

DETECTION AND IDENTIFICATION OF WINE SPOILAGE MICROBES USING PCR-BASED DGGE ANALYSIS

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Thesis presented in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN FOOD SCIENCE



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March 2009

DECLARATION

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ABSTRACT

Grape juice is transformed into wine through the complex processes of alcoholic and malolactic fermentation that is performed by yeasts, lactic acid bacteria and acetic acid bacteria. However, the microbes involved in these processes do not only take part in ensuring the successful production of wine, but also cause spoilage of the wine if their growth is not controlled.

Conventional, culture-dependent methods of microbiology have been used as the main technique in detecting and identifying these spoilage microbes. Culture-independent techniques of molecular biology have recently become more popular in detecting possible spoilage microbes present in must and wine, since it allows the detection and identification of viable, but non-culturable microbes and are not as time-consuming as conventional microbiological methods.

The aim of this study was to investigate the sustainability of polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) analysis in detecting wine spoilage microbes inoculated into sterile saline solution (SSS) (0.85% (m/v) NaCl) and sterile white wine and red wine as single microbial species and as part of mixed microbial populations. Three methods of DNA isolation from SSS, sterile white wine and sterile red wine inoculated with reference microbial strains were compared in terms of DNA concentration and purity, as well as simplicity of the technique. These three DNA isolation methods were the TZ-method, the proteinase K-method and the phenol extraction method. DNA could not successfully be isolated from red wine using any of the three DNA isolation methods. The TZ-method was the method of choice for the isolation of DNA from inoculated SSS and sterile white wine as this technique gave the best results in terms of simplicity, DNA concentration and purity.

PCR and DGGE conditions were optimised for the universal primer pair, HDA1-GC and HDA2, the wine-bacteria specific primer pair, WBAC1-GC and WBAC2, and the yeast specific primer pair, NL1-GC and LS2. DNA from *Acetobacter pasteurianus*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Oenococcus oeni*, *Brettanomyces bruxellensis* and *Saccharomyces cerevisiae* were amplified with the appropriate primers and successfully resolved with DGGE analysis. PCR and DGGE detection limits were successfully determined when 10^6 cfu.ml⁻¹ of the reference microbes, *A. pasteurianus*, *Lb. plantarum*, *Pd. pentosaceus* and *B. bruxellensis* were separately inoculated into SSS and sterile white wine. It was possible to detect low concentrations (10^1 cfu.ml⁻¹) with PCR for *A. pasteurianus*, *Lb. plantarum*,

Pd. pentosaceus, and *B. bruxellensis* in SSS when amplified with the HDA1-GC and HDA2 primer pair. A PCR detection limit of 10^2 cfu.ml⁻¹ was determined in sterile white wine for *Pd. pentosaceus* and 10^3 cfu.ml⁻¹ for *B. bruxellensis* using this primer pair. The results obtained from the PCR amplification with the WBAC1-GC and WBAC2 primer pair compared well with the results of the HDA1-GC and HDA2 primer pair.

The results from the DGGE detection limits indicated that it was possible to detect lower concentrations ($10^1 - 10^2$ cfu.ml⁻¹) of *A. pasteurianus*, *Lb. plantarum* and *Pd. pentosaceus* with the HDA1-GC and HDA2 primer pair than the WBAC-GC and WBAC2 primer pair ($10^2 - 10^4$ cfu.ml⁻¹). Lower detection limits were also determined for *B. bruxellensis* amplified with the HDA1-GC and HDA2 primer pair ($10^3 - 10^4$ cfu.ml⁻¹) than with the NL1-GC and LS2 primer pair (10^5 cfu.ml⁻¹).

PCR and DGGE detection limits for the inoculation of *A. pasteurianus*, *Lb. plantarum* and *B. bruxellensis* at an inoculum of 10^8 cfu.ml⁻¹ as part of mixed populations in SSS and sterile white wine compared well with the results obtained from the reference microbes inoculated as single microbial species. PCR detection limits of 10^1 cfu.ml⁻¹ were determined for all three reference microbes inoculated as part of mixed populations when amplified with the HDA1-GC and HDA2 and the WBAC1-GC and WBAC2 primer pairs. It was observed that similar or higher DGGE detection limits were obtained for the reference microbes inoculated in sterile white wine ($10^1 - 10^7$ cfu.ml⁻¹) than when inoculated into SSS ($10^1 - 10^5$ cfu.ml⁻¹).

PCR-based DGGE analysis proved to be a technique that could be used successfully with the universal, wine-bacteria and yeast specific primer pairs for the detection of *A. pasteurianus*, *Lb. plantarum*, *Pd. pentosaceus* and *B. bruxellensis*. The culture-independent technique makes the early detection of possible spoilage microbes at low concentrations in wine possible.

UITTREKSEL

Druiwesap word omgeskakel na wyn deur die komplekse prosesse van alkoholiese- en appelmelksuurfermentasie wat uitgevoer word deur giste, melksuurbakterieë en asynsuurbakterieë. Die betrokke mikrobies speel egter nie slegs 'n rol in die versekering van die suksesvolle produksie van wyn nie, maar kan ook tot bederf van die wyn lei as die mikrobiële groei nie beheer word nie.

Konvensionele, kultuur-afhanklike mikrobiologiese tegnieke word algemeen gebruik as die hoof metode vir die deteksie en identifisering van hierdie bederfmikrobies. Molekulêre kultuur-onafhanklike tegnieke, wat die deteksie en identifisering van lewensvatbare, maar nie-kweekbare mikrobies toelaat, het onlangs meer gewild geraak vir die deteksie van moontlike bederfmikrobies wat in mos en wyn voorkom. Verder is hierdie tegnieke minder tydrowend as die konvensionele mikrobiologiese tegnieke.

Die doel van hierdie studie was om die suksesvolle toepassing van polimerase ketting-reaksie (PKR)-gebaseerde denaturerende gradiënt jel elektroforese (DGJE) analise vir die deteksie van wyn bederfmikrobies, wat as enkel mikrobiële spesies en as deel van gemengde mikrobiële populasie in steriele fisiologiese soutoplossing (FSO) (0.85% (m/v) NaCl) en steriele witwyn geïnkuleer is, te evalueer. Drie metodes vir die isolasie van DNS vanuit FSO, en steriele wit- en rooiwyn wat met verwysingsmikrobies spesies geïnkuleer, is vergelyk in terme van die DNS-konsentrasie en -suiwerheid, sowel as die eenvoudigheid van die tegniek. Die drie geëvalueerde DNS isolasie metodes was die TZ-metode, die proteinase-K metode en die fenol-ekstraksie metode. DNS kon nie suksesvol vanuit rooiwyn met enige van die drie ekstraksie metodes geïsoleer word nie. Die TZ-metode was die verkose metode vir die isolasie van DNS vanuit geïnkuleerde FSO en steriele witwyn aangesien die tegniek die beste resultate gelewer het in terme van eenvoud, DNS-konsentrasie en -suiwerheid.

PKR en DGJE kondisies is geoptimeer vir die universele inleierpaar, HDA1-GC en HDA2, die wyn-bakterieë spesifieke inleierpaar, WBAC1-GC en WBAC2, en die gis spesifieke inleierpaar, NL1-GC en LS2. DNS vanaf *Acetobacter pasteurianus*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Oenococcus oeni*, *Saccharomyces cerevisiae* en *Brettanomyces bruxellensis* is geamplifiseer met die toepaslike inleiers en is suksesvol geanaliseer met DGJE. PKR en DGJE deteksie limiete is suksesvol bepaal vir die 10^6 kve.ml⁻¹ inokulum van die verwysingsmikrobies, *A. pasteurianus*, *Lb. plantarum*, *Pd. pentosaceus* en *B. bruxellensis* apart in FSO en steriele witwyn. Dit was moontlik om lae konsentrasies (10^1 kve.ml⁻¹) van *A. pasteurianus*, *Lb. plantarum*,

Pd. pentosaceus en *B. bruxellensis*, geïsoleer vanuit FSO, met PKR te bepaal wanneer die DNS met die inleierpaar, HDA1-GC en HDA2, geamplifiseer is. PKR deteksie limiete van 10^2 kve.ml⁻¹ vir *Pd. pentosaceus* en 10^3 kve.ml⁻¹ vir *B. bruxellensis*, in witwyn, wanneer dieselfde inleier paar gebruik is, is bepaal. Die PKR amplifiserings resultate vir die inleierpaar, WBAC1-GC en WBAC2, het goed vergelyk met die resultate verkry vir die inleierpaar, HDA1-GC en HDA2.

Die DGJE deteksie limiet resultate het getoon dat dit moontlik is om laer konsentrasies (10^1 – 10^2 kve.ml⁻¹) van *A. pasteurianus*, *Lb. plantarum* en *Pd. pentosaceus* met die inleierpaar, HDA1-GC en HDA2, te bepaal as wanneer met die inleierpaar, WBAC1-GC en WBAC2, (10^2 – 10^4 kve.ml⁻¹) geamplifiseer word. Laer deteksie limiete (10^3 – 10^4 kve.ml⁻¹) is verder bepaal vir *B. bruxellensis* tydens amplifisering met die inleierpaar, HDA1-GC en HDA2, as wanneer met die inleierpaar, NL1-GC en LS2, (10^5 kve.ml⁻¹) geamplifiseer word.

PKR en DGJE deteksie limiete wat bepaal is vir die inokulasie van *A. pasteurianus*, *Lb. plantarum* en *B. bruxellensis*, teen 'n inokulum van 10^8 kve.ml⁻¹, as deel van 'n gemengde populasie in FSO en steriele witwyn het goed vergelyk met die resultate verkry vanaf die verwysingsmikrobes wat geïnokuleer was as enkel mikrobiëse spesies. PKR deteksie limiete vir al drie verwysingsmikrobes, geïnokuleer as deel van gemengde populasies, en wat met die inleierpare, HDA1-GC en HDA2, en WBAC1-GC en WBAC2 geamplifiseer is, is bepaal as 10^1 kve.ml⁻¹. Vergelykbare of hoër DGJE deteksie limiete is waargeneem vir die verwysingsmikrobes wat in steriele witwyn (10^1 – 10^7 kve.ml⁻¹) geïnokuleer is in vergelyking met die inokulasie van die onderskeie mikrobes in FSO (10^1 – 10^5 kve.ml⁻¹).

Hierdie studie het getoon dat PKR-gebaseerde DGJE analise suksesvol met die universele, wyn-bakterieë en gis-spesifieke inleierpare gebruik kan word vir die deteksie van *A. pasteurianus*, *Lb. plantarum*, *Pd. pentosaceus* en *B. bruxellensis*. Die gebruik van 'n kultuur-onafhanklike tegniek maak die vroeë deteksie van moontlike bederfsmikrobes, teen lae konsentrasies, in wyn moontlik.

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Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following persons and institutions for their invaluable contribution to the successful completion of this study:

My study leaders, Proff. R.C. Witthuhn and M. du Toit for their expert guidance, knowledge, enthusiasm and support;

The National Research Foundation, Stellenbosch University, Winetec and THRIP for financial support;

Lynn Engelbrecht, Talitha Greyling and Elda Lerm at the Department of Viticulture and Oenology and the Institution of Wine Biotechnology at Stellenbosch University;

Staff at the Department of Food Science, for all their support, cherished friendships and valued company during coffee breaks;

Donna Cawthorn and Yvette le Roux for their advice and help, as well as my fellow post graduate students for support and friendships;

Dr. Michelle Cameron for her skilled practical assistance and advice in the lab and her support and love outside of the lab;

My family for their love and support; and

My Heavenly Father for giving me the ability to succeed.

CHAPTER 1

INTRODUCTION

A variety of fermented foods can be found world-wide, including cheese, bread, sauerkraut, pickles, yoghurt, beer and wine. In all of these food products, fermentation plays an important role in the formation of flavour and texture of the product, but is also responsible for the shelf-life and health benefits of the products (Holzapfel, 2002; Giraffa, 2004). During winemaking there are two fermentation stages that play essential roles in ensuring a successful end-product. Alcoholic fermentation is performed by yeasts, with the commercial yeast *Saccharomyces cerevisiae* that is commonly added as a pure starter culture to grape juice. Malolactic fermentation (MLF) is characteristically performed by lactic acid bacteria (LAB) that are generally present in the grape must and during the winemaking process or that are added as MLF starter cultures (Fleet, 1993). Wine is the product of complex microbial interactions between diverse species of yeasts, LAB, acetic acid bacteria (AAB) and filamentous fungi, of which only some are inoculated for the purpose of fermentation (Fleet, 1993). Due to the presence of this diversity of microbial species, it is of greatest importance to have control over the growth of the microbes present on the wine grapes, the must and the wine, and especially those microbes that may cause spoilage (Rankine, 1995). The microbial species that are present play an important role in ensuring a successful end-product, but most importantly, the concentration of these microbial species influence the outcome of the quality of the end-product with the potential to cause spoilage (Giraffa, 2004). The spoilage of wine annually causes economic losses to the wine and grape industry in South Africa, thus it is extremely important to use appropriate techniques for the early detection and identification of possible spoilage microbes present in the must and wine.

The diverse species of yeasts and bacteria that are present in wine are generally identified by culture-dependent techniques of culturing homogenates of wine samples on plates of agar media. The colonies are then enumerated, isolated and identified with the use of standard morphological, biochemical and physiological tests (Fleet, 1992; Deák, 2003). These culture-dependent microbiological methods of detection and identification of microbes present in wine are often time-consuming and expensive and often provides results that are unreliable in assessing the true microbial population present (Ercolini, 2004). However, because of the simplicity and non-specialised

equipment needed for these techniques, it will remain the major approach for the detection and identification of spoilage microbes in the wine industry. Culture-independent molecular techniques are gaining popularity since it has the significant advantage of detecting and identifying viable but non-culturable microbial species that are present in wine and that may potentially cause spoilage (Muyzer & Smalla, 1998; Giraffa & Neviani, 2001). The polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) technique has successfully been applied by other researchers, such as Cocolin *et al.* (2000; 2001) and Mills *et al.* (2002) for the detection and identification of bacteria and yeasts in wine. PCR-based DGGE analysis also has the valuable potential of detecting individual species that are part of a microbial population present in the sample being analysed, as well as the overall profiling of the population changes over time (Lopez *et al.*, 2003).

The aim of this study was to evaluate the performance of PCR-based DGGE analysis for the early detection and identification of possible wine spoilage microbes. Three methods for the isolation of DNA from wine were evaluated and compared. PCR and DGGE conditions were optimised for three primer pairs including a universal primer pair, a wine bacteria specific primer pair and a yeast specific primer pair to ensure that consistent and reliable results are obtained. PCR and DGGE detection limits with the relevant primer pairs were determined for reference wine spoilage microbes inoculated in sterile saline solution (SSS) and sterile white wine as single microbial strains and as part of mixed microbial populations.

References

- Cocolin, L., Bisson, L.F. & Mills, D.A. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiology Letters*, **189**, 81-87.
- Cocolin, L., Heisey, A. & Mills, D.A. (2001). Direct identification of the indigenous yeasts in commercial wine fermentations. *American Journal of Enology and Viticulture*, **52**, 49-53.
- Deák, T. (2003). Detection, enumeration and isolation of yeasts. In: *Yeasts in Food, Beneficial and Detrimental Aspects* (edited by T. Boekhout & V. Robert). Pp. 36-68. Cambridge: Woodhead Publishers.
- Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, **56**, 297-314.
- Fleet, G.H. (1992). Spoilage yeasts. *Critical Reviews in Biotechnology*, **12**, 1-4

- Fleet, G.H. (1993). The microorganisms of winemaking – isolation, enumeration and identification. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 1-26. New York: Taylor & Francis.
- Giraffa, G. (2004). Studying the dynamics of microbial populations during food fermentation. *FEMS Microbiology Reviews*, **28**, 251-260.
- Giraffa, G. & Neviani, E. (2001). DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. *International Journal of Food Microbiology*, **67**, 19-34.
- Holzappel, W.H. (2002). Appropriate starter culture technologies for small-scale fermentation in developing countries. *International Journal of Food Microbiology*, **75**, 197-212.
- Lopez, I., Ruiz-Larrea, F., Cocolin, L., Orr, E., Phister, T., Marshall, M., VanderGheynst, J. & Mills, D.A. (2003). Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, **69**, 6801-6807.
- Mills, D.A., Johannsen, E.A. & Cocolin, L. (2002). Yeast diversity and persistence in Botrytis-affected wine fermentation. *Applied and Environmental Microbiology*, **68**, 4884-4893.
- Muyzer, G. & Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek*, **73**, 127-141.
- Rankine, B. (1995). Microbiology and fermentation. In: *Making Good Wine – A Manual of Winemaking Practice for Australia and New Zealand*. Pp. 118-130. Sydney: Pan Macmillan.

CHAPTER 2

LITERATURE REVIEW

A. Background

Historians believe that wine was first made in the Caucasus and in Mesopotamia as early as 6000 BC (Pretorius, 2000). During the seventeenth century wine was considered to be the only wholesome, readily storable beverage, leading to a rapid and world-wide increase in wine fermentation. In 1863, Louis Pasteur discovered microbial activity in wine and showed that yeasts are the primary catalysts in the fermentation. The yeasts are responsible for the biotransformation of the grape juice sugars, glucose and fructose to ethanol and carbon dioxide (CO₂) (Jolly *et al.*, 2006; Pretorius, 2000).

South Africa, with climatic conditions that are exceptional for the production of wine, has a history of winemaking dating back to 1655 (McDonald *et al.*, 2006). Today, South Africa is the thirteenth largest consumers of wine in the world (SAWIS, 2006). The South African wine industry produced more than an estimate of 686 million litres of wine in 2006, ranking as the ninth largest wine producer in the world. In 2005 the value of fortified, sparkling and natural wine exports accounted for R3 billion, with natural wine accounting for 97.7% of wine exports (SARS, 2005). Domestic wine sales increased from 13.8 to 81.6% and wine exports with 456% from 1994 to 2005 (McDonald *et al.*, 2006).

Wine is the product of complex biochemical and microbial interactions between diverse species of yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and filamentous fungi (Fleet, 1993). While microbial activity is the foundation of winemaking, the final quality is also affected by microbes that cause spoilage during storage in the cellar or after bottling (Fleet, 1998). Only a few genera of microbes can grow in must and on grapes, and play a significant role in winemaking (Rankine, 1995). Although wine and grape juice is a restrictive environment, microbes can cause spoilage and reduce the quality of the wine if microbial growth is not controlled.

