCCDAATTCATCRAA	49°C	1077-1089
pepT	Tripeptidase T	F-TTGATACDGCDGAYTTTAAYGCNGADAATG R-CCRTGCATRTTTTCDBCHCCVGCAAA	53°C	898-907
pepX	X-prolyl dipeptidyl aminopeptidase	F-CTTTTATWDTNTDGNHCTNCAAYTACTNGRNTTT R-ACNGCAAANCCVCGNGMBARAAARTAATBAT	50°C	639-681
metK	S-adenosylmethionine synthase	F-GAAMGMCAYTTATTTACDTCDGA R-AATBCCAGCWGGBCGYAARTCAAA	54°C	1080
metB/metC	Cystathionine γ-lyase/β-lyase	F-ATGAAATTYRAWACMMAAYTWATTCAYGGYGG R-ACCVACHGAKARRCGRATYAGYTCGTCTT	49°C	1080-1083
gshR	Glutathione reductase	F-ATGGCGGAACAGTACGATG R-TTAATACAAATATTGTAAGTCACTAGCCG	49°C	1332

[a] Theoretical amplicon sizes based on the nucleotide gene sequences of several LAB species used as templates for primer design.

Table 8.3. Results of PCR detection of genes with enzyme-specific primers

Species	*dtpT*	*pepC*	*pepI*	*pepN*	*pepM*	*pepO*	*pepT*	*pepX*	*metK*	*metB/C*	*gshR*
Lactobacillus spp.	94 [a] (88 [b])	94 (94)	94 (88)	94 (89)	94 (94)	94 (85)	94 (88)	94 (81)	94 (89)	94 (84)	94 (79)
L. casei	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)
L. curvatus	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)
L. delbrueckii	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)
L. fermentum	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (02)	03 (03)	03 (02)	03 (03)	03 (03)	03 (03)
L. hilgardii	02 (02)	02 (02)	02 (02)	02 (02)	02 (02)	02 (02)	02 (02)	02 (01)	02 (01)	02 (02)	02 (01)
L. malefermentans	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (00)	01 (01)	01 (01)	01 (01)	01 (00)	01 (00)
L. paracasei	05 (05)	05 (05)	05 (05)	05 (05)	05 (05)	05 (05)	05 (05)	05 (04)	05 (05)	05 (05)	05 (03)
L. paraplantarum	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (00)	03 (02)	03 (03)	03 (03)
L. pentosus	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (02)	03 (02)	03 (03)	03 (02)
L. plantarum	71 (66)	71 (71)	71 (66)	71 (67)	71 (71)	71 (66)	71 (65)	71 (66)	71 (69)	71 (64)	71 (63)
L. reuteri	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)
L. sakei	02 (01)	02 (02)	02 (01)	02 (01)	02 (02)	02 (00)	02 (02)	02 (01)	02 (02)	02 (00)	02 (00)
Pediococcus spp.	10 (10)	10 (10)	10 (10)	10 (10)	10 (10)	10 (07)	10 (04)	10 (00)	10 (07)	10 (00)	10 (03)
P. acidilactici	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (01)	01 (00)	01 (00)	01 (00)	01 (00)
P. parvulus	06 (06)	06 (06)	06 (06)	06 (06)	06 (06)	06 (05)	06 (00)	06 (00)	06 (04)	06 (00)	06 (00)
P. pentosaceus	03 (03)	03 (03)	03 (03)	03 (03)	03 (03)	03 (01)	03 (03)	03 (00)	03 (03)	03 (00)	03 (03)

[a] Total number of strains tested.
[b] Number of positive strains.

Table 8.4. Highest homologies found between DNA sequences of *L. plantarum* IWBT B349 and the closest relatives published in the NCBI database

	% identity of *L. plantarum* IWBT B349 gene sequences with those of		
Target gene	***L. plantarum* WCFS1**[a]	***L. plantarum* JDM1**	***L. plantarum* ATCC 14917**
pepC	99.8%[b] (NP_784371[c])	100% (YP_003062126)	99.7% (ZP_04014238)
pepI	100% (NP_784587)	100% (YP_003062296)	99.9% (ZP_04014422)
pepN	99.0% (NP_784655)	100% (YP_003062364)	99.6% (ZP_04014501)
pepM	99.7% (NP_784075)	100% (YP_003061815)	100% (ZP_04015434)
pepO	99.1% (NP_786662)	100% (YP_003064328)	99.1% (ZP_04015003)
pepT	99.9% (NP_785446)	100% (YP_003063184)	99.7% (ZP_04012774)
pepX	99.7% (NP_784590)	100% (YP_003062299)	99.7% (ZP_04014425)
metK	99.5% (NP_784949)	100% (YP_003062685)	99.7% (ZP_04013270)
metB/metC	99.7% (NP_784073)	100% (YP_003061813)	99.9% (ZP_04015432)
gshR	98.9% (NP_784176)	99.8% (YP_003061917)	99.5% (ZP_04015540)

[a] GenBank accession numbers: *L. plantarum* WCFS1 (AL935263); *L. plantarum* JDM1 (CP001617); *L. plantarum* ATCC 14917 (ACGZ00000000).

[b] Percentage identity.

[c] GenBank accession number.

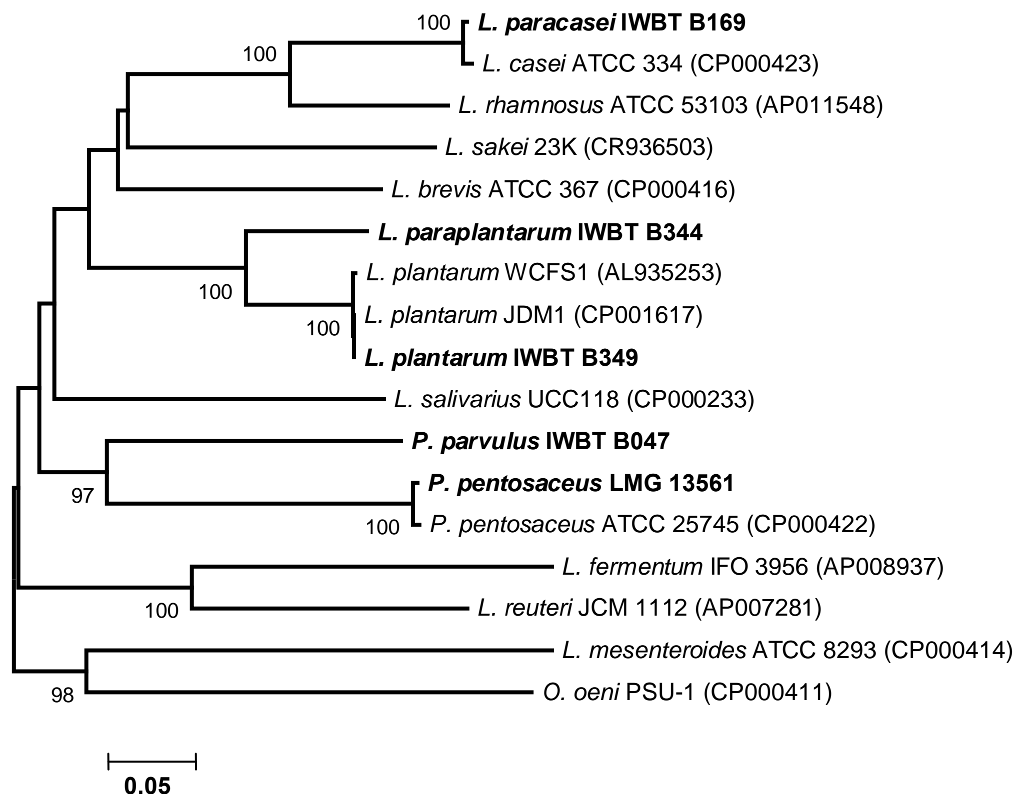


Figure 8.1. Phylogenetic tree showing relationships of various LAB strains tested in this study (indicated in bold) with closely related species (GenBank accession numbers in parentheses) based on partial *pepC* gene sequences. The tree was constructed by the neighbour-joining method with Kimura's two-parameter correction model. Bootstrap values >80% are shown at branching points. The horizontal scale bar represents the number of base substitutions per site.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 9

9.1 GENERAL DISCUSSION

In winemaking, two fermentation processes occur. Malolactic fermentation (MLF) is the secondary fermentation process conducted by lactic acid bacteria (LAB), which plays a crucial role in wine with respect to wine acidity reduction as a result of the conversion of malic acid to lactic acid and CO_2. This reaction is usually accompanied by microbiological stabilisation as well as changes in the sensory attributes of wine (Wibowo *et al.*, 1985; Lerm *et al.*, 2010).

Although wine deacidification is the well known outcome of malate degradation by wine LAB, MLF also confers other desirable traits in wine, such as the modification of wine sensory properties. There are various ways in which the malolactic bacteria effect changes in the sensory profile of wine. One of the mechanisms is by the production of enzymatic activities capable of degrading wine precursor components. The metabolism of these compounds can have a positive or a negative impact in wine, depending on the metabolites formed. Swiegers *et al.* (2005) listed possible pathways by which wine-associated LAB modulate wine aroma. However, there is still limited knowledge regarding the genetics of wine LAB, particularly with regards to the presence of various genes coding for enzymes participating in various metabolic pathways.