B. Wine fermentation

Spontaneous wine fermentations meant that the onset of fermentation, the end results and wine quality were unpredictable. Undesirable flavour and aroma production influenced the wine quality, therefore, the use of pure starter cultures were first

introduced in the 1900s (Rankine, 1995) and today *Saccharomyces cerevisiae* is used to encourage reliable and rapid fermentation and ensure wine with constant quality (Romano *et al.*, 2003). The processes involved in winemaking are complex and two fermentation steps are essential for certain wines. Alcoholic fermentation is the conversion of the sugars, glucose and fructose to ethanol and CO₂ and is performed by yeasts (Boulton *et al.*, 1996). Alcoholic fermentation is followed by malolactic fermentation (MLF), which is the direct decarboxylation of L(-)malic acid to L(+)lactic acid (Boulton *et al.*, 1996), and performed by LAB. MLF is fundamental for all red wines and some white wines and wine colour modification always accompanies this fermentation (Ribéreau-Gayon *et al.*, 2000; Bauer & Dicks, 2004). Colour intensity decreases and the brilliant red colour disappear through reactions that stabilise the colour during MLF (Ribéreau-Gayon *et al.*, 2000).

Alcoholic fermentation is an important stage in winemaking and is performed by yeasts found in wine, must and on the surfaces of grapes (Lambrechts & Pretorius, 2000). The non-*Saccharomyces* yeasts will grow during the early stages of fermentation, but the process becomes dominated by *Saccharomyces* yeasts when ethanol production increases. The more ethanol tolerant and strongly fermenting *Saccharomyces* spp. will take over the fermentation and will dominate until its completion. The number of non-*Saccharomyces* yeasts will decrease because of their lower tolerance to ethanol that is produced by the *Saccharomyces* spp. (Fleet & Heard, 1993). The various yeast species that grow during alcoholic fermentation metabolise grape juice constituents to a variety of volatile and non-volatile end-products that may have an influence on the fermentation bouquet (Rapp & Versini, 1991; Romano *et al.*, 2003). Ethanol and CO₂ make a small contribution to the aroma of wine, although it is the main volatile products of yeast metabolism. From the end of alcoholic fermentation the LAB population including *Oenococcus oeni* (formerly known as *Leuconostoc oenos*), *Lactobacillus* spp. and *Pediococcus* spp. multiply (Lafon-Lafourcade, 1983; Bauer & Dicks, 2004).

MLF improves the organoleptic quality and microbial stability of wine, but the main effect of MLF is deacidification of the wine through the decarboxylation of dicarboxylic L-malic acid (malate) to monocarboxylic L-lactic acid (lactate) and CO₂ (Davis *et al.*, 1985). Deacidification causes a decrease in acidity and an increase in the pH of the wine (Henick-Kling, 1993). MLF wines can be described as malolactic, yeasty, buttery, oaky, lactic, nutty and sweaty. MLF in general enhances the fruity character and decreases the vegetative aromas of wine. Wine colour is also modified

during MLF by the metabolic activity of bacteria on the wine tannins and anthocyanins (Henick-Kling, 1993).

The spoilage of wine by other bacteria decreases when LAB are present in high numbers during MLF (Lonvaud-Funel *et al.*, 1988). This is brought about by the uptake of micronutrients during the growth of LAB, creating a nutritionally poor medium that is incapable of sustaining further growth of fastidious microbes. Furthermore, synthesis of antibacterial compounds such as lactic acid and bacteriocins also play a significant role in the inhibition of spoilage microbes (Henick-Kling, 1993; Lonvaud-Funel & Joyeux, 1993; Boulton *et al.*, 1996). However, MLF is not always favourable and is considered a spoilage defect in some wines. The reduction in the acidity of the wine caused by MLF may negatively contribute to the general wine sensory balance. Furthermore, it increases the pH of the wine to levels that can encourage the growth of spoilage microbes (Fleet, 2007). The use of starter cultures can prevent unpredictable spontaneous MLF which may lead to the spoilage of wine (Henick-Kling, 1993).

C. Microbial population during wine fermentation

Yeasts

Yeasts are primarily responsible for alcoholic fermentation and the diversity of the yeast population contributes to the sensory quality of wine (Romano *et al.*, 2003). *Saccharomyces cerevisiae* is the principal yeast during alcoholic fermentation. However, up to 15 genera of non-*Saccharomyces* yeasts may be present during the fermentation process (Ciani & Picciotti, 1995; Pretorius, 2000), which includes *Brettanomyces* (*Dekkera*), *Kloeckera* (*Hanseniaspora*), and *Candida* (*Metchnikowia*) (Fleet & Heard, 1993; Romano *et al.*, 2003; Fugelsang & Edwards, 2007). *Kloeckera* and *Candida* are the principle non-*Saccharomyces* yeasts in natural and inoculated juice fermentations (Fleet *et al.*, 1984; Heard & Fleet, 1985).

Yeasts originate from the surface of grapes, surfaces of winery equipment and starter cultures, with grapes being the main source of indigenous wine yeasts. *Kloeckera* (*Hanseniaspora*) is the predominant yeast genus on the grape surface and account for 50 – 75% of the total yeast population on grapes. The genera *Candida*, *Cryptococcus*, *Rhodotorula*, *Pichia*, *Kluyveromyces* and *Hansenula* are present in smaller numbers on grape surfaces (Fugelsang & Edwards, 2007). *Saccharomyces* spp. are present at concentrations lower than 50 colony forming units per ml (cfu.ml⁻¹)

on unharmed grapes and prefer the high sugar environments of grape juice (Martini & Vaughan-Martini, 1990).

Damaged grapes encourage the growth of microbes due to an increase in available nutrients. A diverse yeast population develops under these conditions that co-exist with other fungi, LAB and AAB (Fleet & Heard, 1993). Damaged grapes have greater populations of species of *Kloeckera* (*Hanseniaspora*), *Candida* (*Metchnikowia*), *Saccharomyces* and *Zygosaccharomyces* (Fleet *et al.*, 2002).

Prominent non-Saccharomyces and Saccharomyces yeasts present during wine fermentation

The final wine product results from a combined action of several non-*Saccharomyces* yeast species which grow in sequence throughout the fermentation. These include species of *Zygosaccharomyces*, *Kloeckera* and *Candida* and to a lesser extent species of *Hansenula*, *Pichia* and *Brettanomyces* (Fugelsang & Edwards, 2007). Some non-*Saccharomyces* yeasts are sensitive to high ethanol concentrations (above 5% to 6% (v/v)) (Kunkee, 1984) and have an oxidative and poor fermentative metabolism. Temperatures lower than 20°C make these species more tolerant to ethanol (Heard & Fleet, 1988; Fleet, 2007) and may result in a greater contribution from *Hanseniaspora* and *Candida* spp. during alcoholic fermentation. Under these conditions these yeast species will equal *S. cerevisiae* as the dominant species at the end of the alcoholic fermentation and will have an influence on the wine flavour (Heard & Fleet, 1988; Erten, 2002). *Zygosaccharomyces bailii*, *Zygosaccharomyces fermentati* and *Schizosaccharomyces pombe* present in winery environments are tolerant to ethanol levels greater than 10% (v/v) (Fleet, 2000; Romano *et al.*, 1993). They utilise malic acid and can make a positive contribution to the wine quality, but can also be regarded as spoilage microbes.

Species of *Brettanomyces* (*Dekkera*) grow in grape juice and wine and are known for producing volatile phenols in wines, but when produced below their threshold may also play a positive role in wine flavour and bouquet complexity, as well as in imparting aged characters in young red wines. *Brettanomyces* species are strongly acidogenic, and produce large amounts of acetic acid when they metabolise glucose. The production of acetic acid may inhibit the growth of other microbes present (Fugelsang & Edwards, 2007). Spoilage of wine by species of *Brettanomyces* is a global problem (Loureiro, 2000) and significant populations can build up in winery equipment. The growth of *Brettanomyces* (*Dekkera*) populations is caused by

contamination through unsanitary practices. *Brettanomyces* (*Dekkera*) spp. are slow growing yeasts and its presence can easily go undetected since the cells do not form a biofilm or produce visible amounts of CO₂ (Smith, 1998a; 1998b). The growth rate is enhanced when glucose concentrations increase, but significant populations of *Brettanomyces* may grow at glucose levels of less than 0.2% (v/v) (Fugelsang & Edwards, 2007). Unfortunately, low numbers of this yeast species can cause wine spoilage (Smith, 1998a; 1998b).

Zygosaccharomyces bailii, *Z. bisporus*, *Z. rouxii* and *Z. florentinus* have been isolated from grape must and wine (Barnett *et al.*, 1990). *Zygosaccharomyces* spp. are osmophilic and are present in environments with high sugar concentrations [50 – 60% (m/v)]. Species of *Zygosaccharomyces* actively grow over a wide range of sugar concentrations making it osmotolerant or osmoduric (Thomas, 1993). *Zygosaccharomyces rouxii* is capable of growing at a water activity (a_w) ranging from 0.62 in fructose to 0.65 in sucrose or glycerol and up to an a_w of 0.86 in sodium chloride (NaCl). The yeast grows in the thin film of water at the surface of high sugar environments and will grow slowly if the storage temperature is low. Spoilage of wine will only occur when their growth is stimulated by a rise in temperature. Species of *Zygosaccharomyces* are tolerant to alcohol and growth is possible in wines at 10% (v/v) alcohol and higher (Romano & Suzzi, 1993). They are also resistant to preservatives in grape juice, concentrate and wine (Fugelsang & Edwards, 2007), but is sensitive to phenolics and anthocyanins in red wines. Populations of *Zygosaccharomyces* show an increase during and after processing when competition by other microbes is reduced or eliminated. Poor hygiene practices contribute to 95% of the contamination by species of *Zygosaccharomyces* (Fleet, 2003a).

While species of *Zygosaccharomyces* are present as the principle yeasts in grape must and wine, the principal indigenous yeast species on grapes during harvest is *Kloeckera* (*Hanseniaspora*) species. *Hanseniaspora uvarum* and *Kl. apiculata* produce high concentrations of acetic acid and esters before and throughout the early stages of the alcoholic fermentation. The final concentration of esters in wine is directly linked to the population and growth of *H. uvarum* during these early stages (Sponholz, 1993; Fugelsang & Edwards, 2007). Certain strains of *H. uvarum* are also capable of producing killer toxins that may inhibit the growth of *S. cerevisiae* strains (Sponholz, 1993).

A biofilm producer, *Pichia anomala* (formerly known as *Hansenula anomala*), is fermentative and oxidative and is capable of producing 0.2 – 4.5% (v/v) alcohol, as well

as large amounts of acetic acid ($1 - 2 \text{ g.l}^{-1}$), ethyl acetate (2.15 g.l^{-1}) and isoamyl acetate (Sponholz, 1993; Fugelsang & Edwards, 2007). Before and throughout early stages of alcoholic fermentation, low concentrations of esters are produced, which may enhance the sensorial characteristics of wine (Fugelsang & Edwards, 2007). The utilisation of acid by *P. anomala* may lead to a decrease in titratable acidity and an increase in the pH of the wine (Sponholz, 1993). Like *P. anomala*, *Pichia membraeformis* also grows as an oxidative, chalky biofilm in aging wine, as well as during the early phases of alcoholic fermentation (Mora & Mulet, 1991). *Pichia membraeformis*, *Pichia vini* and *Pichia farinosa* may be inhibited by alcohol levels of 10% (v/v) and higher in wine (Heard & Fleet, 1988). These species will then become dominated by other yeasts, for example *S. cerevisiae*, which are more tolerant to high ethanol concentrations and which are more competitive for growth in this environment (Fleet & Heard, 1993).

Factors affecting yeast growth during fermentation

Fermentation and the quality of wine are influenced by a variety of factors that are important in the winemaking process. These include the clarification of grape juice, addition of sulphur dioxide (SO_2), fermentation temperature, composition of the grape juice, inoculation with selected yeasts, sluggish fermentations and the interactions between yeasts and other microbes (Fleet & Heard, 1993).

Grape juice can be clarified by several procedures and include cold-settling, enzyme treatment, centrifugation and filtration (Fleet & Heard, 1993). The reduction of suspended grape solids to levels of 1 – 2% (m/v) prior to fermentation is a common practice (Boulton *et al.*, 1996) since it improves the development of fruit character and reduce the possibility of volatile formation that will affect wine quality negatively (Fugelsang & Edwards, 2007). Clarification could potentially remove indigenous yeasts and may completely eliminate them if the incorrect clarification procedure is used. Clarification may also encourage selective growth of indigenous species that grow well at low temperatures, such as *Kl. apiculata* (Fleet, 2007).

Sulphur dioxide is added to grapes and grape juice to control oxidation reactions, to selectively limit the growth of indigenous non-*Saccharomyces* yeasts, and to enhance the selective growth of *S. cerevisiae* (Fugelsang & Edwards, 2007). The effect that SO_2 may have on the microbes in the grape juice depends on the concentration of SO_2 that is added, the composition of the grape juice, and the tolerance of the yeasts present. Growth of indigenous yeasts, including *Kloeckera* and *Candida* species, have been

found in wine fermentations where standard levels of SO₂ (20 – 50 mg.l⁻¹) have been added to the grape juice. The addition of SO₂ may also potentially influence the chemical properties of wine, by affecting the metabolic activity of the fermenting yeasts present (Fleet & Heard, 1993).

The rate of yeast growth, and thus the duration of the fermentation are influenced by the temperature at which alcoholic fermentation is performed. The rate of alcoholic fermentation and growth of yeast species will increase with an increase in temperature, with the optimum growth rate at temperatures between 20 and 25°C (Fleet & Heard, 1993). Red wines are usually fermented at temperatures between 20 and 30°C and white wines at temperatures between 10 and 20°C (Kunkee, 1984). *Saccharomyces cerevisiae* will dominate alcoholic fermentation at 30°C, while *Kl. apiculata* will dominate fermentations between 10 and 20°C. The non-*Saccharomyces* yeasts are tolerant to ethanol at low temperatures and become dominant at fermentations below 20°C. The metabolism of sugars by non-*Saccharomyces* yeasts does not lead to the production of high ethanol concentrations (Fleet & Heard, 1993).

The composition of the grape juice influences the fermentation, and the chemical composition and sensory quality of the wine. Factors that have an effect on the growth of yeasts include sugar concentration, supply of nitrogenous substrates, availability of sufficient vitamins, concentration of dissolved oxygen, and the concentration of insoluble solids. The growth of *Kloeckera*, *Hanseniaspora*, *Candida* and other non-*Saccharomyces* yeast species during the settling of grape juice or throughout the early stages of fermentation will change the composition of the juice and influence its suitability to support growth of *S. cerevisiae* in the later stages of alcoholic fermentation. The growth rate of yeasts is influenced by the sugar concentration in the grape juice and will thus determine the yeast species that dominate during fermentation. Grape juice contains all the necessary vitamins (inositol, thiamine, biotin, pantothenic acid and nicotinamide) for the yeasts to complete the fermentation (Fleet & Heard, 1993), but alcoholic fermentation alters the vitamin composition and these altered concentrations may then not be able to support the optimal growth of yeasts. The thiamine content (600 – 800 µg.l⁻¹) in wine will become altered and will not be sufficient as a growth factor for the yeasts. Pantothenic acid, pyridoxine and biotin are used and released by yeast and its concentrations are equal in musts, red and white wine. When the pantothenic acid content is not sufficient, yeasts will accumulate acetic acid and this will cause an increase in volatile acidity (Ribéreau-Gayon *et al.*, 2000). The non-*Saccharomyces* yeast species require more vitamins for growth and this may influence their role in the

fermentation. Inadequate amounts of vitamins will lead to incomplete fermentation and may also result in sluggish fermentations and the production of unwanted metabolic end-products, such as acetic acid and hydrogen sulphide (Boulton *et al.*, 1996). Tartaric acid and malic acid are the main compounds that contribute to the pH (between 2.8 and 4.2) of grape juice. It is not known how the pH of grape juice affects the growth of non-*Saccharomyces* yeasts, but the growth rate and fermentation by *S. cerevisiae* decreases when the pH of the grape juice decreases from 3.5 to 3.0. Fungicide residues and substances produced by the growth of microbes on the grapes before harvest may also inhibit or stimulate the growth of the yeasts (Fleet & Heard, 1993).

The size and type of yeast inoculations also has an influence on the duration of the fermentation. Indigenous species of non-*Saccharomyces* yeasts and indigenous strains of *S. cerevisiae* will be dominated by the selected strain of *S. cerevisiae* inoculated into the grape juice during the fermentation (Fleet & Heard, 1993). However, growth of certain non-*Saccharomyces* yeasts (*Kl. apiculata* and *Candida* species) may not be entirely inhibited by inoculation with selected *S. cerevisiae* strains (Heard & Fleet 1985).

Sluggish and stuck fermentation are difficult to control and are thus a major concern for the international wine industry including the South African wine industry (Malherbe *et al.*, 2007). Sluggish fermentations refer to the early termination of the growth of yeasts, and the resultant alcoholic fermentation. Wine with residual and unfermented sugars and less than expected ethanol concentrations is the result of sluggish fermentations (Bisson, 1999; Fugelsang & Edwards, 2007). Stuck fermentations refer to fermentations that have higher than desired residual sugars at the end of alcoholic fermentation (Bisson, 1999). If less than 150 mg.l⁻¹ FAN nitrogen is present during fermentation, it may lead to stuck fermentations, since it will cause a decrease in yeast growth (Monteiro & Bisson, 1991). Medium chain-length fatty acids, decanoic and octanoic acids produced by *S. cerevisiae* play an important role in sluggish fermentations by causing yeast-bacteria antagonism, resulting in the inhibition of bacterial and yeast growth (Lonvaud-Funel *et al.*, 1988; Edwards *et al.*, 1990). At high concentrations, these acids become toxic for the growth of *S. cerevisiae* and other yeast species, and inhibit the growth and survival of the yeasts during fermentation (Lafon-Lafourcade *et al.*, 1984; Fleet & Heard, 1993).

Interaction between yeasts and other microbes

Wine is influenced by the interaction between the microbes present and these interactions include yeast-yeast interactions, yeast-filamentous fungi interactions and yeast-bacteria interactions. These interactions may either have a beneficial or detrimental effect on the quality of the end-product (Fleet, 2003a; 2003b). There are various mechanisms whereby a specific yeast may influence the growth of other yeasts, bacteria or mycelial fungi. The amount of nutrients in grape juice decreases with the early growth of yeasts resulting in wine that is less favourable for microbial growth. Later in the fermentation process, the yeast population will die and autolyse (Fleet, 2003a), releasing amino acids and nutrients that will support microbial growth (Fleet, 2007).

The non-*Saccharomyces* yeasts can grow anaerobically, as well as aerobically and may limit the growth of *Saccharomyces* yeasts. The non-*Saccharomyces* yeasts utilise nutrients during the early stages of fermentation. *Kloeckera apiculata* depletes grape juice of thiamine and other micronutrients, thereby limiting the growth of *S. cerevisiae* (Bisson, 1999).

The principle mycelial fungi present in wine and must include *Botrytis*, *Uncinula*, *Alternaria*, *Plasmopara*, *Aspergillus*, *Penicillium*, *Rhizopus*, *Oidium* and *Cladosporium* (Fleet, 2007; Fugelsang & Edwards, 2007). Various metabolites are produced by mycelial fungi that grow on grapes and may disturb the yeasts during alcoholic fermentation. *Botrytis cinerea*, *Aspergillus* spp. and *Penicillium* spp. produce metabolites that inhibit the growth of yeasts during the fermentation (Ribéreau-Gayon, 1985). During the growth of mycelial fungi on grapes, conditions are created that will encourage the growth of AAB (Ribéreau-Gayon, 1985) and will lead to an increase in the production of acetic acid and other substances that will inhibit the growth of yeasts during alcoholic fermentation (Drysdale & Fleet, 1988).

Interactions between yeasts and wine bacteria may have a positive or negative effect on the production of wine. Generally, bacteria will grow slowly during alcoholic fermentation and will be present in small numbers (populations lower than $10^3 - 10^4$ cfu.ml⁻¹) in grape juice (Fleet, 2007). Growth of LAB and AAB may cause sluggish fermentations if yeast growth is inhibited or delayed. The growth of LAB, AAB, and occasionally, *Bacillus* and *Clostridium* species are encouraged by nutrients released by autolysed wine yeasts after alcoholic fermentation (Fornachon, 1968; Crougneau *et al.*, 2000; Patynowski *et al.*, 2002), but the growth of these bacteria may lead to wine spoilage (Sponholz, 1993; Fugelsang & Edwards, 2007). The interactions

between wine yeasts and bacteria that are present during MLF are important, since it has the possibility to adversely affect the quality and influence bio-deacidification of wines (Alexandre *et al.*, 2004). *Saccharomyces cerevisiae* may inhibit the growth of *O. oeni* and MLF through the production of inhibitory short chain fatty acids, SO₂, peptides and proteins (Wibowo *et al.*, 1988; Markides, 1993; Lonvaud-Funel *et al.*, 1988). Yeasts produce SO₂ that may be inhibitory to the growth of spoilage LAB, including *Lactobacillus hilgardii*, *Lactobacillus brevis* and *Leuconostoc mesenteroides*, and may inhibit MLF as well. Yeasts may also stimulate the growth of LAB and MLF through autolysis after alcoholic fermentation and the release of nutrients that will stimulate the growth of LAB species (Fornachon, 1968; Patynowski *et al.*, 2002).

Lactic acid bacteria

The species and strains of LAB that are commonly associated with wine belong to the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* (Lonvaud-Funel, 1999; Du Toit & Pretorius, 2000). LAB species include *Oenococcus oeni*, *Leuconostoc mesenteroides*, *Pediococcus parvulus*, *Pediococcus pentosaceus*, *Pediococcus damnosus* (previously known as *Pediococcus cerevisiae*) and various species of *Lactobacillus*, such as *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus buchneri*, *Lactobacillus hilgardii* and *Lactobacillus trichodes* (Fleet, 2007). Du Plessis *et al.* (2004) reported that species of LAB present in grape must which include *Lactobacillus* spp., *Pediococcus* spp. and *Leuconostoc mesenteroides* will show a gradual decrease in growth during alcoholic fermentation. Generally, of the LAB associated with wine fermentation, *O. oeni* will dominate in the wine when alcoholic fermentation is completed (Beltramo *et al.*, 2004).

LAB are commonly found in the wine environment and on the surface of grapes and are capable of growing under the anaerobic conditions of grape must (Lonvaud-Funel, 1999). These bacteria are responsible for MLF (Liu, 2002) that occurs spontaneously in wines. Any delay in the onset of MLF can have an adverse effect on the quality of the end-product resulting in wine with a very low pH and increase in wine acidity (Bousbouras & Kunkee, 1971; Henick-Kling, 1995). Wines that contain high residual glucose and fructose concentration will stimulate the growth of LAB and may lead to the production of unacceptable amounts of acetic acid, D-lactic acid and carbon dioxide (Fleet, 2007).

LAB species that are present in wine can be described as strict heterofermenters, facultative heterofermenters or homofermenters depending on how

the LAB utilise glucose to form lactate (Ribéreau-Gayon *et al.*, 2000). *Oenococcus oeni* and *Lactobacillus* spp. are strict heterofermentative, while *Pediococcus* spp. are homofermentative. Homofermentative LAB converts glucose to lactic acid via the Embden-Meyerhof Parnas (EMP) pathway. Heterofermentative LAB, however, lacks the enzyme fructose-diphosphate aldolase and use the 6-phospho-gluconate pathway to produce lactic acid, ethanol, acetic acid and CO₂ (Fugelsang & Edwards, 2007).

Factors affecting lactic acid bacteria growth during fermentation

There are many factors that influence the growth of LAB in wine, but the four main factors are the ethanol content, temperature, pH, and SO₂ concentration (Ribéreau-Gayon *et al.*, 2000). Lactobacilli are more tolerant to ethanol than cocci, since more than 50% of lactobacilli will be tolerant to an ethanol concentration of 13% (v/v), while only 14% of cocci will show resistance. The rosy strains of *Pediococcus damnosus* are more tolerant to ethanol in wine, because of the polysaccharide capsule that may protect the bacterium (Ribéreau-Gayon *et al.*, 2000). An ethanol concentration higher than 5 – 6% will inhibit the growth of *Lb. plantarum*, while *Lb. casei* and *Lb. brevis* will be more tolerant during MLF (Wibowo *et al.*, 1985).

Temperature plays an important role in the growth and inhibition of LAB and the optimum temperature for the growth is between 20 and 37°C. *Oenococcus oeni* grow at an optimum between 27 to 30°C, and at an optimum of 20 to 23°C in wine with high ethanol content. Growth of LAB in wine decreases as the temperature decreases and will become inhibited at temperatures between 14 and 15°C. The optimum temperature for successful MLF is around 20°C (Ribéreau-Gayon *et al.*, 2000).

The pH of wine may inhibit or stimulate the growth of acidogenic LAB and will affect MLF and the final wine quality. LAB grow actively at a pH around 3.5, and growth will become slow at lower pH levels of around 3.0. Wines with high pH levels will stimulate the growth of LAB, which will stimulate MLF. Unfortunately, this may also lead to the growth of spoilage LAB (Ribéreau-Gayon *et al.*, 2000).

The composition and pH of wine determine the effectiveness of SO₂ as an antimicrobial and antioxidant. The molecular form of SO₂ will inhibit the growth of LAB at SO₂ concentrations above 100 mg (m/v) of total SO₂ per litre and 10 mg of free SO₂ (Ribéreau-Gayon *et al.*, 2000).

Acetic acid bacteria

Gram-negative AAB belong to the family Acetobacteriaceae, with 15 recognised genera of which three is associated with grape and wine spoilage. These AAB include *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* (Garrity *et al.*, 2004). The species of *Acetobacter* are more often found in wine, because of its preference for ethanol as a carbon and energy source (Bartowsky & Henschke, 2008). *Acetobacter* has the ability to oxidise ethanol to acetic acid, CO₂ and H₂O, while *Gluconobacter* can only oxidise ethanol (<5% v/v) to acetic acid, and is not capable of growing in the alcoholic environment of wine (Drysdale & Fleet, 1989a; Du Toit & Pretorius, 2002). *Gluconobacter oxydans* is the main species isolated from grapes and grape must. The two species that are most often found in wine are *Acetobacter aceti* and *Acetobacter pasteurianus* (Bartowsky & Henschke, 2008). *Gluconacetobacter hansenii* (formerly known as *Acetobacter hansenii*) and *Gluconacetobacter liquefaciens* (formerly known as *Acetobacter liquefaciens*) are normally present in grapes and wine (Drysdale & Fleet, 1988). Odour- and flavour-active metabolites (such as volatile acids) are formed during the process of acetification, and are one of the main causes of wine spoilage (Drysdale & Fleet, 1988; Fugelsang & Edwards, 2007).

It has been thought that AAB do not play a significant role in the process of winemaking, because of its anaerobic character (Drysdale & Fleet, 1988). However, it has been found that AAB can grow and survive under the semi-anaerobic to anaerobic environments of wine (Du Toit & Pretorius, 2002). *Gluconobacter* can be found in environments that are rich in sugar and with low alcohol concentrations and is thus seldom found in wine (De Ley *et al.*, 1984). *Acetobacter* spp. are commonly found in fermented substrates and in decaying fruit undergoing early fermentation (Fugelsang & Edwards, 2007). The population of AAB is less than 100 cfu.g⁻¹ on healthy grapes, where a single species, *G. oxydans* will dominate (Joyeux *et al.*, 1984a; Drysdale & Fleet, 1988).

Factors affecting acetic acid bacteria growth during fermentation

AAB are the main oxidative microbes that have the ability to grow and survive under the high acidic and ethanol conditions found [between 10 to 14% (v/v)] in wine (González *et al.*, 2005). The major factors that may have an effect on the growth and survival of AAB in wine include the ethanol concentration, low pH, SO₂, dissolved oxygen and temperature (Drysdale & Fleet, 1988).

AAB can oxidise ethanol to acetic acid, but ethanol may also inhibit the growth of AAB if the concentrations are too high (Du Toit & Pretorius, 2002). The ethanol tolerance of AAB is strain dependent and some strains can grow under the normal concentrations of alcohol in wine, while thermotolerant strains are able to grow and oxidise ethanol at 9% (v/v) without a lag phase (Saeki *et al.*, 1997).

AAB have an optimum growth phase at a pH between 5.5 and 6.3 (Holt *et al.*, 1994), but AAB can also grow and survive in a wine environment with a pH between 2.8 and 4.0. Ethanol sensitivity of AAB may vary between different pH values (Du Toit & Pretorius, 2002). The growth of AAB correlate with the must pH value of commercial South African red wine fermentations (Du Toit & Lambrechts, 2002). If the pH of wine is lower, more SO₂ will be available in free molecular form, which is the active form that inhibits microbial growth and survival (Ribéreau-Gayon *et al.*, 2000).

Oxygen is used by AAB during respiration as terminal electron acceptor (Matsushita *et al.*, 1994). AAB have the ability to grow under the unfavourable anaerobic conditions of wine. These bacteria use other phenolic compounds like quinones and reducible dyes as electron acceptors during these conditions, thus contributing to the bacterial presence in wine (Du Toit & Pretorius, 2002). However, oxygen in small concentrations is necessary for polymerisations of tannins and other phenolic compounds, which is essential for sensorial development and stability of red wine (Ribéreau-Gayon *et al.*, 2000).

The AAB, *Acetobacter* and *Gluconobacter* show optimum growth between 25 to 30°C (Holt *et al.*, 1994). *Gluconobacter* and *Acetobacter aceti* do not grow at temperatures above 37°C (De Ory *et al.*, 1998). AAB can still grow and survive at lower temperatures, but lowering the temperatures during storage of wine to between 10 and 15°C may inhibit the growth of these bacteria (Joyeux *et al.*, 1984a)

D. Spoilage of wine by microbes

The quality and acceptability of wine may be adversely affected by microbiological spoilage that can occur during three stages in the winemaking process. The grapes, the raw material, can become spoiled by undesirable growth of potential spoilage moulds, yeasts, AAB and LAB. Indigenous yeasts from the winery environment will contribute to the alcoholic fermentation, even when inoculated with *S. cerevisiae* (Fleet *et al.*, 1984; Fleet, 1990; Sponholz, 1993). The third stage is represented by the wine product after fermentation, since wine is not a stable product and microbiological spoilage may develop. If the wine is not properly handled and stored after fermentation,

it may become a growth substrate for unwanted species of yeasts and bacteria. Uncontrolled growth of microbes during any of these three stages can alter the chemical composition of the wine, and adversely affect the sensorial properties of appearance, aroma and flavour of the wine (Sponholz, 1993).

Wine spoilage by yeasts

Yeasts can cause wine spoilage during alcoholic fermentation, storage and after bottling (Sponholz, 1993; Thomas, 1993; Boulton *et al.*, 1996; Du Toit & Pretorius, 2000; Loureiro & Malfeito-Feirreira, 2003). Wine can become spoiled when unwanted yeast species grow during the fermentation process, leading to high esters content, formation of acetic acid and hydrogen sulphide. Certain yeasts will grow as a biofilm if the wine is exposed to air and these yeast genera include *Candida*, *Pichia* and *Hansenula* (Fleet, 2007). Wines that contain residual sugars after packaging may undergo refermentation, particularly by *S. cerevisiae*, which may cause swelling and explosion of the container (Thomas, 1993).

Indigenous wine yeasts, such as *Hanseniaspora uvarum* (*Kloeckera apiculata*), *Metschnikowia pulcherima*, *Pichia anomala*, as well as *Brettanomyces* spp. produce esters (Berry & Watson, 1987; Fleet, 2007) and the amount formed varies between different yeast species. A concentration of 2 g.l⁻¹ or more of ethyl acetate and concentrations of 0.6 g.l⁻¹ or less of acetic acid may lead to ester taint (Sponholz, 1993). The concentration of esters in wine is related to the growth of *H. uvarum* during the initial stages of alcoholic fermentation. The spoilage of wine by esters can be controlled by limiting the growth of indigenous yeast species during fermentation and damage to grapes must be avoided during harvesting, since damaged grapes promote growth of indigenous yeasts (Sponholz, 1993).

A biofilm of yeasts may grow on the surface of the wine during storage and the changes that they cause are dependant on the yeast species present. The wine will taste less acidic and more oxidised because of high acetaldehyde concentrations (Caputi & Peterson, 1965; Rossi & Singleton, 1966; Sponholz, 1993; Fugelsang & Edwards, 2007) and the smell of acetic acid and ethyl acetate becomes more distinct. Species of *Candida*, *Metschnikowia*, *Pichia* and *Hansenula* are responsible for the spoilage of wine due to biofilm formation. These yeasts are part of the indigenous yeast population of grape musts and may contaminate the winery environment (Sponholz, 1993). The multiplication of biofilm yeasts depend on the presence of oxygen and growth becomes prominent at later stages in the fermentation. Low temperatures

(8 – 12°C), as well as high alcohol levels (10 – 12%) will inhibit the growth of biofilm yeasts (Sponholz, 1993).

Spoilage of wine by *Zygosaccharomyces bailii* (formerly known as *Saccharomyces bailii*) is caused by re-fermentation during storage in tanks and after packaging (Thomas & Davenport, 1985). *Zygosaccharomyces bailii* also causes contamination of grapes and wine cellars (Peynaud & Domercq, 1959; Sponholz, 1993; Fugelsang, 1996; 1998). Several characteristics of *Z. bailii* make it a significant spoilage yeast. These include tolerance of high ethanol concentrations (> 15%), growth at low pH (< 2.0), strong resistance to high concentrations of preservatives (benzoic acid (> 1000 mg.l⁻¹), sorbic acid (> 800 mg.l⁻¹), SO₂ (> 3 mg.l⁻¹) and diethyl pyrocarbamate (> 500 mg.l⁻¹), and the potential to grow in high sugar environments (> 70% v/v) (Thomas & Davenport, 1985; Boulton *et al.*, 1996). The growth of *Z. bailii* in wine causes turbidity and sedimentation (Sponholz, 1993), increases in concentrations of succinic acid and acetic acid (Shimazu & Watanabe, 1981), strong reduction in acidity due to the metabolism of L-malic acid (Sponholz, 1993), as well as a change in the concentration of esters (Soles *et al.*, 1982).

Species of *Brettanomyces*, such as *Brettanomyces bruxellensis* causes spoilage of wine by producing volatile phenols leading to the formation off-odours and losses of the fruity characteristics of wine (Suaréz *et al.*, 2007; Renouf *et al.*, 2008). The compounds, 4-ethylphenol and 4-ethylguaiacol, are responsible for wine spoilage and when present at high concentrations and have been described as animal, medicinal, sweaty leather, barnyard, spicy, clove-like (Suaréz *et al.*, 2007) and the wine becomes unacceptable with the formation of the “Brett” character (Renouf & Lonvaud-Funel, 2007). Species of *Brettanomyces* may also cause wine spoilage by producing haze, turbidity and volatile acidity. *Brettanomyces intermedius* is responsible for 50% of all hazy wines in South Africa (Van der Walt & van Kerken 1958). Growth of *Brettanomyces* spp. is associated with the production of acetic acid, which constitutes more than 90% of the volatile acidity of wine (Van der Walt & van Kerken 1958). It may affect the quality of wine adversely when the level of acetic acid increases, as it produces a vinegary or acetone-like aroma (Eglinton & Henschke, 1999). The production of acetic acid by *Brettanomyces* spp. has also been associated with sluggish and stuck fermentations (Bisson, 1999). Substances that can cause mousiness taint in wines may also be produced (Boulton *et al.*, 1996). *Brettanomyces* spp. can be controlled with the use of 0.5 to 0.8 mg.l⁻¹ molecular SO₂ (Henick-Kling *et al.*, 2000). The effectiveness of the addition of molecular SO₂ to wine in order to control

B. bruxellensis spp. has been found to be affected by the availability of oxygen (Du Toit *et al.*, 2005). Du Toit *et al.* (2005) found that 0.25 mg.l⁻¹ of molecular SO₂ significantly affected the culturability of the strain, but the strain remained viable and numbers increased after exposure to oxygen.

Wine spoilage by lactic acid bacteria

LAB are important in the winemaking process since they are responsible for MLF, but they also may cause wine spoilage (Kunkee, 1991; Fleet, 2007). The growth of unwanted LAB species during the fermentation or after MLF will lead to wine spoilage, and wine with high concentrations of residual sugars (glucose and fructose) will support the growth of these bacteria. Microbiological spoilage in wine caused by LAB includes acidification, mannitol taint, ropiness, diacetyl production, mousiness, acrolein formation, bitterness, tartaric acid degradation, geranium off-odour and biogenic amines formations (Sponholz, 1993).

Acidification

LAB produces acetic acid (volatile acidity) and lactic acid which may cause an increase in the acidity of wine (Sponholz, 1993; Fugelsang & Edwards, 2007). Acidification by LAB can occur in wines containing residual sugars, particularly during storage when nutrients are available for the growth of these bacteria (Wibowo *et al.*, 1985), but can also occur during alcoholic fermentation when a significant amount of fermentable sugars are present in the grape must. The joint production of mannitol and acetic acid by LAB, as well as D-lactic acid is used as indication of wine spoilage by acidification (Sponholz, 1993). Heterofermentative LAB produce acetic acid and D-lactic acid by the fermentation of sugars, while homofermentative LAB produce D-lactic acid, without acetic acid, through the glycolytic metabolism of sugars (Du Toit & Pretorius, 2000). Acidification is more often caused by D-lactic acid, rather than L-lactic acid which are produced during malolactic fermentation (Sponholz, 1993). The formation of D-lactic acid arises from the reduction of pyruvic acid and is performed by homofermentative species of lactobacilli and pediococci. The production of acetic acid by *O. oeni* correlates with the metabolism of fructose (Sponholz, 1993).