This study was therefore aimed at screening wine LAB isolates for the presence of genes of relevance to winemaking. The first part of this project was to screen wine LAB isolates representing different species of *Lactobacillus* for the presence of genes coding for β-glucosidase, protease, esterase, phenolic acid decarboxylase and citrate lyase (α-, β- and γ-subunits). The results of PCR detection indicated that the *Lactobacillus* strains possessed different combinations of the enzymes investigated. It was also worth noting that certain other strains did not possess any of the genes tested. In order to verify if we amplified the correct genes, few strains representing different species were randomly selected from which the corresponding genes were amplified and sequenced. The analysis of gene sequences revealed that these sequences were highly conserved between species. These results are in agreement with the findings of Spano et al. (2005) who reported the similarity in amino acid sequences of β-glucosidase genes from *Lb. plantarum*, *Lb. paraplantarum*, *Oenococcus oeni* and *Pediococcus damnosus*. Of particular interest to note was the fact that β-glucosidase genes from two strains (*Lb. plantarum* 113.1 and *Lb. brevis* 116.3) possessed smaller fragments with 84 nucleotides missing. The presence of this gap is a rare event and it might influence the activity of the enzyme.

In a follow-up study, *O. oeni* strains isolated in South Africa from brandy base wines and from red grape and wine samples were also tested for the presence of different enzyme-encoding genes of oenological relevance. As expected, the strains were shown to possess all the genes investigated (i.e. *mleA*, *bgl*, *estA*, *prtP*, *citD*, *citE*, *citF*, *maeP*, *alsD*, *alsS*, *metK*, *metB*, *metC*, *gshR* and *arcA*), with the notable exception of *arcB*, *arcC* and *pad*. Only two *O. oeni* strains possessed the *pad* gene encoding phenolic acid decarboxylase. From our knowledge, this is the first study to detect the presence of the *pad* gene in *O. oeni* strains. In a previous study, de las Rivas *et al*. (2009) also developed a PCR assay to test various species of wine LAB for the presence of the *pad* gene. Among the species tested, none of the *O. oeni* strains possessed this gene. The verification of PCR-generated amplicons of the *pad* gene from the two *O. oeni* strains tested in this study was performed by sequencing. The obtained nucleotide sequences were compared to other bacterial DNA sequences published in GenBank database and the highest identity with lactobacilli was 74 – 80%. The comparison of these sequences to the genomes of PSU-1, ATCC BAA-1163 and AWRI B429 strains of *O. oeni* also revealed that these strains do not possess the *pad* gene, suggesting that the *pad* gene from the two isolates might have been acquired from another strain/species via the horizontal gene transfer. However, it still remains to be further determined if this gene encodes an active and functional enzyme capable of forming volatile phenols in wine from the degradation of hydroxycinnamic acids as precursor components present in wine during fermentation.

The expression of malolactic enzyme-encoding gene (*mleA*) of the three oenological strains of *O. oeni* was investigated under the winemaking conditions using quantitative real-time PCR. The main aim was to investigate the effect of combined pH and ethanol on the expression of the *mleA* gene under various conditions simulating those of winemaking. Under the conditions tested, the *mleA* gene expression appeared to be negatively affected by high ethanol content of 15% v/v. On the other hand, low pH (i.e. pH 3.2) seemed to have an enhancing effect towards the expression of the gene. The enhancing effect of low pH on *mleA* gene expression was also reported in a previous study by Beltramo *et al*. (2006).

Leuconostoc mesenteroides is also one of the species commonly found in wine during MLF. The impact of this bacterium in wine remains poorly understood. In this study, we aimed at evaluating the presence of a wide array of enzyme-encoding genes in *L. mesenteroides* strains of wine origin. Using a PCR detection method, these strains were screened for the presence of *bgl*, *mleA*, *estA*, *prtP*, *pad*, *alsD*, *alsS*, *metK*, *metC*, *metB*, *ghsR*, *citD*, *citE*, *citF* and *citP*. While the *gshR* and *bgl* genes were only detected in some strains, none of these genes were amplified: *arcA*, *arcB*, *arcC*, *citD*, *citE*, *citF* and *pad*. Sequence verification of PCR-generated fragments also indicated that some strains of *L.*

mesenteroides are genetically divergent from one another. More studies at a genetic level are still required in order to get a better understanding on the impact of this bacterium in wine.

Amongst the species tested in this study, *Lactobacillus florum* represents a new species that has been recently described by Endo *et al*. (2010). A total of 30 wine-associated LAB strains from our culture collection were identified as *Lb. florum* through the taxonomic analysis of their 16S rDNA sequences. Species-specific primers targeting the 16S rDNA of *Lb. florum* were also designed and validated. As expected, the new primers yielded fragments of an expected size from all *Lb. florum* strains. Of particular interest was also to screen these strains for the presence of wine-related enzyme-encoding genes. PCR detection results indicated that the *Lb. florum* strains possessed some of the genes tested for. Since this is the first time that *Lb. florum* is reported in wine, more studies are required to give better insights on the occurrence of this bacterium at various stages of fermentation as well as its impact on wine production. Nevertheless, the preliminary results presented in this study clearly indicate that the strains of this species can have a significant impact – positive or negative – on wine sensory properties due to the presence of a variety of enzyme-encoding genes.

9.2 FUTURE PERSPECTIVES

In summary, this study has demonstrated the existence of different enzyme-encoding genes in various LAB species associated with wine during MLF. Although the expression pattern of the *mleA* gene in *O. oeni* strains was also evaluated, the transcriptional behaviour of other genes under winemaking conditions has not been fully elucidated. For future studies, it will also be of interest to evaluate the regulation of the *mleA* gene in a real wine in order to have a better understanding of the conditions under which this gene is regulated during winemaking. This will help select the best strains adapted for the completion of MLF.

9.3 LITERATURE CITED

Beltramo, C., Desroche, N., Tourdot-Maréchal, R., Grandvalet, C., Guzzo, J., 2006. Realtime PCR for characterizing the stress response of Oenococcus oeni in a wine-like medium. *Res Microbiol* **157**, 267–274.

de las Rivas, B., Rodríguez, H., Curiel, J.A., Landete, J.M., Munoz, R., 2009. Molecular screening of wine lactic acid bacteria degrading hydroxycinnamic acids. *J Agric Food Chem* **57**, 490–494.

Endo, A., Futagawa-Endo, Y., Sakamoto, M., Kitahara, M., Dicks, L.M.T., 2010. *Lactobacillus florum* sp. nov., a fructophilic species isolated from flowers. *Int J Syst Evol Microbiol* **60**, 2478–2482.

Krieling, S.J., 2003. An investigation into lactic acid bacteria as a possible cause of bitterness in wine. Masters Thesis. Stellenbosch University, Stellenbosch, South Africa.

Lerm, E., Engelbrecht, L., Du Toit, M., 2010. Malolactic Fermentation: The ABC's of MLF. *S Afr J Enol Vitic* **31**, 186–212.

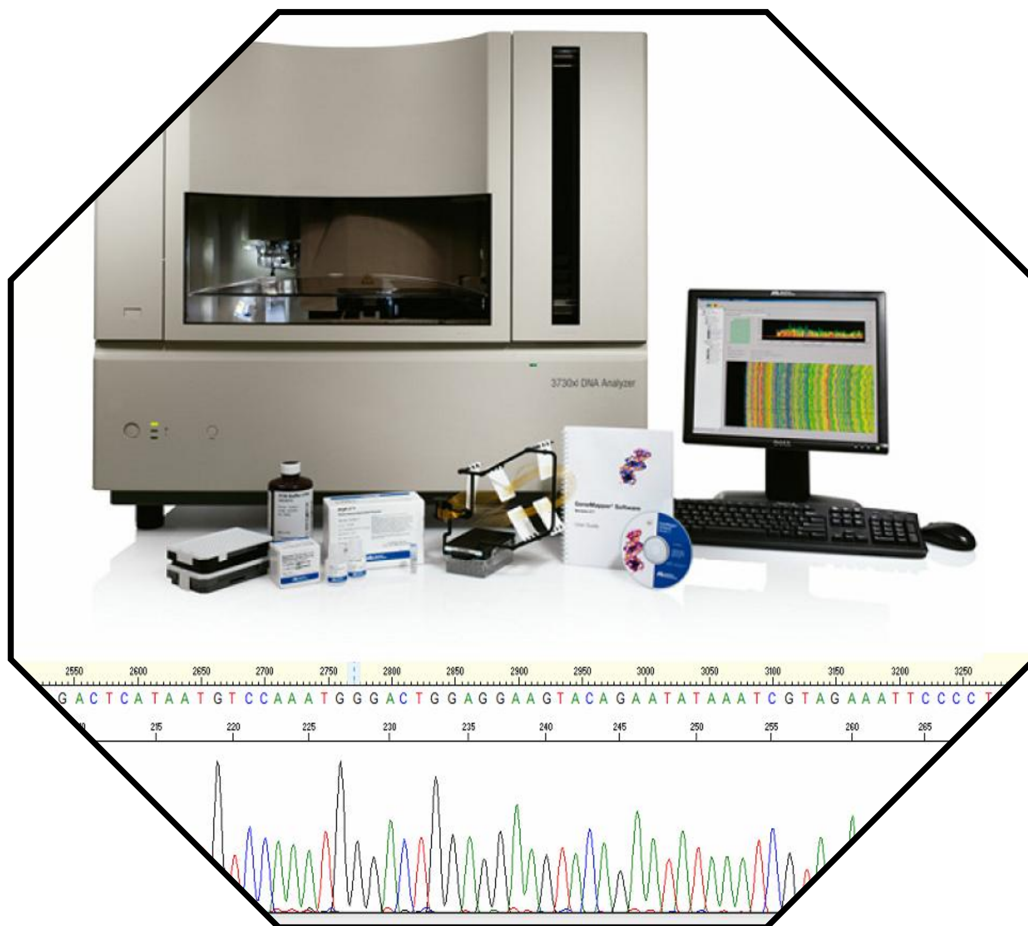
Spano, G., Rinaldi, A., Ugliano, M., Moio, L., Beneduce, L., Massa, S., 2005. A β-glucosidase gene isolated from wine *Lactobacillus plantarum* is regulated by abiotic stresses. *J Appl Microbiol* **98**, 855–861.