Mannitol taint

Heterofermentative LAB can produce mannitol in considerable concentrations by the enzymatic reduction of fructose or fructose-6-phosphate (Martinez *et al.*, 1963;

Sponholz, 1993; Boulton *et al.*, 1996; Fugelsang & Edwards, 2007). Spoilage of wine by mannitol taint is accompanied by high concentrations of acetic acid, D-lactic acid, n-propanol, 2-butanol and often sliminess and diacetyl taint. Wine affected with mannitol taint will have viscous, sweet and acetate-ester taste characteristics (Sponholz, 1993).

Ropiness

Wines with ropiness have a slimy, viscous and oily character (Sponholz, 1993; Du Toit & Pretorius, 2000). Ropiness may be present in low acid wines at the end of alcoholic fermentation, particularly if malic acid degradation has also taken place. The acidity of wine will decrease by yeasts autolysis during storage and when nutrients become accessible for growth of LAB (Sponholz, 1993; Fugelsang & Edwards, 2007). A direct association between the appearance of ropiness and the growth of LAB can be established by the development of viscosity in wine during fermentation (Sponholz, 1993).

Diacetyl production

The presence of unacceptable high diacetyl (2,3-butanedione) concentrations produced by LAB cause an unwanted buttery or whey like aroma and flavour in spoiled wines. Diacetyl is a di-ketone with a very low taste threshold at 1 mg.l⁻¹ (Sponholz, 1993). If produced by yeast activity, diacetyl may be present in wine at concentrations of 0.2 – 0.3 mg.l⁻¹. Growth of *Pediococcus* or *Lactobacillus* species in wine after MLF could produce concentrations of diacetyl (> 5 µg.ml⁻¹) that may cause spoilage of wine with overwhelming buttery flavours (Bartowsky & Henschke, 2004).

Mousiness

Mousiness is not a common problem in the wine industry and occurs in low acid wines that have not been treated with sufficient SO₂. Mousiness, caused by LAB and *Brettanomyces* spp., does not occur in grape must, but wines have a smell suggestive of mouse urine or acetamide as well as a lingering aftertaste (Du Toit & Pretorius, 2000; Costello & Henschke, 2002). The mousey character is linked to the microbial production of two isomers of 2-acetyltetrahydropyridine (Craig & Heresztyn, 1984) produced by *Lb. hilgardii*, *Lb. brevis*, *Lb. cellobiosus* (now synonymous with *Lb. fermentum*) (Du Toit & Pretorius, 2000) and other heterofermentative LAB

(Heresztyn, 1986; Sponholz, 1993; Fugelsang & Edwards, 2007). Bacterial formation of these substances depends on the presence of ethanol or propanol (Heresztyn, 1986).

Acrolein taint

Bacterial degradation of glycerol causes acrolein taint and related bitterness. The bitter sensation is formed when acrolein reacts with the phenolic groups of anthocyanins (Sponholz, 1993). Acrolein itself is not bitter, and red wines with high phenolic levels, more than white wines, are associated with this form of spoilage. Acrolein concentrations of 10 ppm are sufficient to cause a taint (Margalith, 1981). The ability of LAB to utilise and degrade glycerol is not common, but the growth of *Pediococcus parvulus* and *Lactobacillus cellobiosus* has been correlated with the degradation of glycerol in red wine (Davis *et al.*, 1988).

Tartaric acid degradation

Only a few species of LAB are capable of utilising and degrading tartaric acid. Tartaric acid is normally not metabolised in wine, because of its microbiological stability. The ability to metabolise tartaric acid is generally restricted to only a few *Lactobacillus* species (Wibowo *et al.*, 1985). When oxalacetate is converted to pyruvic acid and CO₂ by homofermentative LAB, half of the pyruvic acid is reduced to lactic acid, and the other half is converted to acetic acid and CO₂. The metabolism of oxalacetate by heterofermentative LAB is more complicated and part of the oxalacetate is transformed to succinic acid and the rest is transformed to acetic acid and CO₂ (Sponholz, 1993).

Geranium off-odour

The geranium off-odour that develops in wine may be compared with the odour produced by crushing the leaves of the geranium plant (*Pelargonium* spp.). This form of wine spoilage becomes evident in wine when certain LAB strains metabolise sorbic acid that may be added to wine as an antimicrobial agent to control the growth of yeasts. The concentrations used to inhibit the yeasts are not sufficient to inhibit LAB activity (Edinger & Spiltstoeser, 1986). The substance, 2-ethoxyhexa-3,5-diene (Sponholz, 1993; Fugelsang & Edwards, 2007), is responsible for the geranium odour and has a low sensorial threshold of 0.1 µg.l⁻¹ and the formation of the substance depends on the hydrogenation of sorbic acid to sorbinol by LAB. All *O. oeni* strains and a few heterofermenting *Lactobacillus* strains are capable of reducing sorbic acid to sorbinol

(Edinger & Spilttstoesser, 1986). This form of spoilage is not evident in grape juice, since ethanol is necessary for these reactions to take place.

Biogenic amines

Biogenic amines are formed by certain LAB through the decarboxylation of amino acids. Histamine, tyramine, putrescine, cadaverine, phenylethylamine are only some of the biogenic amines found to be present in wine (Moreno-Arribas *et al.*, 2003). Biogenic amines are formed during and after MLF from precursor amino acids. LAB, such as *Pediococcus* and *Lactobacillus* have been associated with this form of wine spoilage (Moreno-Arribas *et al.*, 2003) and *O. oeni* has also been associated with the production of biogenic amines (Coton *et al.*, 1998). The spoilage of wine by biogenic amines may be reduced with the use of starter cultures for MLF that would not decarboxylate amino acids (Lonvaud-Funel, 2001).

Wine spoilage by acetic acid bacteria

Wine spoilage by AAB are often associated with a characteristic volatility, a vinegar-like sourness on the palate as well as a range of acetic, nutty, sherry-like, solvent or bruised apple aromas that often also lead to reduction in the fruity sensorial characteristics of wine (Bartowsky *et al.*, 2003). Wines that are spoiled by AAB become unacceptable for the consumers and have low commercial value (Bartowsky & Henschke, 2008). AAB may cause spoilage of grapes and wine during any stage of winemaking (Drysdale & Fleet, 1988) and grapes that are physically damaged or infected by mycelial fungi are not acceptable for the use in the wine production if the volatile acidity is too high (Eglinton & Henschke, 1999). Since these bacteria are aerobic and require oxygen for growth, spoilage of wine by AAB may occur in grape must or in stuck and sluggish fermentations if the wine comes into contact with air (Joyeux *et al.*, 1984a).

AAB, also known as the vinegar bacteria, cause vinegary taint of wines through the oxidation of ethanol to acetaldehyde and acetic acid (Du Toit & Pretorius, 2000; Fleet, 2007). Acetic acid is the major volatile acid in wines and this spoilage is often described as volatile acidity and may contribute to 50% of the volatile acid in wines (Du Toit & Pretorius, 2000). Formic acid that is naturally present in the grapes, or that is produced by mycelial fungi on grapes will form most of the volatile acidity (Sponholz, 1993). Small concentrations of ethyl acetate may also contribute to the vinegary taint of wine (Boulton *et al.*, 1996). Wine is considered to be spoiled if the concentration of acetic acid is more than 0.7 – 1.2 g.l⁻¹ depending on the style of wine. The sensory

perception threshold value of acetaldehyde (100 – 125 mg.l⁻¹) is often exceeded in wine, as AAB can produce acetaldehyde at high concentrations of 250 mg.l⁻¹ (Drysdale & Fleet, 1988; Sponholz, 1993; Boulton *et al.*, 1996; Du Toit & Pretorius, 2002). The acetaldehyde will give the wine an unwanted oxidised quality (Du Toit & Pretorius, 2002). Species of AAB that cause vinegary taint include *Gluconobacter oxydans*, *Acetobacter pasteurianus* and *Acetobacter aceti*. These species influence wine by contamination of the grapes, during alcoholic fermentation and during storage in the cellar.

Apart from acetic acid, AAB also produce other compounds that have sensory implications and that can influence the wine quality (Drysdale & Fleet, 1989b). Glycerol is produced by yeasts and mycelial fungi, as a carbon source for the AAB species, *A. aceti* and *G. oxydans* that will convert glycerol to dihydroxyacetone under aerobic conditions. Glycerol contributes to a perception of sweetness and viscosity (body) in wine if present at levels exceeding 4 – 5 g.l⁻¹ (Noble & Bursick, 1984) and dihydroxyacetone also contribute sweet properties to wine and produce a crust-like aroma when it reacts with proline (Margalith, 1981; Drysdale & Fleet, 1988; Boulton *et al.*, 1996). Dihydroxyacetone has the ability to bind to SO₂ and may affect the antimicrobial activity in wine (Edinger & Spilltstoesser, 1986).

Aging wines with high numbers of AAB may contain high amounts of acetaldehyde, the immediate precursor of ethanol during fermentation (Du Toit & Pretorius, 2000). Residual acetaldehyde may reach levels of 100 – 120 mg.l⁻¹ (Drysdale & Fleet, 1988) and is produced by *Acetobacter* spp. as an intermediate in acetic acid production under low oxygen conditions. Acetaldehyde can sensorially be described as nutty and sherry-like or even suggestive of overripe bruised apples (Zoecklein *et al.*, 1995). The aroma threshold in wine is 100-120 mg.l⁻¹ (Berg *et al.*, 1995) and *Acetobacter* may produce concentrations exceeding 160 mg.l⁻¹ (Drysdale & Fleet, 1989b).

Species and strains of *Gluconobacter* and *Acetobacter* are capable of oxidising lactate to acetoin under low oxygen conditions (Du Toit & Pretorius, 2000). Acetoin may reach levels of 3.0 – 31.8 mg.l⁻¹ in wine (Drysdale & Fleet, 1988; Boulton *et al.*, 1996) and has a butter-like aroma and flavour. Apart from influencing the sensory characteristics of wine, acetoin may also bind to SO₂ and affect the antimicrobial activity.

E. Methods for the detection and identification of microbes present in wine

Wine is the product of complex biochemical and microbial interactions between diverse species of yeasts, LAB, AAB and filamentous fungi (Fleet, 1993), but it is essential to have control over the growth of these microbes during winemaking to ensure a successful end-product (Fugelsang & Edwards, 2007). It is important to enhance the fermentative activity and performance of *S. cerevisiae*, but it is also important to control the growth of undesirable microbes that may cause wine spoilage. Yeasts that are present during winemaking play an essential role during fermentation, but the growth of *Saccharomyces* and non-*Saccharomyces* yeasts may also lead to wine spoilage (Deák, 1993). In some cases, the limited growth of *Brettanomyces* is desirable for red winemaking, but any overgrowth may be considered spoilage. Since uncontrolled microbiological growth can occur at any stage during fermentation of the grape must and wine, the early detection and identification of potential spoilage microbes is essential. Microbial stabilisation is necessary after fermentation to prevent the development of spoilage yeasts and bacteria, since these microbes may alter the sensorial characteristics of wine (Renouf *et al.*, 2006).

Conventional methods, such as plate counting are currently used to monitor the growth of wine microbial populations during fermentation (Gracias & McKillip, 2004) and phenotypic methods are used for the identification of microbes. The methods for detection and identification of microbes and identification of species that are based on metabolism, morphology and reproduction are often time-consuming, unreliable and labour-intensive (Hernán-Gómez *et al.*, 2000; Kopke *et al.*, 2000). Methods, such as electrophoresis of soluble proteins (SDS-PAGE) (Izquierdo *et al.*, 1996) and GC analysis of long-chain fatty acids for wine yeasts (Augustyn *et al.*, 1991) have shown contradicting results in the identification of wine microbes (Hernán-Gómez *et al.*, 2000). Methods that are currently used to identify and enumerate *Brettanomyces* contamination in spoiled wines can take 7 to 14 d and is dependent on the growth of this yeast on semi-selective or selective culture media, followed by physiological and biochemical analysis (Smith, 1998b; Cocolin *et al.*, 2004). Selective enrichment media do not always compare well with the particular conditions that microbes require for growth and some microbes are bound to sediment particles and can then not be detected by conventional microscopy (Muyzer *et al.*, 1993). Viable cells that are present in microbial populations are typically enumerated on non-selective as well as selective media, while stressed cells will form colonies on non-selective media, but can not be enumerated on selective media. When adverse conditions are present in the

microbial environment, microbial species often go into a viable but non-culturable (VNC) state. Low temperatures, for example, will damage the microbes and will cause the healthy microbial cells to go into the VNC state in which they are still capable of metabolic activity, but where the microbes will not form colonies that can be enumerated on selective or non-selective media (Fleet, 1999).

Molecular biology methods use DNA-based analysis methods that are not affected by the culture conditions of selective and non-selective media and are extremely useful in taxonomic studies and in distinguishing between strains of the same species (Hernán-Gómez *et al.*, 2000). DNA-based techniques have been developed to directly discriminate specific microbial populations in wine (Cocolin *et al.*, 2000; Gindreau *et al.*, 2001) and novel techniques have been developed for the direct characterisation of microbes in particular environments without enrichment or isolation (Head *et al.*, 1998). These culture-independent methods study the total microbial DNA isolated from mixed microbial populations to detect and identify individual microbes (Hugenholtz & Pace, 1996) without strain isolation, thus eliminating the possible biases intrinsic to microbial enrichment (Cocolin *et al.*, 2002; Cocolin & Mills, 2003).

Culture-dependent methods in the identification and detection of wine microbes

The traditional methods for detection and identification of microbes from food samples are based on culturing, enumeration and isolation of presumptive colonies for further identification analysis. Food samples can be homogenised, concentrated and pre-enriched before culturing on synthetic media that are similar to the conditions of the environment from which the microbes are isolated (Rantsiou & Cocolin, 2006). With culture-dependent methods it is often possible to observe differences in the morphology and colour of the colonies, but almost always the colonies appear to be identical (Rantsiou & Cocolin, 2006). The microbial cells often also become injured or VNC because of survival and growth inhibitors, including heat, cold, acid and osmotic stress during food processing (Kell *et al.*, 1998). These microbial cells may still be able to grow and cause spoilage and methods are necessary to detect them. Pre-enrichment of the microbes in the food sample may be performed by a non-selective or selective broth culture (Zhao & Doyle, 2001) or by the selective agar overlay technique to revive the injured cells (Hurst, 1977; Ray, 1986). The detection of viable microbes can also be improved by concentrating the sample by centrifugation or filtration before plating.

The pre-treated food sample can be plated on differential, non-selective and selective media (Gracias & McKillip, 2004). Microbes that are present in a food sample

can be detected by using non-selective media or standard methods agar. Specific microbes may be inhibited by using selective media that contains an antibiotic, bacteriocin or a growth nutrient, and differential media can be used to differentiate between microbes by various chemical reactions during growth. Differential media contains an indicator, such as a chromogenic or fluorogenic substrate which allows the direct identification of microbes without additional sub-culturing or biochemical tests. The microbes produce specific enzymes for the substrates and the bacterial growth will change colour or fluorescence (Hakovirta, 2008). Although culture methods may be time-consuming, the purification and isolation of microbes may be additionally analysed by subtyping and these isolates can be stored in culture collections.

Phenotypic studies used to study the characteristics of microbes include methods such as biotyping, serotyping and phage typing (Arbeit, 1995). The biochemical growth requirements, environmental conditions (such as pH, temperature, bacteriocin susceptibility and antibiotic resistance) and physiological characteristics (membrane composition, colony and cell morphology, and cell wall composition) of microbes are studied with biotyping methods (Vandamme *et al.*, 1996), while serological and phage typing (Towner & Cockayne, 1993) methods focus on the surface structural differences of microbes. Phages are not only used during the subtyping of microbes, but are also used for the direct identification and detection of pathogens in food samples (Hagens & Loessner, 2007; Kretzer *et al.*, 2007). Unfortunately, these phenotypic methods show limitations as certain microbes have the capability of altering their phenotypic characteristics due to environmental changes or genetic mutations. These limitations can be avoided by identification of microbes by genotypic characteristics.

Culture-dependent traditional wine microbiological techniques rely on various biochemical tests to identify genera and species of microbes present in wine. One of the first microbiological tests done in a winery is to examine the morphology of the microbe. Wine or grape must samples are prepared as a wet mount and are then examined using phase-contrast microscopy. Microscopy provides information on the shape of the microbial cells (cocci, rods, pointed ends, bowling pin, egg, ogival, elongated, lemon and needle-like), the size of the cells and the arrangement of the cells (single, pairs, tetrads, groups or chains). The detection of small and lemon-shaped yeasts early in alcoholic fermentation could indicate the presence of *Kloeckera* or *Hanseniaspora* in the wine. The concentration of cells by centrifugation is often required, since a high population of cells is necessary for detection.

The detection of microbes with the use of microscopy also shows some limitations, since the appearance of the yeasts varies depending on age and culture conditions. When a culture is grown on malt agar for 72 h, it may be clearly different from the cells isolated from a wine sample closer to the end of alcoholic fermentation. Yeasts also show considerable variation in shape and size reflecting the fact that asexual reproduction results from budding. For confirmation, microbes are isolated from wine samples before characterisation and the isolates are re-streaked several times in order to obtain a pure culture that is free from any contaminants. Different media should be used to isolate various microbes that are present in the wine sample. When the various microbes are isolated, classic microbiological tests are used for further characterisation and identification and include assimilation of carbon and nitrogen, presence of ascospores, presence of mycelia and pseudomycelia that is demonstrated by growing fungi as slide cultures, and fermentation of carbohydrates for yeasts. For the identification of bacteria, biochemical tests such as ammonia from arginine, catalase, dextran from sucrose, fermentation of carbohydrates, gas from glucose, Gram stain, ketogenesis, lactate from glucose, malate utilisation, mannitol from fructose, oxidation of ethanol and oxidation of lactate are used (Fugelsang & Edwards, 2007).

Culture-independent methods in the identification and detection of wine microbes

Many culture-independent methods have been developed for the identification and detection of microbes. Techniques and procedures that provide a unique profile of the DNA of a strain or species are especially valuable for the purposes of identification (Deák, 1995). Most bacterial and yeast species that are present in wine have been identified by conventional microbiological techniques relating to cultivation. However, these conventional methods often display bias resulting in an incomplete representation of the true population diversity of yeasts, LAB and AAB present in wine. Stressed and injured cells are also often not recovered in selective media and cells present in low numbers are inhibited by microbial populations present in higher numbers (Amann *et al.*, 1995; Hugenholtz *et al.*, 1998). Culture-independent molecular techniques to monitor the microbial successions of various food and beverage fermentations have shown microbial constituents and microbial interactions not revealed by previous plating analysis (Giraffa & Neviani, 2001). This was done using epifluorescence microscopy to identify populations of VNC bacteria in aging wine (Millet & Lonvaud-Funel, 2001).

The most commonly used of these methods include polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE)/temperature gradient gel electrophoresis (TGGE) analysis, restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE) and fluorescent *in situ* hybridisation (FISH).