Swiegers, J.H., Bartowsky, E.J., Henschke, P.A., Pretorius, I.S., 2005. Yeast and bacterial modulation of wine aroma and flavour. *Aust J Grape Wine Res* **11**, 139–173.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H., Lee, T.H., 1985. Occurrence and growth of lactic acid bacteria in wine: a review. *Am J Enol Vitic* **36**, 302–313.

CHAPTER 10

ADDENDUM



Nucleotide gene sequences to be deposited in GenBank/EMBL/DDBJ databases

CHAPTER 10

10.1 Nucleotide gene sequences of *O. oeni* IWBT B040 strain

A unique GenBank accession number will be assigned to each gene once the nucleotide sequences have been deposited to GenBank/EMBL/DDBJ databases.

> Putative citrate transporter (*maeP*) gene → 984 bp

```
ATGGGTGTTTTTTGGACATCGATTCAGAGTGTTCTCTCAATCGTTATCATCATTGCAATTGGCTAC
GCCTGCCGGGGGGCAGGCTGGTTTGGCGAAGGCTTTCAAAGTGCTTTATCAAAACTGATTATGAAT
GTTGCTTTGCCGGCTTCAATTTTCATGGCGATGCTTTCGCGTTTTAAACCAAAGCAACTTGCTACT
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GTTTTGCCGATTTTGGCTGATACTTATCATTCCGATGTTAAATATGCCACGAATATTGTCGTTATG
ACATCGACTTTATTTATCATTGTTGTACCGATCATAATGAGTATCATCAACTTTATTTGA
```

> α-Acetolactate decarboxylase (*alsD*) gene → 717 bp

```
ATGAAAGATTTAACAAAAGCTTATCAACATGGCACTTTGGCTCAAATTATGGATGGCCAATATGAT
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GAAAGCCTAATTAATCTTGATTCCGATAGTTTTCAAAAAAGGGTTGAAGAAGAATATAAACTTAAA
AATGTCTTTGCCGCAATTAGAGTACACGGAGAATTTACAAATGTTTTGGCGCGATCAGCCGATAAA
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CTGACGGCAACGATGGTTGGCTATTATTCAGCAGCGATGTATGAAGGGACAACCGCGGCCGGCTTT
CACCTTCACATTCTCTCCGATGATCGTCAATTCGGAGGGCACCTATTAGATTTTAAAATCAAAAAA
GCCGACCTCCAGGTTCAGATTTTTCAGGATTTCCAGTTGCATCTACCAATTGAAAATCCCGATTTT
CGCCGACGCGAATTAGACTTGGAAACTTTAAAAAAAGCGATTGAAAAGACAGAATAA
```

> α-Acetolactate synthase (*alsS*) gene → 1683 bp

```
ATGACAGAAAAGAAACGTTTTGGGGCCGATCTGGTTCTTGAGTCCCTATTAAATCATGACGTTAAA
TATGTTTTCGGCATTCCCGGAGCCAAAATAGACCGGCTTTTTGAAGTAATTGAAAATAATTCCAAT
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CTGACCGGAAAACCCGGTGTAGTGCTCGTAACATCCGGACCGGGAGCATCGAATCTGGCAACCGGA
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CTTTATCGCCGAACACACCAATCGACTCCTTCAGTACTTTTGTTCAATGGAATTACAAAATTCACG
ACCGAGGTACAAGATGCAGAAAACTTATCCGAAGTAATTGCAAATGCCTTCGATATTGCCAGCGCG
```

GCCCCTCAGGGAGCCTCTTTCATCAGTCTCCCACAAGACGTTGACGACAGTCCGGTATCGAGTCAA
GCCTTGGAAAAAGTAGAAAATATTTCTGCTGGTCCGGCCAGTCCGGAACAAATCGAGTTCCTGGCA
GGTAAAATACGTCAGGCAAAATTGCCGGTTATTTTGGTTGGCCAACGCGGTTCCGACGAAAAAACC
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GGTGTTGTCGATCGGTCTTTGGTAGAACAATCGTTTTTTGGGCGTGTTGGTTTATTTGCCAACCAA
ACCGGCGATCAACTCTTAAAAGCTTCCGACTTGGTAATTGCACTCGGTTATGATGCCGTTGAATAT
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TTTAATTGGGATCAAGGACCGGTTTTGGTCGATATTCCAGTCGATTATTCGCACGATACCGAACTT
TATTCGGAATTGATCGAAGGAGATATGGATTAA

> Predicted esterase (*estA*) gene → 804 bp

ATGGCATTTTTAGAAGTTAATTATTATTCACGGGTTTTGGGAATGAATCGGGTGATGAACGTTCTT
TTGCCTGAAGAATCTGATCATAATCCAAATTGGACAAATGACAGTTTGAAAGATTTGCCGGTACTT
TATTTATTACATGGCATGTCCGGCAATCATTTTGATTGGCAGAGAAAAAGCGATATTGAACGTTTA
CTCCGGCAGACAAAGTTGGCAGTTATTATGCCTGCGGCCGATTTGGCCTGGTATACAAATACTGAT
TACGGTATGAATTATTTCGATGCAATATCTCAGGAGCTGCCCAGAAAAGTTGCCAGTTTATTTCCA
CAAATTTCAACTAAAAGGAAGAAACATTTTGTCGCCGGCATGTCGATGGGTGGCTATGGCGCTTTT
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TTAAATTATCCTGGTTTTTTGGATATGGAAAAACAAGCTTATTGGAAAGGAATTTTTGGTGATCTT
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GAACTACCGAAACTTTATGCTTGGGTTGGACAACAGGATTTTTTTATGGTGCTAATGAAAAGGCT
ATTCCTCGTCTGCGCAAAATGGGATACGATGTTTCTTATGAAACTAACCCCGGCGATCATGAATGG
TATTACTGGAGTAAATATATAGAAAATATTTTGCAATGGCTGCCAATTAATTATCAAGCAGAAAAA
CGTTTGTCATAG

> Serine protease (*prtP*) gene → 1278 bp

GTGACTGAAGAACAAGACCAAGGAAAAACGCAAAAAGATATCCAAGGCAAAAAGCCGAAAAAAAAT
CGTCCCATTGGTCGGATTATTGCAACTGCTCTTTTGGCTGGTCTTCTCGGTGGTGGTGTCGCTGTT
GGAGCAGGCTATATTTATACGCAAACGACTGATTTTATTGGAAAATCGACCGGTGCCTTAAGCGAT
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AAAGTCGGTGATACAGTTAAAATTACTTATTATCGAGCTTCGACTGAAAAAACAGTCAAGGTTAAG
ATGACTGAAACTTTGAAACAATAA

> S-adenosylmethionine synthase (*metK*) gene → 1167 bp

ATGAAAAAGTTTTTTACGAGTGAGTCGGTCGCGATTGGTCATCCAGACAAAATTGCTGATCAGATT
GCGGACGCCATTTTAGATGAGGTTCTAAAACAGGACCCTCTAGCGAGAAGTGCGATTGAAGTTACC
GTCTCGACCGGAGATGTTTCTATCTTTGGTGAATTATCAACGAAAGCTTATGTCAACGTTAGGGAT
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GAGGCGGGTGACTCGAACAGCTACTTGCGTCCCGATGGAAAAGGAGAGGTTTCTGTTGAATATGGG
GACGATAATCGTCCAAAACGTATTTCGGCAGTTGTTCTTTCTGCACAGCACATCGAAGGAATCGAA
TTGGAAGATTTGCGGGCCCGCATCAGCGAAGATATTATTGCTCCTGTATTGCCGACAGAATTGGTC
GATGAAAATACAAAATTTTTCATTAATCCGTCTGGATTATGGTCCTTGGGCGGTCCACAAGCCGAT
TCTGGTTTAACCGGCAGAAAAATAATTGTTGATACATACGGTGGTGCTGCCCATCATGGCGGCGGA
GCTTTTTCCGGTAAAGATGCTACAAAGGTCGACCGCTCAGGTGCCTATTACGCGCGTTACGTTGCT
AAGAACCTGGTGGCAGCCGGTTTAGCCGATAAACTGGAAATTCAGGTAGGCTATGCAATCGGTGTC
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TCTATTGTCGATCAGGTTTTTGATTTTCGGCCTTTATCAATTATTAATCAGCTTGATTTACGGCGT
CCTATTTACTTGCAAACAGCTGCTTTTGGTCATTTTGGCAGGTCCGATTTGGATCTTCCTTGGGAG
AAGCTCGATCAAGTTGAAAAAATTAAAGCTCTTTTGGCAAATTAA