Polymerase chain reaction-based denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis analysis of microbial populations in wine

The culture-independent methods, DGGE and TGGE are also used for the separation of bacterial 16S and yeast 26S ribosomal DNA (rDNA) amplicons and are common methods to characterise microbial communities from specific environmental niches (Muyzer & Smalla, 1998). These approaches are attractive since they enable detection of individual species, as well as the overall profiling of community structure changes with time.

DGGE/TGGE is frequently used for the detection and identification of microbial populations and is based on the separation of polymerase chain reaction (PCR) amplicons of the same size but with different base-pair sequences on polyacrylamide gels. PCR-DGGE/TGGE is used to determine the microbial consortium in environmental samples without cultivation and to determine the community dynamics in reaction to variation in the environment (Ercolini, 2004). The DGGE technique is based on the electrophoretic separation of PCR-generated double stranded DNA in a polyacrylamide gel containing a gradient of a denaturant. The DNA fragments will encounter an appropriate denaturant concentration and a sequence-dependant partial separation of the double strands will occur. The TGGE technique separates DNA fragments amplified with PCR on a polyacrylamide gel as a result of differing electrophoretic mobilities that is caused by partial denaturing along a linear temperature gradient (Riesner *et al.*, 1991). When the molecule reaches its melting point (T_m), like with DGGE, the double helix will undergo a conversion to a partially denatured molecule and will stop migrating (Lerman *et al.*, 1984; Hernán-Gómez *et al.*, 2000). With two fragments of the same size, the DNA melting point is dependent on the proportion and position of the guanine and cytosine bases (Hernán-Gómez *et al.*, 2000). The conformational change in the tertiary structure of the DNA fragments causes a reduced migration rate and after staining results in a DNA band pattern or a fingerprint that is representative of the sampled microbial community (Satokari *et al.*, 2003; Sigler *et al.*, 2004).

PCR-based DGGE/TGGE is widely used in molecular ecological studies to assess the diversity and community dynamics in microbial populations (Muyzer *et al.*, 1993; Muyzer & Smalla, 1998; Muyzer, 1999). Typically, DGGE/TGGE only detect the microbes that make up at least 1% of the total population (Muyzer & Smalla, 1998) and to improve the sensitivity of the detection of these microbes, PCR reactions that target restricted microbial groups can be used. DGGE/TGGE can also be used to monitor the complexity of microbial populations and changes that take place, while individual microbes of the population can be identified by subsequent cloning and sequencing of the fragments. The DGGE/TGGE profiles can also be hybridised with phylogenetic probes in order to obtain further information on the specific species or microbial groups (Felske *et al.*, 1997; Satokari *et al.*, 2003).

Hernán-Gómez *et al.* (2000) PCR amplified 18S rDNA from 74 wine yeast strains, where after the fragments were analysed with TGGE. It was difficult to differentiate between species in some cases and in others difficult to differentiate between genera because of the similar mobility of the fragments that were analysed.

Lopez *et al.* (2003) used the PCR-DGGE technique to investigate the bacteria that are present during wine fermentation and found that several PCR primers described in the literature to amplify bacterial 16S rDNA, also co-amplify yeasts, mycelial fungi, or plant DNA that are present in the samples. The amplification of such non-bacterial DNA can mask the microbial populations in the DGGE profiles. With the use of primer sets that specifically amplify the bacterial 16S rDNA in wine samples, without the subsequent amplification of eukaryotic DNA, it is possible to overcome this problem. The specificity and efficacy of two primer sets, WLAB1 and WLAB2, and WBAC1 and WBAC2, were examined with DNA isolates from various wine bacteria, yeasts, and mycelial fungi. Both these primer sets successfully distinguished between bacterial species in wine containing a mixed population of yeasts and bacteria.

Yeasts have a defining impact on the quality of wine and since they perform alcoholic fermentation, they also contribute to the essential chemical structure of wine aroma and flavour. Unfortunately, yeasts can also cause spoilage of wine during the later stages of wine fermentation (Fleet, 2003a; 2007). Thus, it is important to have reliable knowledge about the ecology of yeasts (Fleet *et al.*, 2002). Prakitchaiwattana *et al.* (2004) compared DGGE with results based on cultural isolation on malt extract agar (MEA) for the detection of the yeasts that are associated with wine grapes. The detection limit for yeasts with PCR-DGGE was determined as 10^2 cfu.ml⁻¹, although the value was affected by the culture age, as well as the relative populations of the species

present in mixed culture. These researchers found that PCR-DGGE was less sensitive than culture on MEA for the determination of the yeast ecology of grapes and could not reliably detect the species present at populations less than 10^4 cfu.g⁻¹, but PCR-DGGE could detect a larger diversity of species than agar plating.

Manzano *et al.* (2005) used the PCR-TGGE technique and could successfully distinguish between *S. cerevisiae* and *S. paradoxus* as well as between different strains of *S. cerevisiae*. The researchers also performed direct analysis of *S. cerevisiae* and *S. paradoxus* ecology in musts with PCR-TGGE and found that this technique could be used to confirm the presence of starter cultures during fermentation. With the use of PCR-TGGE on must samples, without the interference from other yeast genera in the amplification of *S. cerevisiae*, it is possible to immediately modify the parameters of fermentation if problems with *S. cerevisiae* activity take place during fermentation.

Amplified fragment length polymorphism analysis of microbial populations in wine

AFLP is a molecular technique for the fingerprinting of DNA from many origins. There is a wide range of applications for AFLP, which include the monitoring of inheritance of agronomic traits in plant and animal breeding, pedigree analysis, forensic typing, diagnostics of genetically inherited diseases, parentage analysis, screening of DNA markers linked to genetic traits and microbial typing (Blears *et al.*, 1998). The AFLP technique shows many advantages over other molecular DNA fingerprinting techniques, but probably the most important of these are the capacity to investigate a whole genome (Vos *et al.*, 1995; Lin *et al.*, 1996; Olive & Bean, 1999) for polymorphism. A further advantage of this technique is its reproducibility. AFLP can be applied to DNA enzymatically digested from larger human, animal and plant genomes to smaller microbial genomes (Blears *et al.*, 1998; Masiga *et al.*, 2000).

The principle of AFLP is based on the selective amplification of a subset of restriction fragments from a digest of mixed genomic DNA fragments using the PCR technique (Blears *et al.*, 1998; Van der Vossen *et al.*, 2003). This results in a unique, reproducible fingerprint for each individual (Mueller & Wolfenbarger, 1999). The markers that make up the fingerprint are widely distributed throughout the genome, allowing assessment of genome-wide variation. The anonymous markers are often concentrated in centromeric regions (Alonso-Blanco *et al.*, 1998; Saliba-Colombani *et al.*, 2000) and consist largely of non-coding DNA (Wong *et al.*, 2001; Shirasawa *et al.*, 2004). Molecular genetic polymorphisms are identified by the presence or the absence of DNA fragments following restriction and amplification of genomic DNA. The genomic

DNA is digested by two restriction enzymes, whereafter double-stranded oligonucleotides adapters are ligated to the DNA fragments. Oligonucleotide adapters are homologous to one 5'- or 3'-end generated during restriction digestion. The ligated DNA fragments are then amplified by PCR with the use of primers that are complimentary to the adapter and restriction site sequence with supplementary selective nucleotides at the 3'- end. Only the fragments that are completely matched, with complementary nucleotides extending beyond the restriction site will be amplified and this technique results in 30 – 40 DNA fragments, with some that are species specific and others that are strain specific (Janssen *et al.*, 1996; Jackson *et al.*, 1999; Melles *et al.*, 2007). With the use of selective primers the complexity of the mixture is reduced and the fragments are amplified by the selective primers under rigorous annealing conditions. Polymorphisms are shown by analysis of amplified fragments and by comparison of the pattern generated for each sample on a denaturing polyacrylamide gel (Bleas *et al.*, 1998).

AFLP is not sensitive to the DNA template concentration, but the technique procedure is affected by the quality of the DNA template. There are also some factors that may affect the reproducibility of the AFLP technique. Genomic DNA of a high purity is required to ensure complete digestion by the restriction endonucleases. High molecular weight (100 – 1000 ng) DNA that is not degraded, and that is free of contaminants or inhibitors that could interfere with digestion, ligation and amplification is essential for successful AFLP analysis (Vos *et al.*, 1995; Bensch & Åkesson, 2005; Benjak *et al.*, 2006). The incomplete restriction of DNA will produce partial fragments of a high molecular weight and amplification of fragments that are not fully digested produces an altered banding pattern, which may lead to the misinterpretation of the results (Vos *et al.*, 1995). The amplification reaction will stop when the labelled primer is consumed (Vos *et al.*, 1995) and this will ensure that fingerprints of equal intensity are produced although variations in the concentration of the DNA template may exist (Bleas *et al.*, 1998). At very high template dilutions the nucleotide sequences along the restriction site will not be random for a small pool of restriction fragments and variations in the banding patterns may occur.

AFLP show many advantages over other molecular DNA analysis techniques. Small sequence variations can be detected with the use of small quantities (0.05 – 0.5 µg) of genomic DNA. The ability to expose many polymorphic bands is a major advantage of AFLP markers and the numerous bands on a polyacrylamide gel can be analysed at the same time, making this technique very efficient. AFLP is also

advanced in the number of sequences amplified per reaction and the markers produced are consistent and reproducible within and between laboratories and are relatively easy and inexpensive to produce (Blears *et al.*, 1998).

Restriction fragment length polymorphism analysis of microbial populations in wine

The RFLP fingerprinting technique is one of the most sensitive methods used for strain identification and several bacterial strains have been studied using this technique. RFLP is a molecular technique that involves the isolation of DNA where after the DNA is cleaved by restriction enzymes at specific nucleotide sequences. The resulting DNA fragments that are obtained are separated by gel electrophoresis and the fragments are then transferred to either a nitrocellulose or nylon membrane (Deák, 1995). Different binding patterns (polymorphisms) may be observed after hybridisation with radioactively labelled known DNA sequences (probes). Probes are used to visualise a small portion of the genome and also allows comparison of similar sequences from the different samples. One or multiple probes that are specific for a certain sequence or gene are used to hybridise the membrane bound fragments (Hakovirta, 2008). The probes can also be labelled with enzyme-chemiluminescent substrates, enzyme-colorimetric substrates or detectable moieties, such as radioactive isotopes (Arbeit, 1995; Olive & Bean, 1999). Because of species and strain differences in the position of the restriction enzyme sites and with the specificity of the probe, the fingerprint is simplified (Hakovirta, 2008). The rDNA probe is applicable to a diversity of bacteria (Towner & Cockayne, 1993) and the use of the rRNA probe for characterisation is referred to as ribotyping. The probe can be either one of the rRNA genes or a mixture or parts of the rRNA genes and the spacer sequences, because the ribosomal operons in bacteria are organised into 16S, 23S and 5S rRNA and are often separated by non-coding spacer DNA (Towner & Cockayne, 1993). Labelled probes containing *Escherichia coli* 23S, 16S and 5S rRNA sequences are usually used for ribotyping (Saunders *et al.*, 1990).

The isolation of DNA in sufficient amounts for RFLP analysis is time-consuming and labour-intensive and PCR is often used to amplify small amounts of DNA (Masneuf *et al.*, 1996; Smole Mozina *et al.*, 1997). The technique is reproducible and (Baleiras Couto *et al.*, 1996) is useful for the characterisation of microbial species and strains (Coakley *et al.*, 1996).

Fernández *et al.* (1999) used PCR-RFLP to comparatively identify non-*Saccharomyces* yeast isolates from musts in spontaneous fermentation using physiological and molecular tests. The region between 18S and 28S rRNA genes was

amplified and the 47 non-*Saccharomyces* isolates produced nine different phenotypic profiles and 13 different genetic profiles. The results showed that PCR-RFLP can be more discriminating. PCR-RFLP can confirm identifications by phenotype, and in some cases to achieve intra-species differentiation.

Sato *et al.* (2000) used PCR-RFLP for the identification of LAB isolated from red wine and found that *O. oeni* strains showed unique RFLP patterns after *HaeIII*-digestion of 12 reference strains. These researchers concluded that PCR-RFLP could be used as a rapid and direct method for the identification of LAB in red wine and that analysis of the RFLP profile of 16S rRNA should enable a rapid control over the microbial population during MLF. It was also found that DNA isolation for subsequent PCR amplification is complicated due to the presence of interfering components in grape must.

DNA corresponding to 16S rDNA and the 16S-23S intergenic rDNA (ITS) from 22 reference strains of AAB and 24 indigenous AAB isolates from wine fermentations were analysed by Ruiz *et al.* (2000) using PCR-RFLPs. This technique is reliable and can be used to identify AAB at the genus level. PCR-RFLP of the 16S-23S rDNA ITS is not a useful method for the identification of AAB isolates at the species level, but it can be used for the detection of intraspecific differentiation.

Pulsed-field gel electrophoresis and the detection and identification of wine microbes

With the culture-independent molecular technique PFGE, chromosomal DNA is digested with restriction enzymes, which is then subjected to electrophoretic separation (Arbeit *et al.*, 1990; Finney, 1993; Kelly *et al.*, 1993). DNA macrorestriction analysis uses restriction enzymes that cuts the chromosomal DNA infrequently and generates a small number of restriction fragments (Sutton, 2005). Since, the DNA fragments are too large it can not be separated by gel electrophoresis (Hakovirta, 2008). The PFGE technique uses alternating electric fields that are consecutive and which will allow the DNA fragments to continuously change the direction of migration. The larger DNA fragments will change migration direction more slowly than smaller DNA fragments. As a new electric field is applied the DNA fragment will re-orient itself. The pulse time (ramping) and electron force (gradient) may constantly be increased to achieve better separation of all the different DNA fragment sizes (Towner & Cockayne, 1993). When a fingerprint pattern is obtained it can be compared to other microbial fingerprints and the DNA fragments can be analysed using size standards. PFGE has the capability of separating DNA molecules from 50 – 12 000 kilo base pairs (Towner & Cockayne,

1993) and can differentiate between species and strains making it extremely useful in epidemiological studies.

Guerrini *et al.* (2003) used PFGE to phenotypically and genotypically characterise *O. oeni* strains isolated from Italian wines. *Oenococcus oeni* is most commonly associated with MLF in wine, which can either have a positive or negative impact on the sensorial quality of wine. On the basis of *ApaI* PFGE restriction patterns, 84 isolates were grouped into five different clusters at 70% similarity, but no correlation was established with the phenotypic groups. The researchers combined the phenotypic and genotypic data and found a relationship between the 84 isolates of *O. oeni*, grouped into eight phenotypic-genotypic combined profiles, so that strain specificity could be predicted for each.

Wine yeasts play an extremely important role in winemaking and also influences the fermentation performance and quality of the wine end-product (Fleet & Heard, 1993). The identification of wine yeasts is often difficult to achieve and has been conducted by morphological and physiological properties, such as flocculation and film formation. Molecular methods have been developed for the differentiation of industrial yeast strains and Yamamoto *et al.* (1991) described the use of PFGE for the electrophoretic karyotyping of wine yeasts. These researchers examined the chromosomal DNA of 77 wine yeast strains by PFGE and found that the wine yeasts showed extensive variation in the PFGE patterns. The strains that showed different PFGE patterns could evidently be differentiated, but it could also be possible that the strains with the same PFGE patterns were the same or similar strains, if the PFGE was run on the same gel. The researchers also stated that further RFLP of genomic DNA and restrictive fingerprinting of mtDNA would be necessary for verification of the identification of the yeast strains. The researchers concluded that PFGE could be used as a reliable and valuable technique for the differentiation of yeast strains present wine.

Fluorescent in situ hybridisation analysis for microbial detection in wine

In molecular detection methods rDNA molecules are targeted, because they are universally distributed, contain conserved and variable sequence regions and are naturally amplified within microbial cells as integral parts of the ribosome.

The molecular technique, FISH uses short sequences of fluorescently labelled oligonucleotide probes for the detection of DNA within microbial cells and the degree of conservation of the probe target sequence determines the phylogenetic depth (Amann *et al.*, 1995). The FISH reaction is dependent on the hybridisation with a

complementary probe (Fugelsang & Edwards 2007). Microbes in environmental samples can be detected by using these probes with the recommended hybridisation conditions within a few hours after sampling when using epifluorescence or confocal laser scanning microscopy (Daims *et al.*, 2005). It is also recommended using more than one probe for the detection of microbes to ensure reliable results and multiple probe hybridisations is possible since probes labelled with different fluorescent dyes can be simultaneously applied during the analysis (Amann *et al.*, 1996). A positive FISH signal from a microbial cell in an environmental sample is used to identify the microbe, but the microbe may still be present in the sample even when there is not a FISH signal, because of the high detection limit of FISH. A requirement of $10^3 - 10^4$ cfu.ml⁻¹ per sample is necessary, but the detection limit can be lowered by a pre-enrichment step to induce growth of the microbes (Fang *et al.*, 2003). The FISH technique has the advantage of being carried out on a microscope slide with whole-cell preparations. When hybridisation is complete, the fluorescent molecules can be visualised and the location of the DNA molecule on the chromosome can be identified.

FISH has been used for the rapid monitoring of LAB (Sohier & Lonvaud-Funel, 1998; Blasco *et al.*, 2003), as well as for the detection of the spoilage yeast *Dekkera bruxellensis* (Stender *et al.*, 2001). Stender *et al.* (2001) used a new FISH method using peptide nucleic acid (PNA) probes for the identification of *Brettanomyces/Dekkera*. This method is based on fluorescein-labeled PNA probes that target a species-specific sequence of the rRNA of *Dekkera bruxellensis*. The researchers tested 127 different yeast strains, with 78 isolates of *Brettanomyces* from wine. The other yeast strains represented yeast species potentially found in wine, five type strains representing the five *Brettanomyces/Dekkera* species and 10 reference strains representing synonyms of *D. bruxellensis*. The results of this study showed that spoilage yeast *Brettanomyces* belongs to the species of *D. bruxellensis* and that this method of FISH can identify *Brettanomyces (D. bruxellensis)* with 100% sensitivity and 100% specificity.

Xufre *et al.* (2006) designed specific fluorescein-labelled oligonucleotide probes targeted to the D1/D2 region of the 26S rRNA of different yeast species that is commonly found in wine fermentations. The probes were used to identify isolates from wine musts, as well as the evolution of the yeast populations in two winery fermentations of white and red grape must. A diverse population of non-*Saccharomyces* species were detected in both studies. Strains isolated from industrial

musts were also used to perform two laboratory microvinifications in synthetic grape juice and similar results were obtained as in the previous study.

F. Conclusion

Winemaking involves the interaction between different microbes which influence the aroma and quality of the wine through fermentation. The spoilage of wine by yeasts, AAB and LAB cause severe economic losses for the wine industry. There are several methods for the detection and identification of spoilage microbes in wine. The current conventional culture-based techniques used to identify different microbes in wine are time consuming, expensive and may produce inaccurate and unreliable results. It is also often difficult to cultivate microbes since various microbes are known to be difficult to grow on synthetic growth media, even though the cells are viable.