> Cystathionine β-lyase (*metC*) gene → 1137 bp

ATGACAGAATCCGATTGGACAAAATTAATTAAATCAACTACTAAAATTGGTCCATTAAGCGGAGCG
GTCAATACGCCGATTCAGTTTTCCAGTACTTTTCATCAATCGAATTTTGATCAGTTTGGCGAATCC
GATTATGCACGTTCCGGTAATCCAACAAGAAAAGTTGCCGAATATGCCATTGCTGAATTAGAAAAC
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CTGCCGCGTTTTGGAATAAACCATAGTTTTGTTGATTTTTCGGATTTGTCAGCGATTGAAAACTCA
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GTTTACTTTGTTCAAAATGCGATTGGAGCGACATTGGGAGTTACAGATGCGTGGCTGCTTTTACGA
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TTTGAGAAATCAGGGAAAAAAGTCTTTTATCCAGGTTTGCCGAGTAATCCTGGATACGAAATCCAC
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AAATTTGTTGAAAAAATCAAGATTCCTGTATTTTCGGTCAGTTTAGGTGGAGTGGAAACAATTGTC
AGTTATCCGCCGAAAATGAGTCATGCTGAATTATCGGCCGATGATCTGGCTGCCGATGGTATCACA
CCAGGTCTTTTGAGAATTTCAGTCGGCCTGGAAAATGCCGACGATTTAATTGATGATTTTAATCAA
GCATTGGAGGATTAA

> Cystathionine γ-lyase (*metB*) gene → 1140 bp

ATGAAATTCAATACAAAACTTATTCATGGCGGTATTAGCGAAGATTCATCAACCGGGGCAGTTTCA
ATCCCTATCTATCGTTCTTCGACTTTTCATCAAAACAAGGTCGCTGGAAATGCAAAGTGGGAATAC
GGGCGCAGTGGAAATCCAACCCGTGCGGCTTTGGGAAAACTGATTGCCGATTTAGAAGAAGGGAAA
GCCGGTTTTGCTTTTGCCTCCGGTTCGGCGGCGATTCATGCGGTTTTTCATTGTTCTCTTCCGGT
GATCACATTGTTGTTGGCGATGATGTTTACGGAGGTACTTTCCGTTTGATAGATCAGGTGTTAAAA
CGCTTCGGTTTGGAATTTACTGTTGTTGATACCCGAGATCTGTCCGCGGTTGAAAATGCCATACAA
AAAAATACCCGGGCAATTTATTTGGAAACGCCGACCAATCCCTTATTGAGAATCACCGATATTAAA
AAAATTGCCGAGATTTCAAAACACTACCAACTTCACACAATTGTCGATAATACCTTTGCGACCCCT
TATAATCAAAATCCGTTGGTTTTGGGAGCGGATATTGTTGTTCATAGCGCAACGAAATATTTGGCT
GGGCACAGTGATCTTGTGGCCGGTCTGGCAGTGACCAACGATCCTGAAGTTGCCGACAAATTAGCT
TTTCTTCAAAACTCGATTGGTAGTGTTTTAGGTCCTGATGATAGTTGGTTATTGCAGCGGGGGATT
AAAACTTTAGCTGCCAGAATGGAAATTCATCATAAAAATACTCAGCTTATTTATGATTATTTTTCT
CGCAATGACAAGGTCGCGCGGATATATTATCCTGGTGATCCTGCTTCTCAAGGATATGAAATTGCA
AAACGACAAATGCGTGGTTTTGGCGGCATCATATCCTTTGAACTGAAAAAAGGCTTGGACCCGAAG
AAATTTGTTGAGAGTCTGCGGATCATTGATTTAGCGGAAAGCTTGGGCGGTGTTGAAAGTTTGATC
GAAATTCCGGCCTTGATGACACATGCCTCGATTCCTCGAGATATTCGTTTGAAGAACGGTATTAAA
GACGAGCTGATCCGTCTATCGGTTGGTTTGGAAGATGGTCAGGATTTACTGGACGACTTAAAACAG
TCATTCAGCAAGATTTAG

> Glutathione reductase (*gshR*) gene → 1341 bp

ATGAAAAACCAGCAATATGATTATGATGTTTTATACATTGGCAGCGGACACGGCACTTTTGATGGT
GCGATTCCATTGGCTGCAAAGGGATTTAAGGTCGGAATCGTTGAATACGATTTGGTTGGCGGCACC
TGTCCCAACCGTGGCTGCAACGCAAAAATAACTTTAGATGCGCCGGTCGCTTTACAACGCCAATTT
GAAAATTTAAATGGTGTGATTGAGGGGGAAGCCAAGATTAATTGGTCGGCCAACCTGACTCACAAG
CAGGAAGTTATTGGAAAATTACCTGATATGATCGCCGGTTTGGCAAAATCAGTTCATATTGATATT
CTGTCCGGACACGGTGTTTTGGATGATCCACATACTGTTTTGGTTGACGGAACTCCTAAAACTGCT
GAAAAAATTGTTATCGCAACCGGATTGCGTCCACATCGGCTCGATATTTCCGGCAGCGAATTGGCT
CACGACAGCAGTGATTTTATGAATCTCTCAGCTATGCCAAAACGTCTGACTGTTATAGGTTCCGGT
TATATAGCAATGGAATTTGCAACGATGGCTAATGCGGCCGGATCAGAAGTTACTGTGATTACGCAT
GGCAATCGCGCTTTACGTAAATTCAATCAGGATTTCGTCGAAAAAATTATTGATGATTTGCAAAAG
CGCGGTGTTAAATTTGTCCGTAATACGGAAGTTACTTCTTTTGAGAAGACAGGAACTGCTTTGACT
GTTAACGCGGAAGACAACTTTCAACTTGAAACCGATTGGATTTTAGATGCAACCGGTCGAATTCCA
AATGTTGAAAAAATCGGTTTGGACAAGCTAGGAGTTGAATACAATAAAAATGGTGTGGTTGTTAAT
GATCATTTGCAAACCAATGTTCCAAATATCTATGCTTCAGGTGATGTAATCGACAAAATACAGCCC
AAATTAACTCCAACTGCCGTTTTTGAATCAACTTATTTAATGCATCAATTTGCGGGTGATAGCTCG
TCAGCGATCGATTATCCGGCAATTCCTTCGGTTGTCTTTACATCGCCACGAATTGCTCAGGTTGGT
GTGACTCCCGAAGAAGCAAAGAAGAATCCTGACAAATACACAATCGAAACTCATCACACTCCTGAT
GATTGGTATCGGCAGGTTGATAAAGAACAGCTCGGTGATAATGCCCTTATTTTCGATAAGGAACAT
CATTTGGTTGGCGCCAGCGAGTTTAGTGATAAGGCTGATGATGCTATTAATACCTTGCTGCCAGCG
ATTGAATTCAAACTTGGTCCAGAACAATTGGGACGCTTGATTTATCTCTTCCCGTCGATTTCGTCT
TCGGCAGCCGGCCAATTGTAA

10.2 Nucleotide gene sequences of *Leuc. mesenteroides* IWBT B290 strain

A unique GenBank accession number will be assigned to each gene once the nucleotide sequences have been deposited to GenBank/EMBL/DDBJ databases.

> Malolactic enzyme (*mleA*) gene → 950 bp

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TGTGTATGATCCTATTGTCGCAGAGTCTATTGAACAATATAATGAAATTTACACTAATCCTCAAAA
TGCAGCATTTTTGTCAATCGATCATCCAGAAAATATTGAAAGTACATTGAAAAATGTCGCTGACGG
TAGAGATATAAAGTTAGTTGTTGTGACTGATGCTGAAGGTATATTAGGCATGGGAGATTGGGGTGT
CAATGGTGTTGATATTGCGGTTGGTAAATTGATGGTTTACACAGCAGCAGCTGGAATAGACCCGGC
AACAGTATTACCAGTAAGCATTGATGCAGGTACGAATAACAAAATATTATTAGAAAATCCTTTGTA
TTTAGGGAACAAACATGAACGTATTGCTGGTGAAAAGTATCTTGAATTCATAGATAAGTTTGTAAC
TGCTGAACAAAAATTGTTCCCAGAATCATTATTGCATTGGGAAGATTTTGGACGTTCAAATGCACA
AGTAATTTTGGATAAATATAAAGACAGCATTGCCACATTTAACGATGATATTCAAGGAACCGGAAT
GATTGTTTTGGCAGGAATATTCGGAGCTCTAAATATATCGAAAGAAAAACTAGTTGATCAAAGATT
CCTAACGTTCGGTGCTGGTACAGCTGGTATGGGTATTGTTAATCAGATTTTTTCAGAATTAAAACA
AGCTGGGCTATCCGATTCAGAGGCTCGCAGTCATTTCTATCTTGTGGATAAGCAAGGATTATTATT
TGATGACACTGAAGATTTAACTGAAGCGCAAAAGCCGTTCACACGTTCAAGAAAAGAATTTGTTAA
CTCTGAACAACTAGACAATTTGGAAGCAGTGGTCAATGAGATACGTCCCACAGTTTTGATTGGTAC
GTCAACACAGCCAGGCACATTTACGGAAGCAATTGTAAAATCGATGGCACAAAATACAGAACGCCC
AATTATTTTTCCTTTGTCAAATCCAA
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> β-Glucosidase-related glycosidase (*bgl*) gene → 667 bp

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CGGGGAAAGATTTCTGGTTTACCGCGGAAAATATTGAAAATGATATACCAAAAATCATGGTAACAG
ATGGTCCTTCAGGATTGCGAAAACAAGCAAGTAGTGCAGACGCACTAGGCTTGAATCAAAGTGTGG
AAGCCATTGCTTTTCCAAGTTCAGCTTTGATGGCTAGTTCATTTAATGTGGACATGCTTTATGAAT
TAGGTCAAAATCTTGGAACAGCATCTAGAGCTGAAAATGTGTCAGTTTTATTGGGGCCAGGTATTA
ATATTAAGCGTTCTCCATTGGCAGGAAGGAATTTTGAATATTTTTCCGAAGATCCATATCTAACTG
GAGAACTAGGAAGCGCTTATGTGAAGGGCGTGCAATCGCAAGGTGTTGGCGTGAGTGTCAAGCACT
TTGCAGCCAATAATCGAGAAGATCAACGTTTTACCTCGTCCTCGAATGTTGATGAACGTGCTTTAC
GTGAGATATACTTGCTGGCTTTTGAAAAGATTGTCAAAGAGGCACATCCAGCAACGTTAATGTGCT
CTTACAACGCGATTAATGGTGTGCTCAATTCTCAAAATTATCGTTTGTTAACCGAAATACTGCGTA
ATGAATGGGGATATACTGGCGTCGTTATGTCAGATTGGGGAGCTGTAGCCGATAATATTGCTTCGC
TAAAAGC
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> Predicted esterase (*estA*) gene → 792 bp

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ATGGCTTTTTTAGAAGTTAATTATTATTCAAAAGTACTAGGTATGGATCGTGTGATGAATGTCATT
CTACCCGAATTATCAGATCATAACCCAACTTGGACAACAGAAACCTTGAAGGATATTCCTGTATTG
TACCTTCTCCATGGTATGTCAGGTGATCATGCAATTTGGCAACGGCGGACATCAATTGAACGTTTA
GTAAGGCAAACACCTGTAGCAATTGTAATGCCGTCTACTGACTTAGCTTGGTATACTAATACAACC
TATGGATTGAACTACTTTGATGCATTAGCACGTGAGCTACCTGAAAAAGTTGCTAGCTTATTTCCA
CAAATATCAACTAAAAGAGAAAAAAATTTCGTAGCCGGACTGTCAATGGGTGGTTACGGTGCGTTT
AAGTTGGCCTTGGGAACAAATCAATTCAGCTATGCCGCCTCTCTTTCCGGTGCATTAGTAGGTAAT
CCGAGACAAGAAGACTTTTTAAAGATGGAAAAGCTTTCATATTGGCAAGGAATTTTTGGCGATTTT
GATAGTTTCGCTGGATCTAAAAATGATATTTTAGCTCTCGCTAAAACGTGTCACAAGCGACCAAAA
CTATATGCATGGATAGGAGAACAAGATTTTTTGAAGCCCATTAATGATGTTGCCATATCAACCTTG
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CAGCAATTAAATTATGATATCACATATGAAACAGCACCTGGCACACACGAATGGTATTATTGGAAC
AAACAAATTGAACGCGTGTTAGAGTGGTTACCGATTAACTATGTTCAGGAAGAGAGATTGAGTTAA

> Serine protease (*prtP*) gene → 861 bp

ATGAAAAAGCATTCTTTAATTATCGTTGCTATTGTTTCAGCATTGGTGGCAAGTTTTGTTGTTTAC
TCAGGCTTGCAACCAAATTCCTGGTTTCAACAGCATTCTTATGCAACAAAAACAACGAATTCAGTT
GGTACAACAACAGTAGCCAAAACAGCATATACAAGTAATGATACGGCAACAACTGCCTATAATAAA
GTTAAAAATGCAGTTGTTACAGTACAAAATTTGCAGAAAACATCAACATCAAGTAGCGGTTGGTCT
TCTTATTTTCAACAAAATCAACAAGAAAGTAGTTCAGAATTAGAGACGGCGTCAGAAGGTTCAGGC
GTTGTTTATAAAATTTCTGGTGGCTATGCGTATATTATTACTAATAATCACGTGGTAGCTGATTCT
GATGAATTACAATTAATTACCGCTAGCGGTAAAAAAATTGAAGCAACTATCGTTGGCACAGATTCA
AGTAAAGACTTGGCTCTGTTAAAGGCAAAAACCACAGATATCAAAACATCAGCATCCTTTGGCAAT
GCCAAAAAACTGCAGTCAGGTCAACAGGTCTTAGCTATCGGCTCTCCTTTGGGTTCTGATTATGCT
ACTTCTTTGACTAGTGGTATTGTTTCAGCTCCACGCCGTACACTTTCGGCTGAAGAAACGGGTTCT
TCAGCAACTACTGCTATTCAGACAGATGCTGCTATCAATCCTGGTAATTCTGGTGGACCATTGATT
AACCTAAAAGGGGAAGTAGTTGGTATTAACTCATCAAAAATAGCTTCTTCTACGGATGGTACGAGT
GTTGAAGGAATGGGATTTGCTATTCCAGCAGATATTGTTCAGACATTTATTAAGAATACTGAAAAG
TAG

> α-Acetolactate decarboxylase (*alsD*) gene → 720 bp

ATGACAACAATATATCAACATGGTACATTAGCACAATTAGTAGCGCGCCAAATGTCAGGGACAATA
ACAGTCGCTGAAATGTTGGAACATGGGGACACTGGTATTGGTACTTTTGAGGGTCTTAACGGCGAA
GCTATTTTTCTAAATGGGGAAGCCTATCAAGCTGATAGTACAGGAAAAGTCCACCACATAACTGAT
AAACAAACTACACTACCTTTTGCATCAATACATTTTGATCAACCAGAGGCAAGTCAAAAATTACCT
TTTAAAAAAATAAAATATAGTAATTTGACTCAGAACTTGAAAGATGAGCAGTTATTTAACGTTTTC
TCTGCCTTAAAAATGCATGGTGAGTTTGCCCACGTTCACGTTCGTATTGTAACAAAACAAGAAAAA
CCATATCCAAGTTTGTTACAAGTAGCTGAACAGCAGCCTGAATTCAAAGCAGACAACATAACTGGG
ACATTAGTTGGATATTATGCACCGAAAGTTTTTGGCGGTCCAACCGCAGCAGGGTGGCATTTACAC
TTTTTGTCAGATGATTTAACCTTTGCTGGGCACGTTTTGGATTTTGAAGCAACAGATGTGGATGGT
ACTTTAGAAATTTTTGATAACTTTTTGCAACATCTGCCTATTAATAATGCTGACTTTAGAAGCATG
AATCAGGATATAGTTGGTTTGGATAAAGCCATTGAGGCCAGTGAAGGCGGAAAAAATTAG

> α-Acetolactate synthase (*alsS*) gene → 1686 bp

ATGGCAAATAAAAAATATGGTGCAGATATTGTTACTGAGAGTTTAGTCAATCATGGTGTTGATTTG
GTTTTTGGAATTCCAGGTGCCAAAATTGATCGCTTATTTGAAACATTAGAACATCCAGCCGAAGGT
CAAAGAGTACCTAAATTAGTTGTTGCACGTCACGAACAAAACGCAGCTTTTATGGCACAGGCATTT
GCTCGTATAACAGGGAAAACAGGTGTTGTGATTGCCACCTCTGGCCCTGGTGTCGGCAACTTAGCT
ACTGGATTAATGACAGCAACTGCTGAAAGCGATCCTATTGTAGCCATTGGTGGTCAAGTACCGAGA
AATGATTTATATCGTTTGACTCATCAATCAACAAATTCAGTGGCATTGTTTAGTCCGATTACAAAC
CTTGCTTCAGAAATTCAAGATCCAAATAATATTTCAGAAATTATTGCTAACGCTTTTGCAGCCGCT
AATGGTGCCAAAAAAGGTGCGACTTTTGTTTCATTGCCACAAGATGTAGACGATGCACAAGTAACT
ATTGACGCACTTCCTGAAATTACACCTGCACAGCAAGGCGCGGCCGCTATTAAGGATATTGATTGG
CTGGCTGAACAAATTAAGGCTGCAAAATTACCGGTGTTGCTTGTGGGATCACGTGGATCTGATGAT
GCTACCGTTACTGCGCTACATCAATTGCTGAAACAAACGACTTTGCCAGTCGTTGAAACTTTCCAA
GGCGCTGGTGTCATTTCACGTGAATTAGAACCGGAAACATTTTTCGGTCGTATTGGCCTATTCCGT
AATCAAACTGGTGACAAACTGCTAAAGCAATCAGATTTAGTGGTTACATTGGGTTATGACGCGATT
GAATATGAGCCACGTAACTGGAACAAAGAAAACAATCTGAACATTGTCGCTTTGGATACAACGCCA