Molecular methods are an attractive alternative to culture-based techniques since it provides more reliable and rapid results. The culture-independent PCR-DGGE technique is one of the most commonly used of molecular fingerprinting techniques. With PCR-DGGE it is possible to detect and identify spoilage microbes in wine and for the rapid analysis of diverse microbial populations present in the wine sample.

PCR-based DGGE analysis has enormous potential as a fingerprinting technique in the wine industry for microbial analysis, however, detection limits with relevant primer sets need to be determined before this technique can be used for routine testing. Furthermore, conditions for PCR-DGGE analysis also needs to be optimised for the primer sets to ensure that consistent and reliable results are obtained.

G. References

- Alexandre, H., Costello, P.J., Remize, F., Guzzo, J. & Guilloux-Benatier, M. (2004). *Saccharomyces cerevisiae*–*Oenococcus oeni* interactions in wine: current knowledge and perspectives. *International Journal of Food Microbiology*, **93**, 141-154.
- Alonso-Blanco, C., Peeters, A.J.M., Koorneef, M., Lister, C., Dean, C., van den Bosch, N., Pot, J. & Kuiper, M.T.R. (1998). Development of an AFLP based linkage map of *Ler*, *Col* and *Cvi* *Arabidopsis thaliana* ecotypes and construction of a *Ler/Cvi* recombinant inbred line population. *The Plant Journal*, **14**, 259-271.

- Amann, R.I., Ludwig, W. & Schleifer, K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews*, **59**, 143-169.
- Amann, R.I., Snaidr, J., Wagner, M., Ludwig, W. & Schleifer, K.H. (1996). *In situ* visualization of high genetic diversity in a natural microbial community. *Journal of Bacteriology*, **178**, 3496-3500.
- Augustyn, O.P.H., Ferreira, D. & Kock, J.L.F. (1991). Differentiation between yeast species, and strains within species by cellular fatty acids analysis. IV: *Saccharomyces sensu stricto*, *Hanseniaspora*, *Saccharomycodes* and *Wickerhamiella*. *Systematic and Applied Microbiology*, **14**, 324-334.
- Arbeit, R.D. (1995). Laboratory procedures for the epidemiologic analysis of microorganisms. In: *Manual of Clinical Microbiology* (edited by P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Tenover & R.H. Tenover), 6th ed. Pp. 190-208. Washington DC: American Society of Microbiology.
- Arbeit, R.D., Arthur, M., Dunn, R., Kim, C., Selander, R.K. & Goldstein, R. (1990). Resolution of recent evolutionary divergence among *Escherichia coli* from related lineages: the application of pulsed field gel electrophoresis to molecular epidemiology. *Journal of Infectious Diseases*, **161**, 230-235.
- Baleiras Couto, M.M., Hartog, B.J., Huis in't Veld, J.H.J., Hofstra, H. & van der Vossen, J.M. (1996). Identification of spoilage yeast in a food-production chain by microsatellite polymerase chain reaction fingerprinting. *Food Microbiology*, **13**, 59-67.
- Barnett, J.A., Payne, R.W. & Yarrow, D. (1990). *Yeasts: Characterisation and Identification*. Pp. 338-339, 379, 431-432, 460-461, 503-504, 595-597. Cambridge: Cambridge University Press.
- Bartowsky, E.J. & Henschke, P.A. (2004). The buttery attribute of wine – diacetyl – desirability, spoilage and beyond. *International Journal of Food Microbiology*, **96**, 235-252.
- Bartowsky, E.J. & Henschke, P.A. (2008). Acetic acid bacteria spoilage of bottled red wine – a review. *International Journal of Food Microbiology*, **125**, 60-70.
- Bartowsky, E.J., Xia, D., Gibson, R.L., Fleet, G.H. & Henschke, P.A. (2003). Spoilage of bottled red wine by acetic acid bacteria. *Letters in Applied Microbiology*, **36**, 307-314.
- Bauer, R. & Dicks, L.M.T. (2004). Control of malolactic fermentation in wine. A review. *South African Journal of Enology and Viticulture*, **25**, 274-288.

- Beltramo, C., Grandvalet, C., Pierre, F. & Guzzo, J. (2004). Evidence for multiple levels of regulation of *Oenococcus oeni clpP-clpL* locus expression in response to stress. *Journal of Bacteriology*, **186**, 2200-2205.
- Benjak, A., Konradi, J., Blauch, R. & Forneck, A. (2006). Different DNA extraction methods can cause different AFLP profiles in grapevine (*Vitis vinifera* L.). *Vitis*, **45**, 15-21.
- Bensch, S. & Åkesson, M. (2005). Ten years of AFLP in ecology and evolution: why so few animals? *Molecular Ecology*, **14**, 2899-2914.
- Berg, H.W., Filipello, F., Hinreiner, E. & Webb, A.D. (1995). Evaluation of thresholds and minimum difference concentrations for various constituents of wines, I, water solutions of pure substances. *Food Technology*, **9**, 23-26.
- Berry, D.R. & Watson, D.C. (1987). Production of organoleptic compounds. In: *Yeast Biotechnology* (edited by D.R. Berry, I. Russell & G.G. Stewart). Pp. 345-368. London: Allen & Unwin.
- Bisson, L.F. (1999). Stuck and sluggish fermentations. *American Journal of Enology and Viticulture*, **50**, 107-119.
- Blasco, L., Ferrer, S. & Pardo, I. (2003). Development of specific fluorescent oligonucleotides probes for *in situ* identification of wine lactic acid bacteria. *FEMS Microbiological Letters*, **225**, 115-123.
- Bleas, M.J., De Grandis, S.A., Lee, H. & Trevors, J.T. (1998). Amplified fragment polymorphism (AFLP): a review of the procedure and its applications. *Journal of Industrial Microbiology & Biotechnology*, **21**, 99-114.
- Boulton, R.B., Singleton, V.L., Bisson, L.F. & Kunkee, R.E. (1996). *Principles and Practices of Winemaking*. Pp. 132-133, 245, 247. New York: Chapman & Hall.
- Bousbouras, G.E. & Kunkee, R.E. (1971). Effect of pH on malo-lactic fermentation in wine. *American Journal of Enology and Viticulture*, **22**, 121-126.
- Caputi, A. & Peterson, R.G. (1965). The browning problems in wines. *American Journal of Enology and Viticulture*, **16**, 9-13.
- Ciani, M. & Picciotti, G. (1995). The growth kinetics and fermentation behaviour of some non-*Saccharomyces* yeasts associated with wine-making. *Biotechnology Letters*, **17**, 1247-1250.
- Coakley, M., Ross, R.P. & Donnelly, D. (1996). Application of the polymerase chain reaction to the rapid analysis of brewery yeast strains. *Journal of the Institute of Brewing*, **102**, 349-354.

- Cocolin, L. & Mills, D.A. (2003). Wine yeast inhibition by sulphur dioxide: a comparison of culture-dependent and independent methods. *American Journal of Enology and Viticulture*, **54**, 125-130.
- Cocolin, L., Bisson, L.F. & Mills, D.A. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiology Letters*, **189**, 81-87.
- Cocolin, L., Manzano, M., Rebecca, S. & Comi, G. (2002). Monitoring of yeasts population changes during a continuous wine fermentation by molecular methods. *American Journal of Enology and Viticulture*, **53**, 24-27.
- Cocolin, L., Rantsiou, K., Iacumin, L., Zironi, R. & Comi, G. (2004). Molecular detection and identification of *Brettanomyces/Dekkera bruxellensis* and *Brettanomyces/Dekkera anomalous* in spoiled wines. *Applied and Environmental Microbiology*, **70**, 1347-1355.
- Costello, P.J. & Henschke, P.A. (2002). Mousy off-flavor of wine: precursors and biosynthesis of the causative N-heterocycles 2-ethyltertrahydropyridine, 2-acetyltetrahydropyridine, and 2-acetyl-1-pyrroline by *Lactobacillus hilgardii* DSM 20176. *Journal of Agriculture and Food Chemistry*, **50**, 7079-7087.
- Coton, E., Rollan, G.C., Bertrand, A. & Lonvaud-Funel, A. (1998). Histamine-producing lactic acid bacteria in wines: early detection, frequency and distribution. *American Journal of Enology and Viticulture*, **49**, 199-204.
- Craig, J.T. & Heresztyn, T. (1984). 2-ethyl-3,4,5,6-tetrahydropyridine – an assessment of its possible contribution to the mousy off-flavours of wines. *American Journal of Enology and Viticulture*, **35**, 46-48.
- Crouigneau, A.A., Feuillat, M. & Guilloux-Benatier, M. (2000). Influence of some factors on autolysis of *Oenococcus oeni*. *Vitis*, **39**, 167-171.
- Daims, H., Stoecker, K. & Wagner, M. (2005). Fluorescence *in situ* hybridization for the detection of prokaryotes. In: *Molecular Microbial Ecology* (edited by A.M. Osborn & C.J. Smith). Pp. 213-222. UK: Taylor & Francis.
- Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H. & Fleet, G.H. (1985). Practical implications of malolactic fermentation – a review. *American Journal of Enology and Viticulture*, **36**, 290-301.
- Davis, C.R., Wibowo, D., Fleet, G.H. & Lee, T.H. (1988). Properties of wine lactic acid bacteria: their potential enological significance. *American Journal of Enology and Viticulture*, **39**, 137-142.
- Deák, T. (1993). Simplified techniques for identifying foodborne yeasts. *International Journal of Food Microbiology*, **19**, 15-26.

- Deák, T. (1995). Methods for the rapid detection and identification of yeasts in foods. *Trends in Food Science & Technology*, **6**, 287-292.
- De Ley, J., Swings, J. & Gossele, F. (1984). Genus I. *Gluconobacter beijerinck* (1898). In: *Bergey's Manual of Systematic Bacteriology*, Volume 1, 9th ed. Pp. 268-274. Baltimore: Williams and Wilkens.
- De Ory, I., Romero, L.E. & Cantero, D. (1998). Modeling the kinetics of growth of *Acetobacter aceti* in discontinues culture: influence of the temperature of operation. *Applied Microbiology and Biotechnology*, **49**, 189-193.
- Drysdale, G.S. & Fleet, G.H. (1988). Acetic acid bacteria in winemaking: a review. *American Journal of Enology and Viticulture*, **39**, 143-154.
- Drysdale, G.S. & Fleet, G.H. (1989a). The growth and survival of acetic acid bacteria in wines at different concentrations of oxygen. *American Journal of Enology and Viticulture*, **40**, 99-105.
- Drysdale, G.S. & Fleet, G.H. (1989b). The effect of acetic acid bacteria upon the growth and metabolism of yeast during the fermentation of grape juice. *American Journal of Applied Bacteriology*, **67**, 471-481.
- Du Plessis, H.W., Dicks, L.M.T., Pretorius, I.S., Lambrechts, M.G. & du Toit, M. (2004). Identification of lactic acid bacteria isolated from South African brandy base wines. *International Journal of Food Microbiology*, **91**, 19-29.
- Du Toit, W.J. & Pretorius, I.S. (2000). Microbial spoilage and preservation of wine: using weapons from nature's own arsenal – a review. *South African Journal of Enology and Viticulture*, **21**, 74-96.
- Du Toit, W.J. & Pretorius, I.S. (2002). The occurrence, control and esoteric effect of acetic acid bacteria in winemaking. *Annals of Microbiology*, **52**, 155-179.
- Du Toit, W.J. & Lambrechts, M.G. (2002). The enumeration and identification of acetic acid bacteria from South African red wine fermentations. *International Journal of Food Microbiology*, **74**, 57-64.
- Du Toit, W.J., Pretorius, I.S. & Lonvaud-Funel, A. (2005). The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated form wine. *Applied and Environmental Microbiology*, **98**, 862-871.
- Edinger, W.D. & Splittstoesser, D.F. (1986). Production by lactic acid bacteria of sorbic alcohol, the precursor of the geranium odor compound. *American Journal of Enology and Viticulture*, **37**, 34-38.

- Edwards, C.G., Beelman, R.B., Bartley, C.E. & McConell, A.L. (1990). Production of decanoic acid and other volatile compounds and the growth of yeast and malolactic bacteria during vinification. *American Journal of Enology and Viticulture*, **41**, 48-56.
- Eglinton, J. & Henschke, P. (1999). The occurrence of volatile acidity in Australian wines. *Australian Grapegrower and Winemaker*, Annual technical issue, 7-12.
- Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, **56**, 297-314.
- Erten, H. (2002). Relations between elevated temperatures and fermentation behaviour of *Kloeckera apiculata* and *Saccharomyces cerevisiae* associated with winemaking in mixed cultures. *World Journal of Microbiology and Biotechnology* **18**, 373-378.
- Fang, Q., Brockmann, S., Botzenhart, K. & Wiedenmann, A. (2003). Improved detection of *Salmonella* spp in foods by fluorescent in situ hybridization with 23S rRNA probes: a comparison with conventional culture methods. *Journal of Food Protection*, **66**, 723-731.
- Faria, M.A., Magalhaes, R., Ferreira, M.A., Meredith, C.P. & Ferreira-Monteiro, F. (2000). *Vitis vinifera* must varietal authentication using microsatellite DNA analysis (SSR). *Journal of Agricultural Food Chemistry*, **48**, 1096-1100.
- Felske, A., Rheims, H., Wolterink, A., Stackebrandt, E. & Akkermans, A.D.L. (1997). Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiology*, **143**, 2983-2989.
- Fernández, M.T., Ubeda, J.F. & Briones, A.I. (1999). Comparative study of non-*Saccharomyces* microflora of musts in fermentation, by physiological and molecular methods. *FEMS Microbiology Letters*, **173**, 223-229.
- Finney, M. (1993). Pulsed-field gel electrophoresis. In: *Current Protocols in Molecular Biology* (edited by F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith & K. Struhl), Volume 1, Pp. 2.5.9-2.5.17. New York: Green-Wiley.
- Fleet, G.H. (1990). Growth of yeasts during wine fermentation. *Journal of Wine Research*, **1**, 211-223.
- Fleet, G.H. (1993). The microorganisms of winemaking – isolation, enumeration and identification. In: *Wine Microbiology and Biotechnology* (edited by G.H. Fleet). Pp. 1-25. Switzerland: Harwood Academic Publishers.

- Fleet, G.H. (1998). The microbiology of alcoholic beverages. In: *Microbiology of Fermented Foods* (edited by J.B. Wood), Volume 1, 2nd ed. Pp. 217-262. London: Blackie Academic & Professional.
- Fleet, G.H. (1999). Microorganisms in food ecosystems. *International Journal of Food Microbiology*, **50**, 101-117.
- Fleet, G.H. (2000). Schizosaccharomyces. *Applied and Environmental Microbiology*, **50**, 727-728.
- Fleet, G.H. (2003a). Yeasts in fruit and fruit products. In: *Yeasts in Food. Beneficial and Detrimental Aspects* (edited by T. Boekhout & V. Robert). Pp. 267-288. Cambridge: Woodhead Publishing.
- Fleet, G.H. (2003b). Yeast interactions and wine flavour. *International Journal of Food Microbiology*, **86**, 11-22.
- Fleet, G.H. (2007). Wine. In: *Food Microbiology Fundamentals and Frontiers* (edited by M.P. Doyle & L.R. Beuchat), 3rd ed. Pp. 863-890. Washington DC: ASM Press.
- Fleet, G. H. & Heard, G.M. (1993). Yeast-growth during fermentation. In: *Wine Microbiology and Biotechnology* (edited by G.H. Fleet). Pp. 27-54. Switzerland: Harwood Academic Press.
- Fleet, G.H., Lafon-Lafourcade, S. & Ribéreau-Gayon, P. (1984). Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Applied and Environmental Microbiology*, **48**, 1034-1038.
- Fleet, G.H., Prakitchaiwattana, C., Beh, A.L. & Heard, G. (2002). The yeast ecology of wine grapes. In: *Biodiversity and Biotechnology of Wine Yeasts* (edited by M. Ciani). Pp. 1-17. India: Research Signpost.
- Fornachon, J.C.M. (1968). Influence of different yeasts on growth of lactic acid bacteria in wine. *Journal of the Science of Food and Agriculture*, **19**, 374-378.
- Fugelsang, K.C. (1996). *Zygosaccharomyces*, a spoilage yeast isolated from wine. *Viticulture and Enology Research Center*, CATI publication, August 1996.
- Fugelsang, K.C. (1998). *Zygosaccharomyces*, a spoilage yeast isolated from grape juice. *Viticulture and Enology Research Center*, CATI publication, September 1998.
- Fugelsang, K.C. & Edwards, C.G. (2007). *Wine Microbiology, Practical Applications and Procedures*, 2nd ed. Pp. 3-19, 29-35, 45-47, 83-101, 163-179. New York: Springer Science and Business Media.

- García-Beneytez, E., Moreno-Arribas, M.V., Borrego, J., Polo, M.C. & Ibez, J. (2002). Application of a DNA analysis method for the cultivar identification of grape musts and experimental and commercial wines of *Vitis vinifera* L. using microsatellite markers. *Journal of Agricultural Food Chemistry*, **50**, 6090-6096.
- Garrity, G.M., Bell, J.A. & Lilburn, T.G. (2004). Taxonomic outline of the prokaryotes. In: *Bergey's Manual of Systematic Bacteriology* (edited by N.R. Krieg & J.G Holt). New York: Springer-Verlag.
- Gindreau, E., Walling, E. & Lonvaud-Funel, A. (2001). Direct polymerase chain reaction detection of ropy *Pediococcus damnosus* strains in wine. *Journal of Applied Microbiology*, **90**, 535-542.
- Giraffa, G. & Neviani, E. (2001). DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. *International Journal of Food Microbiology*, **67**, 19-34.
- González, A., Hierro, N., Poblet, M., Mas, A. & Guillamón, J.M. (2005). Application of molecular methods to demonstrate species and strain evolution of acetic acid bacteria population during wine production. *International Journal of Food Microbiology*, **102**, 295-304.
- Gracias, K.S. & McKillip, J.L. (2004). A review of conventional detection and enumeration methods for pathogenic bacteria in food. *Canadian Journal of Microbiology*, **50**, 883-890.
- Guerrini, S., Bastianini, A., Blaiotta, G., Granchi, L., Moschetti, G., Coppola, S., Romano, P. & Vincenzini, M. (2003). Phenotypic and genotypic characterization of *Oenococcus oeni* strains isolated from Italian wines. *International Journal of Food Microbiology*, **83**, 1-14.
- Hagens, S. & Loesner, M.J. (2007). Application of bacteriophages for detection and control of foodborne pathogens. *Applications of Microbiological Technology*, **76**, 513-519.
- Hakovirta, J. (2008). Modern techniques in detection, identification and quantification of bacteria and peptides from foods. PhD in Microbiology Thesis, University of Helsinki, Finland.
- Head, I.M., Saunders, J.R. & Pickup, R.W. (1998). Microbial evolution, diversity, and ecology: a decade of ribosomal analysis of uncultivated microorganisms. *Microbial Ecology*, **35**, 1-21.
- Heard, G.M. & Fleet, G.H. (1985). Growth of natural yeast flora during the fermentation of inoculated wines. *Applied and Environmental Microbiology*, **50**, 727-728.