GTTCAAATTGATAATAATTTTGTACCGCAACGGCAGTTGGTCGGGGATTTAGCACAGAGCCTGCGT
TTGTTGATGGAACGTCTTAACGGATATGAATTGCCAACCACTAGCAAAGAAGTATTAAAAAAATTG
AAAGAAGATCTACGAGCATCTGACGAACCTTCTTATACACCAGCACAAGGGAATTTGAATCATCCG
TTGGATATTATTAAGTCGATCCAAGCCCATGTGACAGATGATATGACAGTATCAACAGACATCGGT
TCACACTATATTTGGATGGCACGTCACTTCAAGTCTTATGTTGCGCGTCATTACCTTATCTCTAAT
GGCATGCAAACGCTTGGGGTAGGGTTACCTTGGGCTTTAGCGGCGGCAATGGTTCGTCCTAATGCC
AAATCGGTATCAGTATCTGGTGACGGGGGTTTCTTCTTCTCGGCGATGGAATTGGAAACGGCAGTA
CGTTTAGGATTAAATACAGTTCATATCGTTTGGAATGATAATGCATATTACGACATGGTTAAGTTC
CAAGAAGAAATGAAGTACAACGGCCAGTCAGCAGGAGTTAAGTTTGGTAATATTGATTTGGTTAAG
TACGCTGAAAGCTTTGGGGCCAAAGGCTTACGTGTTGAAACACCAGATGAGCTTGATACTGTGTTA
GACGAGGCATTTGCAACACAAGGACCTGTTGTTGTAGATATTCCGGTAGATTATTCACATAACTAT
GAGCTTGGTTCACAATTGATTGGTTCTGAAGGATAA

> S-adenosylmethionine synthase (*metK*) gene → 1158 bp

ATGGCAAAGTATTTCACATCGGAATCAGTTTCTGCTGGGCATCCAGATAAGATAGCTGATCAAATA
GCCGATGCTATTTTAGATGCAGTTCTCGAACAAGATCCGAAGGCACGTTCAGCGGTTGAAGTGACT
ACTTCAACAGGAGATGTATCCATTTTTGGTGAATTATCCACGAATGCTTATGTTAATATTCGCAAA
ATCGCGACGGATACAATTCGTGAAATTGGATATAATCATGCTGAATTAGGGTTTACTGCCGATTCA
GTCAACGTTTCTAATAAAATTGTTGAGCAATCAGGGGATATTGCACAAGCTGTCGATAATGCAGAA
GATGATCCAGACCAACTTGGAGCTGGCGATCAAGGTATGGTATTTGGCTATGCTACGAACGAAACA
GACAGTTATTTACCATTGACGTTGGCTTTGTCACATCGTCTAATGCGCAAGATTCGTGATGCACGT
GAAAACGAAATTTTACCATATTTAAGACCAGATGCTAAAGGTGAAGTAACAGTTGAATTAGATGAT
AACGATAAAGTTAAGCGCATCGCTGCTGTGGTTATTTCAACACAACATGATGACGAGGTCACACTA
GAACAATTGCGAGCTGATATTCGTAAACATGTCATTGATGAAGTGTTACCACAAGATTTGGTAGAC
GAAGACACGATTTATTATATTAATCCATCTGGAAGATTTGTTTTAGGTGGGCCACAAGCCGATTCA
GGATTAACAGGTCGTAAAATTATTGTGGACACTTATGGCGGTGCTGCCCACCATGGTGGTGGTGCC
TTTTCAGGTAAAGACGCTACAAAAGTGGATCGTTCTGCTGCTTACTATGCTCGCTATGTTGCAAAA
AACATGGTCGCAGCTGGTGTCGCTGATAAATTGGAGCTGCAAGTATCATATGCAATTGGTGTTGCA
CGTCCTGTATCATTGAATGTTGATTCATTTGGTACAGCCAAAGTTTCTGAAGAAAAAATCAATGAA
ATTATAACTAAGTTATTTGACTTCAGACCATTAGCTATTATTAACAACTTAAATTTGCGTCGTCCA
ATCTATAAGCAGACAGCTGCATTCGGACATTTCGGACGTACGGATATCGATCTTCCTTGGGAATCA
CTAGATAAGGTGAAAGAAATCAAAAACTTACTTTAA

> Cystathionine β-lyase (*metC*) gene → 1140 bp

ATGAGTGATTGGACAAATATTATTGATGCAGCAACAACAAATGATCCACTGTCAGGTGCAATTAAT
ACACCGATTCAACTAAGTTCAACCTTTAGTCAAAAATCTTTTGATGAATTTGGAGAATATGACTAC
GCTCGCTCTGGAAATCCAACTCGTGATGCGGGTGAAAAAGCAGTTGCACAGCTTGAACATGGAAAT
TATGGGTACCTTTTTAGTACTGGAATGGCAGCAATTAGTAGTGTGCTATTCACCTTATCAGCTGGC
GATCATATTGTTGTTAGTAAACATGTTTATGGTGGCACATTTCGAGTTTTAGAAGATGTTTTGCCA
CGCTGGGGTATCACACACGACTTTGTTGACTTTAGCGATTTGGCAGCAATCGAGAAAGCTATTAAA
CCAGAGACCAAAGCATTGTATATTGAAACACCCTCAAACCCAGTTTTGAACATTACTGATATTCGT
GCGGTAGTTGGTATCGCAAAGAAACATCAGTTGTTTACAATTGCTGACAACACATTCTTATCACCA
TTCTTGCAGAAGCCTTTGGATCTAGGTGTTGATATTGTGGTTCATTCGGCAACAAAGTTTCTGGCA
GGACATTCTGATATCCTTGCTGGTGCTGTTGTAGTTAATGATAAAAAGTTAGCAGACCAAATTTAT
TTTGTTCAAAATGCGGTTGGGGCAACACTTAGCGTTTTTGATACGTGGCTGTTGTTGCGTGGTATA
AAGACACTTGGTGTTCGTATGACACATTCAAGTGAATCAGCGTATAAAATAGCTGAGCATTTAGAG
GCACATGAAAAAGTATCAAATGTCCTATACCCAGGGTTAAAAACACATAAAGGTTATGAAATTCAT
GCTTCACAAGCTAAAAATGGTGGGGCGGTGTTGAGCTTTGATGTGGGCAGTCAAGAAAATGCTAAG

AAAGTAGTGGAGTCCTTACATATCCCAGTATTTTCGGTTAGTTTAGGAGCGGTGGAAACAATTATC
AGTTACCCGCCAAAAATGAGCCACGCAGAGTTAAACGTTGATGAGTTGGCTAAATGCGGTATTACT
CCTGGTTTGTTACGCTTCTCGGTTGGATTAGAGGATGCCGATGATTTGATTGCAGATTTGGATAGT
GCATTAGCCTTAATTTAG

> Cystathionine γ-lyase (*metB*) gene → 1140 bp

ATGAAATTTGATACACAACTTATTCATGGTGGCATTAGTCTTGACCAATCAACTGGCGCCGTATCT
GTCCCAATTCATATGGCTTCAACCTTTAAGCAAACTAAAATTGGCGAGGCAAAATATGAATATTCA
AGATCTGGTAACCCAACTCGTGAAGCCGTAGAAAGCTTAATTGCAGACTTGGAAAATGGTACTGCT
GGCTTTGCTTTTGCATCAGGATCTGCGGCGATAAGTACTATTTTTTCACTTTTTTCATCTGGGGAT
CACATTATTGTTGGAAACGATGTTTATGGTGGTACATTTAGGCTAATTGACAATGTTCTAAAAAGA
ACAGGTCAGACATTTACAATTGTCGATACCCGTGATTTATCTGCTATTCAGGAAGCTATTCAAGAT
AATACTGTCGCCATTTACCTTGAAACACCAACTAATCCTCTATTACGCATTAGTGATATCAAGGCA
ATTTCTGAACTTGCTCATCGTCATAATTTATTAAGTATTGTTGACAACACATTTGCTTCTCCCTAT
GTACAAAAACCAATTGATTTAGGTGTGGATATTGTTGTTCACAGTGCTTCAAAGTATTTAGGTGGT
CATAGCGACCTTATTGCTGGCTTAGTTGTTACCAAAGGCGAGGAACTTAGTGAGAAAATTAAGTTC
TTACAAAATGCGATTGGTGCAATCCTGGCCCCTCAGGAAAGTTGGCTCCTACAACGAGGTATGAAA
ACGCTCAGTTTAAGAATGCGTGCTCACCAGTCAAATGCTCAAACAATATTTGACTACCTTAAAACA
CAAGATAAAGTTGCTAAGATATATTTCCCTGGTGATCCTGATAATCCTGACCACGCTTTAGCCAAA
CAACAGATGAATGGTTTTGGTGCCATGATTTCATTTGAGCTAAAGGTTGGGCTAGATCCAGAACAA
TTCATTAGTAACTTAAAAATCATTACCTTAGCAGAGAGTTTAGGTGCACTTGAAAGCCTAATTGAA
ATTCCAGCTAAAATGACTCACGGCGCCATTCCTCGTAATATCCGAATTTCTCATGGTATTCAAGAC
GAACTAATCCGTCTCTCTGTGGGTGTTGAAGATATGCAAGATTTAATTGAAGATCTAGAACAAGGT
TTTAATCAATTGAAATAA

> Glutathione reductase (*gshR*) gene → 1332 bp

ATGGCGGAACAGTACGATGTTGTTGTGATTGGTGGCGGACCAGCCGGCAATGCCATGGCTAGCGGA
TTAAAGGCTCAGGGCAAGACAGTGTTGATCGTTGAAGCGGATCTGTGGGGCGGCACTTGTCCTAAC
CGCGGTTGTGACCCTAAGAAAATCCTATTAAGCGCCGTCGAAGCGCGACAAGCAGCGCAACATTTA
CAAGGGCAGGGCCTGATTGGTGCGCCCAAAATTGATTGGCCAGCACTGATGGCGCATAAACGAGGC
TATACGGATGGCATCAACGATGGGACGTTGAACGGACTAAAGGGGCAAGATATTACGACGTTACAT
GGTCAAGCGCACTTTCAATCCGACAATCAGTTAGCGGTCGGGGATCGAGTAGTCAGTGCGACTGAT
TACGTGATTGCCACTGGTCAGCGTCCGGCGATTCTACCGATTACCGGGCACGAATACTTTAAGACG
AGCACTGACTTCTTAGATTTGGACCAGATGCCTAAACGCGTGACATTCGTAGGTGGTGGCTACGTA
GGCTTTGAATTGGCGACGATTGCGAATGCCGCTGGCGCTGATGTGCACGTGATTCTTCATAATGAC
CGCCCGTTAAAAGCTTTTGATGCAGATTTGGTTAAGGATTTGATGGCCGCAATGACGGCTGATGGA
ATCACGTTTGACTTGAATACGGATGTCCAAGCAATTACTAAAACGGCGACCGGTCTACAATTGACA
GCTGATAATTTCGAGCTGACAACGGATCTGGTCATCAGCTCAGCGGGACGGATTCCGAACGCGGAC
CAGTTAGGTCTAGCCAACGTGGGCGTTACCTTTGATCGGCATGGGATTCAAGTCAACGATCATTTG
CAGACGGCCAACCCGCACATTTATGCCATTGGGGATGTCAGCGATACACCGGTACCGAAGTTAACG
CCAGTTGCAGGTTTTGAAGCGCGTTATCTGGTCGGTGAGTTGACGCATCCCGGCGCAGCCATAAAG
TATCCCGTTGTGCCAACGCAGGTTTTTGCAGCGCCCAAGTTAGCGCAAGTCGGGATCAGCGCGGCC
GCGGCGACTGAGCATCCAGATGAGTATCGTGTCAATACACTTGATATGACGAAGTGGTTCACTTAT
TACCGCTTTAGCGCACAACAAGCCCAAGCTAAAGTAGTGGTTGCTAAAGCGAGTGGGCAGGTTGTG
GGTGCTACCCTTCTAAGTGATGTTGCCGACGAGATGATTAACTACTTCACGTTGTTAATTGAAAAA
CACGTGACTTTACCAGATTTACAACGGTTGGTATTGGCTTACCCAACGCCGGCTAGTGACTTACAA
TATTTGTATTAA

10.3 Nucleotide gene sequences of *Lb. florum* IWBT B322 strain

A unique GenBank accession number will be assigned to each gene once the nucleotide sequences have been deposited to GenBank/EMBL/DDBJ databases.

> 16S ribosomal DNA gene → 1479 bp

ACATGCAAGTCGAACGAGGCTGCCCAGTTGCTAGTCGGTGCTTGCACTGACGAACAATTGGATCCA
GCCGAGTGGCGAACTGGTGAGTAACACGTGGGTAACCTGCCCAGCAGAAGGGGATAACACCTGGAA
ACAGATGCTAATACCGTATAAACCTGAAAACCGCCTGGTTTTCAGCTAAAAGATGGTGTACGCTAT
CGCTGCTGGATGGACCCGCGGCGTATTAGCTAGTTGGCGAGATAATAGCTCACCAAGGCGATGATA
CGTAGCAGACCTGAGAGGGTAATCTGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAG
GCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAG
GGTTTCGGCTCGTAAAACTCTGTTGTTAGAGAAGAACGATCGTAAGAGTAACTGCTTACGGTGTGA
CGGTATCTAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAA
CGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTCTAAGTCTGATGTGAAAGCCTT
CGGCTTAACCGAAGAAGTGCATCGGAAACTGGGGAACTTGAGGGCAGGAAAGGATAGTGGAACTTC
ATGTGTAGCGGTGAAATGCGTAGATATATGAAGGAACACCAGTGGCGAAGGCGGCTATCTGGTCTG
CATCTGACGCTGAGGCTCGAAAGCATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCG
TAAACGATGAATGCTAGGTGTTGGGAGGTTTCCGCCTCTCAGTGCCGGAGCTAACGCATTAAGCAT
TCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGATGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTCTGTTAGCC
TAAGAGATTAGGTGTCCCCTTCGGGGGCAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTTTTAGTTGCCAGCATTTAGTTG
GGCACTCTAAAGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAATCATCATGCC
CCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTTGCGAAACCGCGAGGTCA
AGCTAATCTCTTAAAGCCGTTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTTGGAA
TCGCTAGTAATCGTGGATCAGCATGCCACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGT
CACACCATGAGAGTTTGTAACACCCAAAGTCGGTTGGATAACCTTCGGGAGTCCGCCGCCTAAGGT
GGGACAGATGATTAGGGTGAAGTCGTA

> Malolactic enzyme (*mleA*) gene → 953 bp

TATGATCCAACGATTGCGGATTCAATTGAAAACTACAGTCGCCTATACGTTAATCCCCAAAATGCG
GCCTATTTATCAATCAATGATCACAGTCGCGCAACGATTCGGGAAAGTTTAATCAATGCTGCTGAA
GGGCGCAACGTTAAATTGTTAGTTGTGACTGATGGTGAAGGAATCCTTGGCATTGGTGATTGGGGA
ACTCAGGGAATTGATATTCCGGTTGGTAAGCTGATGGTTTATACAGCTGCTGCGGGGATTGACCCG
TCAGAAATTTTACCAGTGGTCTTAGATGCAGGGACTACTCGTGCATCATTAAAGGATGATCCTTTG
TACGTTGGTCTTGATCAAGATCGGGATTATTCTGATAATTACTATGAATTTGTGGATAACTTTGTT
CAGGAAGCAGAATCACTTTTCCCTAATCTTTATTTACACTTTGAAGATTTTGGTCGAGCTAATGCT
GCTAAGATTCTGGAAAAATATCAGGATCAATTCTTGGTCTTTAATGATGACATTCAGGGAACTGGA
ATTATCGTTTTAGCTGGGGTACTCGGGGCATTGAACATTTCTGGTGAGTCAATGACGGATCAGAAA
TATCTATGCTTTGGTGCTGGTACTGCTGGGGTCGGAATTGCGCAGCGAGTTGCTGAAGAAATGGTT
CAAGCAGGCTTGAGCGAAGCAGAGGCTAAAAAGCACTTCTACATGGTCGACAAACAAGGACTCTTG
TTTGATGACATGCCAGATTTGACACCGGGACAACAGGAATTTGCTAGAAGTCGTTCCGAATTTGAT
AATGCCGATGAATTAACGGATTTACTCTCGGTTGTACAAGCGGTTCATCCAACCATTATGGTAGGA
ACTTCAACTGTTCATGGTGCCTTCACACAGGAAGTTGTTACGGAAATGGCAGCGCACACGAACCGT
CCAATTATTTTGCCAATTTCAAACCCAAC