- Heard, G.M. & Fleet, G.H. (1988). The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. *Journal of Applied Bacteriology*, **65**, 23-28.
- Henick-Kling, T. (1993). Malolactic fermentation. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 289-326. Switzerland: Harwood Academic Publishers.
- Henick-Kling, T. (1995). Control of malolactic fermentation in wine: energetics, flavour, modification, and methods of starter culture preparation. *Journal of Applied Bacteriology, Supplement*, **79**, 29S-37S.
- Henick-Kling, T., Egli, C., Licker, J., Mittrakul, C. & Acree, T.E. (2000). *Brettanomyces* in wine. In: *Proceedings of the 5th International Symposium on Cool Climate Viticulture and Oenology*. Melbourne, Australia, 16-20 January.
- Heresztyn, T. (1986). Formation of substituted tetrahydropyridines by species of *Brettanomyces* and *Lactobacillus* isolated from mousy wines. *American Journal of Enology and Viticulture*, **37**, 127-132.
- Hernán-Gómez, S., Espinosa, J.C. & Ubeda, J.F. (2000). Characterization of wine yeasts by temperature gradient gel electrophoresis (TGGE). *FEMS Microbiology Letters*, **193**, 45-50.
- Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. & Williams, S.T. (1994). Genus *Acetobacter* and *Gluconobacter*. In: *Bergey's Manual of Determinative Bacteriology*, 9th ed. Pp. 71-84. Maryland: Williams & Wilkens.
- Hugenholtz, P. & Pace, N.R. (1996). Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. *Trends in Biotechnology*, **14**, 90-97.
- Hugenholtz, P., Goebel, B.M. & Pace, N.R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology*, **180**, 4765-4774.
- Hurst, A. (1977). Bacterial injury: a review. *Canadian Journal of Microbiology*, **23**, 936-944.
- Izquierdo, P.M., Briones, A.I. & Ubeda, J.F. (1996). Relationship between the chromosomal profile and whole-cell protein patterns of wine yeast strains. *Lebensmittel-Wissenschaft und Technologie*, **29**, 738-742.

- Jackson, P.J., Hill, K.K., Laker, M.T., Ticknor, L.O. & Keim, P. (1999). Genetic comparison of *Bacillus anthracis* and its close relatives using amplified fragment length polymorphism and polymerase chain reaction analysis. *Journal of Applied Microbiology*, **87**, 263-269.
- Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M. & Kersters, K. (1996). Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology*, **142**, 1881-1893.
- Jolly, N.P., Augustyn, O.P.H. & Pretorius, I.S. (2006). The role and use of non-*Saccharomyces* yeasts in wine production. *South African Journal of Enology and Viticulture*, **27**, 15-39.
- Joyeux, A., Lafon-Lafourcade, S. & Ribéreau-Gayon, P. (1984a). Evolution of acetic acid bacteria during fermentation and storage of wine. *Applied and Environmental Microbiology*, **48**, 153-156.
- Kell, P., Kalif, A., Schupp, J., Hill, K., Travis, S.E., Richmond, K., Adair, D.M., Hugh-Jones, M., Kuske, C.R. & Jackson, P.J. (1998). Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie Van Leeuwenhoek*, **73**, 169-197.
- Kelly, W.J., Huang, C.M. & Asmundson, R.V. (1993). Comparison of *Leuconostoc oenos* strains by pulsed-field gel electrophoresis. *Applied and Environmental Microbiology*, **59**, 3969-3972.
- Kopke, C., Cristovão, A., Prata, A.M., Silva Pereira, C., Figueiredo Marques, J.J. & San Romão, M.V. (2000). Microbiological control of wine. The application of epifluorescence microscopy method as a rapid technique. *Food Microbiology*, **17**, 257-260.
- Kunkee, R.F. (1984). Selection and modification of yeasts and lactic acid bacteria for wine fermentation. *Food Microbiology*, **1**, 315-332.
- Kunkee, R.F. (1991). Some roles of malic acid in the malolactic fermentation in wine making. *FEMS Microbiology Review*, **88**, 55-72.
- Kretzer, J.W., Lehman, R., Schmelcher, M., Banz, M. Kim, K.P., Korn, C. & Loesner, M.J. (2007). Use of high-affinity cell wall-binding domains of bacteriophage endolysins for immobilization and separation of bacterial cells. *Applications of Environmental Microbiology*, **73**, 1992-2000.
- Lafon-Lafourcade, S., (1983). Wine and brandy. In: *Biotechnology, Food and Feed Production with Microorganisms* (edited by G. Reed), Volume 5. Pp. 81-164. Weinheim: Verlag Chemie.

- Lafon-Lafourcade, S., Geneix, C. & Ribéreau-Gayon, P. (1984). Inhibition of alcoholic fermentation of grape must by fatty acids produced by yeasts and their elimination by yeast ghosts. *Applied and Environmental Microbiology*, **47**, 1246-1249.
- Lambrechts, M.G. & Pretorius, I.S. (2000). Yeast and its importance to wine aroma – a review. *South African Journal of Enology and Viticulture*, **21**, 97-129.
- Lerman, L.S., Fischer, S.G., Hurley, I., Silverstein, K. & Lumelsky, N. (1984). Sequence determined DNA separations. *Annual Review of Biophysics and Bioengineering*, **13**, 399-423.
- Lin J.-J., Kuo, J. & Ma, J. (1996). A PCR-based DNA fingerprinting technique: AFLP for molecular typing of bacteria. *Nucleic Acids Research*, **24**, 3649-3650.
- Liu, S.-Q. (2002). Malolactic fermentation in wine – beyond deacidification. *Journal of Applied Microbiology*, **92**, 589-601.
- Lodhi, M.A., Ye, G.N., Weeden, N.F. & Reisch, B.I. (1994). A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and *Ampelopsis*. *Plant Molecular Biology Reporter*, **12**, 6-13.
- Lonvaud-Funel, A. (1999). Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie van Leeuwenhoek*, **76**, 317-331.
- Lonvaud-Funel, A. (2001). Biogenic amines in wines: role of lactic acid bacteria. *FEMS Microbiology Letters*, **199**, 9-13.
- Lonvaud-Funel, A. & Joyeux, A. (1993). Antagonisms between lactic acid bacteria of wines: inhibition of *Leuconostoc oenos* by *Lactobacillus plantarum* and *Pediococcus pentosaceus*. *Food Microbiology*, **10**, 411-419.
- Lonvaud-Funel, A., Joyeux, A. & Dessens, C. (1988). Inhibition of malolactic fermentation of wines by products of yeast metabolism. *Journal of the Science of Food and Agriculture*, **44**, 183-191.
- Lopez, I., Ruiz-Larrea, F., Cocolin, L., Orr, E., Phister, T., Marshall, M., VanderGheynst, J. & Mills, D.A. (2003). Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, **69**, 6801-6807.
- Loureiro, V. (2000). Spoilage yeasts in foods and beverages: characterisation and ecology for improved diagnosis and control. *Food Research International*, **33**, 247-256.
- Loureiro, V. & Malfeito-Ferreira, M. (2003). Spoilage yeasts in the wine industry. *International Journal of Food Microbiology*, **86**, 23-50.

- Malherbe, S., Bauer, F.F. & du Toit, M. (2007). Understanding problem fermentations – a review. *South African Journal of Enology and Viticulture*, **28**, 169-186.
- Manzano, M., Cocolin, L., Iacumin, L., Cantoni, C. & Comi, G. (2005). A PCR-TGGE (temperature gradient gel electrophoresis) technique to assess differentiation among enological *Saccharomyces cerevisiae* strains. *International Journal of Food Microbiology*, **101**, 333-339.
- Matsushita, K., Toyama, H. & Adachi, O. (1994). Respiratory chains and bioenergetics of acetic acid bacteria. *Advances in Microbial Physiology*, **36**, 247-301.
- Margalith, P.Z. (1981). *Flavor Microbiology*. Pp. 173-224. Illinois: Charles C. Thomas.
- Markides, A. (1993). Factors influencing the growth of malolactic bacteria and malolactic activity in wine – interactions between wine yeast and lactic acid bacteria. *Australian Grapegrower and Winemaker*, **352**, 108-111.
- Martini, A. & Vaughan-Martini, A. (1990). Grape must fermentation: past and present. In: *Yeast Technology* (edited by J.F.T.D. Spencer & M. Spencer). Pp. 105-123. Berlin: Springer Verlag.
- Martinez, G., Barker, H.A. & Horecker, B.L. (1963). Specific mannitol dehydrogenase from *Lactobacillus brevis*. *Journal of Biological Chemistry*, **238**, 1598-1603.
- Masiga, D.K., Tait, A. & Turner, C.M.R. (2000). Amplified fragment length polymorphism in parasite genetics. *Paritology Today*, **16**, 350-353.
- Masneuf, I., Aigle, M. & Dubourdieu, D. (1996). Development of a polymerase chain reaction/restriction fragment length polymorphism method for *Saccharomyces cerevisiae* and *Saccharomyces bayans* identification in enology. *FEMS Microbiology Letters*, **138**, 239-244.
- McDonald, S., Punt, C. & Bhanisi, S. (2006). The impact of an increase in wine industry export on the SA economy, focusing on the Western Cape. *PROVIDE Project Working Paper 2006: 3*, Elsenburg, South Africa.
- Melles, D.C., van Leeuwen, W.B., Snijders, S.V., Horst-Kreft, D., Peeters, J.K., Verbrugh, H.A. & van Belkum, A. (2007). Comparison of multilocus typing (MLST), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) for genetic typing of *Staphylococcus aureus*. *Journal of Microbiological Methods*, **69**, 371-375.
- Millet, V. & Lonvaud-Funel, A. (2001). The viable but non-culturable state of wine microorganisms during storage. *Letters of Applied Microbiology*, **30**, 136-141.

- Monteiro, F.F. & Bisson, L.F. (1991). Biological assay of nitrogen content of grape juice and prediction of sluggish fermentations. *American Journal of Enology and Viticulture*, **42**, 47-57.
- Mora, J. & Mulet, A. (1991). Effect of some treatments of grape juice on the population and growth of yeast species during fermentation. *American Journal of Enology and Viticulture*, **42**, 133-136.
- Moreno-Arribas, M.V., Polo, M.C., Jorganes, F. & Munoz, R. (2003). Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. *International Journal of Food Microbiology*, **84**, 117-123.
- Mueller, U.G. & Wolfenbarger, L.L. (1999). AFLP genotyping and fingerprinting. *Trends in Ecology Evolution*, **14**, 389-394.
- Muyzer, G. (1999). DGGE/TGGE a method for identifying genes from natural ecosystems. *Current Opinion in Microbiology*, **2**, 317-322.
- Muyzer, G. & Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) on microbial ecology. *Antonie van Leeuwenhoek*, **73**, 127-141.
- Muyzer, G., de Waal, E.C. & Uitterlinden, A.G., (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, **59**, 695-700.
- Noble, A.C. & Bursick, G.F. (1984). The contribution of glycerol to perceived viscosity and sweetness in white wine. *American Journal of Enology and Viticulture*, **35**, 110-112.
- Olive, D.M. & Bean, P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. *Journal of Clinical in Microbiology*, **37**, 1661-1669.
- Patynowski, R.J., Jiranek, V. & Markides, A. (2002). Yeast viability during fermentation and *sur lie* ageing of a defined medium and subsequent growth of *Oenococcus oeni*. *Australian Journal of Grape and Wine Research*, **8**, 62-69.
- Peynaud, E. & Domercq, S. (1959). A review of microbial problems in wine-making in France. *American Journal of Enology and Viticulture*, **10**, 69-77.
- Prakitchaiwattana, C.J., Fleet, G.H. & Heard, G.M. (2004). Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Research*, **4**, 865-877.
- Pretorius, I.S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast*, **16**, 675-729.

- Rankine, B. (1995). Microbiology and fermentation. In: *Making Good Wine – A Manual of Winemaking Practice for Australia and New Zealand*. Pp. 118-130. Sydney: Pan Macmillan.
- Rantsiou, K. & Cocolin, L. (2006). New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: a review. *International Journal of Food Microbiology*, **108**, 255-267.
- Rapp, A. & Versini, G. (1991). Influence of nitrogen compounds in grapes and aroma compounds in wine. In: *Proceedings of the International Symposium on Nitrogen in Grapes and Wines* (edited by J. Rantz). Pp. 156-164. Davis, California, American Society for Enology and Viticulture.
- Ray, B. (1986). Impact of bacterial injury and repair in food microbiology: its past, present and future. *Journal of Food Protection*, **49**, 651-655.
- Renouf, V. & Lonvaud-Funel, A. (2007). Development of an enrichment medium to detect *Dekkera/Brettanomyces bruxellensis*, a spoilage wine yeast, on the surface of grape berries. *Microbiological Research*, **162**, 154-167.
- Renouf, V., Claisse, O., Miot-Sertier, C. & Lonvaud-Funel, A. (2006). Lactic acid bacteria evolution during winemaking: use of rpoB gene as a target for PCR-DGGE analysis. *Food Microbiology*, **23**, 136-145.
- Renouf, V., Strehaiano, P. & Lonvaud-Funel, A. (2008). Effectiveness of dimethyldicarbonate to prevent *Brettanomyces bruxellensis* growth in wine. *Food Control*, **19**, 208-216.
- Ribéreau-Gayon, P. (1985). New developments in wine microbiology. *American Journal of Enology and Viticulture*, **36**, 1-10.
- Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. & Lonvaud-Funel, A. (2000). *Handbook of Enology, The Microbiology of Wine and Vinifications* (edited by P. Ribéreau-Gayon), Volume 1. Pp. 149-169. Chichester: John Wiley & Sons.
- Riesner, D., Henco, K. & Steger, G. (1991). Temperature-gradient gel electrophoresis: a method for the analysis of conformational transitions and mutations in nucleic acids and proteins. In: *Advances in Electrophoresis* (edited by A. Chrambach, M.J. Dunn & B.J. Radola), Volume 4. Pp. 169-250. Weinheim: VCH Verlagsgesellschaft.
- Romano, P. & Suzzi, G. (1993). Potential use for *Zygosaccharomyces* species in winemaking. *Australian Journal of Grape and Wine Research*, **4**, 89-94.

- Romano, P., Suzzi, G., Zironi, R. & Comi, G. (1993). Biometric study of acetoin production in *Hanseniaspora guilliermondii* and *Kloeckera apiculata*. *Applied and Environmental Microbiology*, **59**, 1838-1841.
- Romano, P., Fiore, C., Paraggio, M., Caruso, M. & Capece, A. (2003). Function of yeast species and strains in wine flavour. *International Journal of Food Microbiology*, **86**, 169-180.
- Rossi, J.A. & Singleton, V.L. (1966). Contribution of grape phenolics to browning and oxygen absorption in wines. *American Journal of Enology and Viticulture*, **17**, 231-233.
- Ruiz, A., Poblet, M., Mas, A. & Guillamón, J.M. (2000). Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. *International Journal of Systematic and Evolutionary Microbiology*, **50**, 1981-1987.
- Saeki, A., Theeragool, G., Matsushita, K., Toyama, H., Lotong, N. & Adachi, O. (1997). Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at higher temperatures. *Bioscience, Biotechnology, and Biochemistry*, **61**, 138-145.
- Saliba-Colombani, V., Causse, M., Gervais, L. & Philouze, J. (2000). Efficiency of RFLP, RAPD, and AFLP markers for the construction of an intraspecific map of the tomato genome. *Genome*, **43**, 29-40.
- SARS (2005). South African Postal Code Data for 2005. South African Revenue Service, Pretoria.
- Sato, H., Yanagida, F., Shinohara, T. & Yokotsuka, K. (2000). Restriction fragment length polymorphism analysis of 16S rRNA genes in lactic acid bacteria isolated from red wine. *Journal of Bioscience and Bioengineering*, **90**, 335-337.
- Satokari, R.M., Vaughan, E.E., Smidt, H., Saarela, M., Mättö, J. & de Vos, W.M. (2003). Molecular approaches for the detection and identification of Bifidobacteria and Lactobacilli in the human gastrointestinal tract. *Systematic and Applied Microbiology*, **26**, 572-584.
- Saunders, N.A., Harrison, T.G., Haththotuwa, A., Kachwalla, N. & Taylor, A.G. (1990). A method for typing strains of *Legionella pneumophila* serogroup I by analysis of restriction fragment length polymorphism. *Journal of Medical Microbiology*, **31**, 45-55.
- SAWIS (2006). South African Wine Industry Statistics No 30. South African Wine Industry Information and Systems (SAWIS), Paarl.

- Shimazu, Y. & Watanabe, M. (1981). Effects of yeast strains and environmental conditions on forming of organic acids in must during fermentation. *Journal of Fermentation Technology*, **59**, 27-32.
- Shirasawa, K., Kishitani, S. & Nishio, T. (2004). Conversion of AFLP markers to sequence-specific markers for closely related lines in rice by use of the rice genome sequence. *Molecular Breeding*, **14**, 283-292.
- Sigler, W.V., Miniaci, C. & Zeyer, J. (2004). Electrophoresis time impacts the denaturing gradient gel electrophoresis-based assessment of bacterial community structure. *Journal of Microbiological Methods*, **57**, 17-22.
- Siret, R., Boursiquot, J.M., Merle, M.H., Cabanis, J.C. & This, P. (2000). Toward the authentication of varietal wines by the analysis of grape (*Vitis vinifera* L.) residual DNA in must and wine using microsatellite markers. *Journal of Agricultural Food Chemistry*, **48**, 5035-5040.
- Smith, M.T. (1998a). *Dekkera*. In: *The Yeasts, A Taxonomic Study* (edited by C.P. Kurtzman & J.W. Fell), 4th ed. Pp. 174-177. NewYork: Elsevier Science Publisher.
- Smith, M.T. (1998b). *Brettanomyces*. In: *The Yeasts, A Taxonomic Study* (edited by C.P. Kurtzman & J.W. Fell), 4th ed. Pp. 450-453. NewYork: Elsevier Science Publisher.
- Smole Mozina, S., Dlačhy, D., Deák, T. & Raspor, P. (1997). Identification of *Saccharomyces sensu stricto* and *Torulaspota* yeast by PCR ribotyping. *Letters in Applied Microbiology*, **24**, 311-315.
- Sohier, D. & Lonvaud-Funel, A. (1998). Rapid and sensitive *in situ* hybridization method for detecting and identifying lactic acid bacteria in wine. *Food Microbiology*, **15**, 391-397.
- Soles, R.M., Ough, C.S. & Kunkee, R.E. (1982). Ester concentration difference in wine fermented by various species and strains of yeast. *American Journal of Enology and Viticulture*, **33**, 94-98.
- Sponholz, W. R. (1993). Wine spoilage by microorganisms. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 395-413. Switzerland: Harwood Academic Publishers.