> Cysteine aminopeptidase (*pepC*) gene → 1072 bp

GCGGAGCCAAATTGAAAACCAATTTAATGTGTCAAAGACTTCGAACTTTCTCAAGTATTTGTATTC
TTCTGGGATAAATTTGAAAAAGCAAATTATTTCTTAAATAACGTGCTTGCGACTGCTGATCGACCA
CTGACTGATCGCAAAGTCAACTTTCTTTTGCAACAACCACAACAAGATGGTGGTCAGTGGGATATG
TTATGTGCTCTCGTTGAAAAGTATGGCATTGTTCCCAAAGATGCCATGCCAGAAACTGCTAACAGC
GCTAATTCCAGCGAAATTAACCGAACGCTCAATACCAAGTTGCGCCACGATGCTGTTATTTTAAGA
AAGATGCAGGCAGCTAATGCCACTGCTTCAGAAATTGCACATCAACAAGAAAGTATGTTGGCCGAA
GTTTATCGTATGCTGGTTCTAGCATTTGGCGAGCCGGTCGAAAGCTTTGATTTTGAATATCGTGAT
CAACAAAATCATTACCAGATTGATCGTCATCTGACTCCCAAAACTTTCTTTAAAAAGTATATTAAC
CTTGATTTAGAGGATTATCTTTCAATTATTAATTCACCAACCGCCGACAAACCATTTGAAAAAACT
TACACGGTTGAGTTATTAGGTAACGTCGTCGGTGGTCGTCCCGTTAAACATTTCAACCTCAGTATC
GAACGACTCAAAGAACTTACCATCAAACAACTCCAAGCTAATGAAACGGTCTGGTTTGGCAGTGAC
GTTACTCAAGCATCTGATCGACAAGCCGGACTGTTGGATCCCGAACTATACCAGGTTGACGAACTA
ATGGGGACAAATCTCTCGCTCTCAAAGGCAGAACGATTAGATTACGGCGAAAGTGTTATGGATCAT
GCCATGGTAATTACTGGGGTTGACCTGGTCGATGGTCAACCCACCAAGTGGAAAATCGAAAACAGT
TGGGGTCCCAAAGTTGGAACCAAGGGCTACTTCGTAATGAGCGATCAGTGGTTCGAGCAATTTGTT
TACCAAGTTGTAATTAACAAAAAATATTTAAGTGCTACCGAACAAGCTGCTCAGCAGCAGACCAAC
GGTGCTTGCCCCGTGG

> Proline iminopeptidase (*pepI*) gene → 685 bp

ACACCGCACAACAACTCAAAAAACAGGGACTAGATGTCCAGGTTCACATGTATGATCAACTTGGAT
CATGGTATTCAGACACCCCTGATTGGGACAATCCGGAAGTTGCCAGCCAGATTCAAACCTACGATT
ATTATGTTGATGAGATTGAAGAAGTCCGTCAAAAGCTTGGAATCGACCAGTTTTACCTGATTGGTC
AGTCTTGGGGAGGAGCCCTCGTTCAACTGTATGCTGCTAAATACGGTCAACATTTGAAGGGAGCTA
TCATTTCTTCTATGGTTGATCGCATCTCAGACTATACAGACCATCTCAACCAGATTCGCAAGACTG
CTTTAAGCCCTACTGAACTTGAATACATGCAACAGTGTGAAGCAAATAATGATTACGATAATGATC
ACTATCAGCAGCTTGTCGAAAAATTAAATGACGCTTATGTTGATCGCAAAAAACCAGCTGCAATTG
CTCACCTAGTCAACACCATGAGCGTCCCCCTTTACAATGCATTTCAAGGTGACAACGAATTCGTAA
TCACCGGTAAACTTGGGGAATGGAACTTTACTGACCACCTTAAGGATATCAAGGTTCCAACGTTAG
TCACCTTTGGCGAACATGAAACCATGCCACTAGCGACGGGACGCCGAATGGCTGAAATGATCCCTA
ATGCGCAGTTTGTTTCAACCCCAGA

> Membrane alanine aminopeptidase (*pepN*) gene → 512 bp

CGAGAAGCATATCTACTGATCGATCCAGAAAATACTTCATTTGACGTGAAACGTTTAGTTGCTACC
GTAATTACGCATGAATTAGCTCATCAGTGGTTTGGTGATTTAGTTACTATGAAGTGGTGGGACGAT
CTGTGGTTGAATGAAAGCTTTGCTAATATGATGGAATATGTGGCGGTTGATGCCCTAGAACCAACG
TGGAAGATTTGGGAGTTGTTCCAGGTATCTGATGTTCCAGCCGCCCTAGAACGTGATGCGACTGAT
GGAGTCCAACCAGTACACGTTGAAGTTCGCAACCCGGCTGAAATTGATGCGCTTTTTGATCCAGCG
ATTGTCTATGCCAAGGGAGCACGGATGCTGGTGATGGTCCGGGCACTCTTAGGAGATGACGCCTTA
AGAGCTGGGCTGAAACAGTATTTTGCTGATCATCAATATGGAAATGCTCAGGGATCTGATTTGTGG
CAAGCACTTGAGGATGCCTCTGGTTTAAAAATCGGTGAAATTATGCATTC

> Citrate lyase β-subunit (*citE*) gene → 857 bp

TTTGTTCCTGGAAACAATGCGGGGATGCTGAAAGACGCTGGTATTTACGGAGCTGACTCGATTATG
TTTGATTTAGAGGATGCGGTCTCACTGGCAGAAAAGGATTCTGCTCGGACATTAGTCTATGAGGCA
TTGAAAACGGTTGACTACGGTGATACCGAACTGGTGGTTCGAGTTAATGGTTTGGATACTGAATTT

GTCAAAGCTGATGTTTTGGCAATGGTTAAAGCCGGAATTGATGTCATCCGGATTCCTAAAACTGAA
AATGCTGCAATGATTAAAGAGATGGAGTCACTAGTTGCTGAGGCAGAACAACGTTTCGGTCGACCA
GTGGGGAGCACGCACATTATGGCAGCCATTGAAAGTGCTGAAGGCGTCTTAAATGTACCCGCAATT
GCTAAGGCTTCTGAGCGGATGATTGGGGTAGCGTTATCAGCAGAAGACTATACTACTGATTTGCAG
ACGCACCGTTATCCAGATGGCAAGGAATTAGAATTCGCTAGAAACATGATTATTCATGCGGCGCGG
GCAGCGCATATTTCTGCGTTTGACACGGTATATACGGATGTTGATAATACGCAGGGATTGATTGAT
GAAACCGAGTATATTCATCAATTAGGTTACGATGGAAAGTCGGTTATTAATCCGCGACAAATTCCA
GTAATTAATTCGGTTTTTGAACCAACTGAGGCCCAAGTTAAAAACGCTCAAAATGTTATTGCTGCC
ATTGAACGAGCACATCAAGCTGGTTCTGGAGTTATTTCTATGAACGGTCAGATGGTTGATCGACCT
GTGGTTTTGCGGGCTGAACGAGTTATGAAACTAGCCCAAGCATCTGGAATTGTCGACAAGGAGGG

> Citrate lyase α-subunit (*citF*) gene → 1238 bp

CTTTGTTTTTACCAAGTAATGAAAATTATTATGGAACTGGGAATCAAGAACCTCACCTTGGCCCCT
TCTTCACTAACAGGAGTTATGAATGACGTTGCAATTGAAGCAATCAAACAGGGAACGGTAACTGCC
ATCACAACTTCTGGAATGCGGGGCTCGCTAGGAGATGCAGTCTCACATGGTTTGCTAGCTAAGCCG
GTGATTTTCCGGTCACATGGTGGTCGGGCACGAGCCATCGAAAATGGCGAAATTAAGATTGACGTT
GCTTTCTTGGGAGTTCCTAATGCTGATCGTTGTGGAAATGCCAATGGTAGTTATGGCGATGAAGCC
TTTGGTTCTTTAGGCTATGCTTTGATGGATGCCAACTATGCTAACAAAGTGGTTTTGCTAACCGAT
AATCTAGTTGCTTATCCCAACACCCCTGCTTCTATCAAACAAACTCAAGTTGATTACGTGGTAGAG
GTTGATCAGGTTGGTGATCCGGATAAAATTGGTTCAGGAGCTACTCGCTTTACGAAAGATCCTAAA
AATTTAAAGATTGCTAACTTAGTAAGTGATGTAATTACTAATTCAAGCTACTTTAAAGATGGATTC
TCGTTTCAAACTGGTTCTGGTGGAGCTGCTTTGGCAGTTACTCGTTACTTGCGAGCTGCCATGGAA
GCAAGAAATATCCATGCTTCGTTTGGATTAGGTGGATTACTAATCCAATGGTGGAGCTACTAGAA
GCGGGCTTGATTGATAAATTGATGGACGTCCAAGACTTTGATAAAGCGGCAGCGCAGTCCATGAAA
AACAATTTAAATCAACAAGAAATCGATGCTTCTTGGTATGCTGATCCCGATAATAAAGCCGCCATG
GTGGATCAGTTGGATGTTTCCATTTTGAGTGCGCTAGAAATTGATACGGACTTTAATGTCAATGTC
TTGACTGGTGCTGATGGAATTATTCGCGGAGCGGTCGGTGGTCATCAAGATGCAGCCACTGCCAAG
CTAACCATCATTTGTACTCCATTAGTACGAGGTCGTATTCCCTCAGTTGTTAAGAGTGCGAATACC
GTGGTCACTCCCGGGAGCTCCGTTGATGTTTTAGTAACAGAACGCGGAATTGCTGTAAATCCGCAA
CGACCAGATTTGTTAGCACAGTTGCAAAACGTACCGGGACTCCAAATCTATTCGATTGATGAGTTA
GCCGAGCTAGCCCAACAAATTGTTGGTACAGAAGCCCCACTAGAGTTACT