- Stender, H., Kurtzman, C., Hyldig-Nielsen, J., Sørensen, D., Broomer, A., Oliveira, K., Perry-O'Keefe, H., Sage, A., Young, B. & Coull, J. (2001). Identification of *Dekkera bruxellensis* (*Brettanomyces*) from wine by fluorescence in situ hybridization using peptide nucleic acid probes. *Applied and Environmental Microbiology*, **67**, 938-941.
- Steenkamp, J., Wiid, I., Lourens, A. & van Helden, P. (1994). Improved method for DNA extraction from *Vitis vinifera*. *American Journal of Enology and Viticulture*, **45**, 102-106.
- Suaréz, R., Suaréz-Lepe, J.A., Morata, A. & Claderón, F. (2007). The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: a review. *Food Chemistry*, **102**, 10-21.
- Sutton, S.V.W. (2005). Pulsed-field gel electrophoresis – a new genetic technology for microbial identification. *Rapid Microbiology Newsletter*, **4**, Issue 1.
- Thomas, S.D. (1993). Yeasts as spoilage organisms in beverages. In: *The Yeasts, Yeast Technology* (edited by A. Rose & J. Harrison), Volume 5, 2nd ed. Pp. 517-561. London: Academic Press.
- Thomas, S.D. & Davenport, R.R. (1985). *Zygosaccharomyces bailii* – a profile of characteristics and spoilage activities. *Food Microbiology*, **2**, 157-169.
- Towner, K.J. & Cockayne, A. (1993). *Molecular Methods for Microbial Identification and Typing*, 1st ed. Pp. 1-202. London: Chapman & Hall.
- 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.
- Van der Vossen, J.M., Rahaoui, H., de Nus, M. & Hartog, B. (2003). PCR methods for tracing and detection of yeasts in the food chain. In: *Yeasts in Food, Beneficial and Detrimental Aspects* (edited by T. Boekhout & V. Robert). Pp. 123-138. Cambridge: Woodhead Publishers .
- Van der Walt, J.P. & van Kerken, A.E. (1958). The wine yeasts of the Cape. Part V. Studies on the occurrence of *Brettanomyces intermedius* and *B. schanderlii*. *Antonie van Leeuwenhoek*, **27**, 81-90.
- Vos, P., Hogers, R., Bleeker, M., Rijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. & Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acid Research*, **23**, 4407-4414.

- Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H. & Lee, T.H. (1985). Occurrence and growth of lactic acid bacteria in wines: a review. *American Journal of Enology and Viticulture*, **36**, 302-313.
- Wibowo, D., Fleet, G.H., Lee, T.H. & Eschenbruch, R. (1988). Factors affecting the induction of malolactic fermentation in red wines with *Leuconostoc oenos*. *Journal of Applied Bacteriology*, **64**, 421-428.
- Wong, A., Forbes, M.R. & Smith, M.L. (2001). Characterization of AFLP markers in damselfies: prevalence of codominant markers and implications for population genetic applications. *Genome*, **44**, 677-684.
- Xufre, A., Albergaria, H., Inácio, J., Spencer-Martins, I. & Gírio, F. (2006). Application of fluorescence in situ hybridisation (FISH) to the analysis of yeast population dynamics in winery and laboratory grape must fermentation. *International Journal of Food Microbiology*, **108**, 376-384.
- Yamamoto, N., Yamayoto, N., Amemiya, H., Yokomori, Y., Shimizu, K. & Totsuka, A. (1991). Electrophoretic karyotypes of wine yeasts. *American Journal of Enology and Viticulture*, **42**, 358-363.
- Zhao, T. & Doyle, M. P. (2001). Evaluation of universal pre-enrichment broth for growth of heat-injured pathogens. *Journal of Food Protection*, **64**, 1751-1755.
- Zoecklein, B.W., Fugelsang, K.C., Gump, B.H. & Nury, F.S. (1995). *Wine Analysis and Production*. Pp. 115-151, 179-19, 221-222, 282-286, 328-329, New York: Chapman & Hall.

CHAPTER 3

PCR-BASED DGGE OPTIMISATION AND DETECTION LIMITS FOR SPOILAGE
MICROBES IN WINE**Abstract**

In this study the culture-independent technique, polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) was investigated for the early detection and identification of possible spoilage microbes in wine. PCR and DGGE conditions were successfully optimised with the universal primer pair, HDA1-GC and HDA2, the wine bacteria specific primer pair, WBAC1-GC and WBAC2 and the yeast specific primer pair, NL1-GC and LS2. Three DNA isolation methods were compared and it was determined that the TZ-method produced the best results in terms of reliability, consistency and also the simplicity of the technique. PCR and DGGE detection limits were successfully determined for the reference microbes, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Acetobacter pasteurianus* and *Brettanomyces bruxellensis* when each microbe was separately inoculated at 10^6 cfu.ml⁻¹ into sterile saline solution (SSS) (0.85% (m/v) NaCl) and sterile white wine. The PCR detections were more sensitive ($10^1 - 10^2$ cfu.ml⁻¹) than the DGGE detections ($10^1 - 10^4$ cfu.ml⁻¹), with the exception of *B. bruxellensis* that had higher PCR and DGGE detection limits than the other reference microbes. PCR and DGGE detection limits were then determined for the inoculation of *Lb. plantarum*, *A. pasteurianus* and *B. bruxellensis* at a concentration of 10^8 cfu.ml⁻¹ as part of mixed populations in SSS and sterile white wine. PCR detection limits of 10^1 cfu.ml⁻¹ were determined for all three reference microbes inoculated as part of mixed populations when amplified with the HDA1-GC and HDA2 primer pair and the WBAC1-GC and WBAC2 primer pair. The DGGE detection limits were higher when the reference microbes were inoculated as part of mixed populations than when the microbes were inoculated as single microbial strains. DGGE conditions were optimised for the reference wine microbes, *Lb. plantarum*, *Pd. pentosaceus*, *A. pasteurianus*, *Oenococcus oeni*, *B. bruxellensis* and *Saccharomyces cerevisiae*. PCR-based DGGE analysis can successfully be used for the detection and identification of spoilage microbes present in wine at low contamination levels, to prevent possible spoilage of the wine product.

Introduction

Wine is the product of complex microbiological processes with interactions between diverse species of yeasts, bacteria and mycelial fungi (Querol & Ramón, 1996; Fleet, 1993). Yeasts are important in winemaking since they are responsible for alcoholic fermentation. However, yeasts can also cause spoilage during storage in the cellar and after bottling (Fleet, 1993). The main fermenting yeast that eagerly grows in grape juice is *Saccharomyces cerevisiae* and it is mostly added as a selected pure starter culture during grape juice fermentation.

Not only yeasts are important in winemaking. The bacteria that contribute to the aroma-enrichment of wine (Andorrà *et al.*, 2008) can be found in two groups, namely lactic acid bacteria (LAB) and acetic acid bacteria (AAB). *Oenococcus oeni* is mainly responsible for malolactic fermentation (MLF), but other genera of LAB such as *Lactobacillus*, *Leuconostoc* and *Pediococcus* also play a key role in MLF and may cause wine spoilage under specific conditions (Rankine, 1995). AAB, such as *Acetobacter pasteurianus* are present in wine and may cause volatile acidity through the oxidation of ethanol to acetaldehyde and acetic acid (Fleet, 1993).

Most microbial species have been identified using conventional microbiological methods, which involve cultivation and microscopy (Lopez *et al.*, 2003). However, conventional microbiological methods have limitations in the identification and classification of microbes (Muyzer, 1999) and are often time-consuming and labour-intensive (Heard & Fleet, 1986; Hernán-Gómez *et al.*, 2000; Kopke *et al.*, 2000). It is often difficult to assess the true microbial diversity in an ecosystem (Giraffa & Neviani, 2001) and to cultivate all the viable microbes, because of the complex conditions under which these microbes grow in their natural environment (Muyzer, 1999).

Culture-independent molecular techniques make it possible to study the total microbial DNA isolated from mixed microbial populations in order to detect, identify and characterise individual microbes in food ecosystems (Hugenholtz & Pace, 1996). Genetic fingerprinting of complex microbial populations (Muyzer, 1999) is currently extensively used to study the microbial ecology of wine fermentations (Cocolin *et al.*, 2000; Mills *et al.*, 2002; Di Maro *et al.*, 2007; Renouf *et al.*, 2007). Polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) analysis is used, since it allows the detection and identification of individual species, as well as the overall profiling of microbial populations (Stahl & Chapman, 1994).

The aim of this study was to compare three methods of DNA isolation from inoculated sterile saline solution (SSS), sterile white wine and sterile red wine. Three

primers pairs, including universal, wine bacteria specific and yeast specific primers were evaluated for the identification and detection of microbes present in wine. The detection limit for each wine spoilage microbe (*Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Acetobacter pasteurianus* and *Brettanomyces bruxellensis*) was determined with the three primer pairs using PCR-based DGGE analysis. SSS and sterile white wine were then also inoculated with mixed microbial populations (*Lb. plantarum*, *A. pasteurianus* and *B. bruxellensis*) in order to determine the detection limits of the reference microbes when present in mixed populations.

Materials and methods

Microbial strains, media and growth conditions

The reference microbial species and strains selected for the study were the LAB *Pd. pentosaceus* LMG 1361, *Lb. plantarum* LMG 13556 (Culture Collection of the Laboratory of Microbiology, Belgium), and *O. oeni* NCDO 2122 (National Collection of Dairy Organisms, Reading, UK). The AAB *A. pasteurianus* DSM 3509^T (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany), and the yeasts *B. bruxellensis* ISA 1649 (ISA Culture Collection, Instituto Superior de Agronomia, Portugal) and *Saccharomyces cerevisiae* VIN13 (commercial yeast strain, Anchor Yeast, South Africa) were used in the study. All microbial strains were provided by the Institute of Wine Biotechnology (IWBT), Stellenbosch University, South Africa. The microbes and their specific growth requirements are summarised in Table 1.

Standard growth curves

The optical density (OD) of each reference strain for which PCR and DGGE detection limits were determined (*Lb. plantarum*, *Pd. pentosaceus*, *A. pasteurianus* and *B. bruxellensis*), was determined spectrophotometrically at 500 nm (Beckman Coulter DU 530 Life Sciences UV/Vis Spectrophotometer, Beckman Instruments Inc., USA). A dilution series was made in SSS (0.85% (m/v)) NaCl (Merck, Cape Town, South Africa) and the growth curves were all prepared in triplicate.

DNA isolation

Three methods were used and compared for the isolation of DNA from the pure cultures of the reference microbes used in the study. DNA was extracted from SSS, as well as

Table 1 Growth media, incubation times and temperatures used for the cultivation of the wine reference microbes

Microbe	Growth media	pH^c	Incubation time (h)	Incubation temperature (°C)
<i>Pediococcus pentosaceus</i>	MRS ^a broth	6.5	48	30
<i>Lactobacillus plantarum</i>	MRS broth	6.5	48	30
<i>Oenococcus oeni</i>	MRS broth supplemented with 20% (v/v) apple juice (Appletiser)	5.2	96	25
<i>Acetobacter pasteurianus</i>	MRS broth supplemented with 2% (v/v) ethanol	5.5	72	30
<i>Brettanomyces bruxellensis</i>	YPD ^b broth	6.5	96	25
<i>Saccharomyces cerevisiae</i>	YPD broth	6.5	48	25

^aMRS = de Man, Rogosa and Sharpe broth (Biolab Diagnostics (Pty) Ltd., Wadeville, Gauteng, SA supplied by Merck, Cape Town, SA).

^bYPD = Yeast Peptone Dextrose broth (Biolab Diagnostics).

^cpH adjusted with 1 M HCl according to The South African Wine Laboratories Association (2002).

white wine and red wine sterilised with Velcorin (Sigma-Aldrich, Gauteng, South Africa) 48 h before inoculation of the reference microbes and DNA isolation. Velcorin (Sigma-Aldrich) was prepared and diluted in a 1:4 ratio with 100% ethanol (Merck) and 200 $\mu\text{l.l}^{-1}$ of the dilution was added to the wine. All experiments were completed in triplicate.

Phenol extraction method

DNA was isolated according to the modified method of Van Elsas *et al.* (1997). Two ml of the inoculated broth was centrifuged (Eppendorf Centrifuge 5415D, Merck) for 10 min at 5 900 x *g* where after the supernatant was discarded. The pellet was vortexed for 2 min with 0.6 g sterile glass beads (0.2 – 0.3 mm in diameter) (Sigma-Aldrich), 800 μl phosphate buffer (1 part 120 mM NaH_2PO_4 (Merck, Cape Town, South Africa) to 9 parts 120 mM Na_2HPO_4 (Merck); pH 8), 700 μl phenol (Fluka, supplied by Sigma-Aldrich) and 100 μl 20% (m/v) sodium dodecyl sulphate (SDS) (Merck). The microcentrifuge tubes were then incubated for 20 min at 60°C, and the incubation was repeated twice. After incubation, the samples were centrifuged for 5 min at 1 500 x *g*. The aqueous phase was collected and the proteins were extracted with 600 μl phenol (Fluka). Further extraction was performed with a 600 μl phenol:chloroform:iso-amylalcohol (25:24:1) mixture and repeated until the interphase was clear. The DNA was then precipitated with 0.1 volume 3 M sodium acetate (NaOAc) (pH 5.5) (Saarchem, supplied by Merck) and 0.6 volume isopropanol (Saarchem) on ice for 60 min. The mixture was centrifuged for 10 min at 15 000 x *g*, where after the pellet was washed with 70% (v/v) ethanol and air-dried. The DNA was re-suspended in 100 μl 1 x TE (10mM Tris (Fluka), 1mM EDTA (Merck); pH 8).

Proteinase K-method

A modified lytic method using proteinase K (Sigma-Aldrich) for digestion (Cocolin *et al.*, 2006) was used to isolate DNA. A colony of each strain was placed in separate microcentrifuge tubes containing 200 μl SSS and 10 μl 25 mg.ml^{-1} proteinase K was added. The sample tubes were subjected to a heat treatment for 1 h at 65°C, followed by a 10 min heat treatment at 100°C.

TZ-method

The third method used for DNA isolation was carried out according to the modified method of Wang & Levin (2006). Two ml of the growth medium was centrifuged for 10 min at 6 000 x *g* after which the supernatant was discarded. The

pellet was re-suspended in 250 μ l SSS and 250 μ l of the suspension was mixed with 250 μ l double strength TZ (2 x TZ), consisting of 4% (v/v) Triton X-100 (Merck) and 5 mg.ml⁻¹ sodium azide (Merck) in 0.1 M Tris-HCl (Fluka); pH 8.0. The sample tubes were boiled for 10 min in a waterbath to lyse the cells, where after the microcentrifuge tubes were placed on ice for 5 min. The microcentrifuge tubes were then centrifuged for 5 min at 10 000 x g and 200 μ l of the supernatant was extracted and purified using a Micropure-EZ column (Millipore, supplied by Microsep, Cape Town, South Africa).

DNA purity and concentration

The three DNA isolation methods were compared in terms of simplicity of the method, as well as the DNA concentration and DNA purity by measuring the extracted DNA spectrophotometrically at 260 nm (DNA concentration) and 280 nm (DNA purity) (Johnson, 1994). In order to compare the different methods, SSS, sterile white wine and sterile red wine were inoculated with 10⁶ cfu.ml⁻¹ of *A. pasteurianus* (representative wild AAB), *Lb. plantarum* (representative wine LAB) and *B. bruxellensis* (representative wine yeast), respectively. The DNA was isolated using the three methods. The extracted DNA was suspended in SSS and 2 ml was pipetted into matched quartz cuvettes and the absorbance was measured at 260 and 280 nm (Beckman Coulter DU 530 Life Sciences UV/Vis Spectrophotometer, Beckman Instruments Inc., USA). The value obtained from the measurement at 260 nm was divided by 20 to convert the concentration from molarity to mg.ml⁻¹ [Concentration (mg.ml⁻¹) = A₂₆₀/20]. For the determination of DNA purity, the measured absorbance at 260 nm was divided by the measured absorbance at 280 nm. The extracted DNA was considered pure if the value was ≥ 1.8 and if the value was < 1.8 the extracted DNA was contaminated with protein (Johnson, 1994).

PCR

The 5'-end of the V3 variable region of the 16S ribosomal RNA (rRNA) gene for the bacterial reference strains and the 5'-end of the 26S rRNA gene for the yeast reference strains was amplified using the universal primers HDA1-GC (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G ACT CCT ACG GGA GGC AGC AGT-3') and HDA2 (5'- GTA TTA CCG CGG CTG CTG GCA C-3') (Lopez *et al.*, 2003). To facilitate DGGE separation, a GC-rich sequence (GC clamp sequence is underlined) was attached to the forward primer. The PCR reactions were performed in a total volume of 40 μ l containing 1 x reaction buffer free from MgCl₂, (Super-Therm, supplied

by Southern Cross Biotechnologies, Cape Town, South Africa), 4 μl (2.5 mM) MgCl_2 (25 mM, Super-Therm), 3.2 μl (0.8 mM) dNTPs (10 mM AB gene, supplied by Southern Cross Biotechnologies), 2 μl (500 nM) of each primer (10 μM), 0.3 μl (1.5 U) *Taq* DNA polymerase (5U. μl^{-1} , Super-Therm) and 2 μl of DNA template. Thermal cycling was carried out with a Thermal cycler (Eppendorf Mastercycler Personal, Merck, Hamburg, Germany) at an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and elongation at 68°C for 60 s. A final elongation at 68°C for 7 min was also performed.

The 5'-end of the V7 – V8 variable region of the 16S rRNA gene for the bacterial reference strains was amplified using the wine bacteria specific primers WBAC1-GC (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCC CCC GGTC GTC AGC TCG TGT CGT GAG A-3') and WBAC2 (5'-CCC GGG AAC GTA TTC ACC GCG-3') (GC clamp sequence is underlined) (Lopez *et al.*, 2003). According to Lopez *et al.* (2003) no specific primers have been reported for AAB and that the WBAC1 and WBAC2-GC primers could successfully be used for the amplification of both LAB and AAB found in wine. The PCR reactions were performed in a total volume of 50 μl containing 1 x reaction buffer free from MgCl_2 , (Super-Therm), 3 μl (1.5 mM) MgCl_2 (25 mM, Super-Therm), 4 μl (0.8 mM) dNTPs (10 mM AB gene), 1 μl (200 nM) of each primer (10 μM), 0.5 μl (2.5 U) *Taq* DNA polymerase (5U. μl^{-1} , Super-Therm) and 3 μl of DNA template. Thermal cycling was carried out with an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 60 s, annealing at 57°C for 30 s and elongation at 72°C for 60 s. A final elongation at 72°C for 5 min was also performed during the reaction.

The 5'-end of the 26S rRNA gene of the yeast reference strains was amplified using the yeast specific primers NL1-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA GGA AAA G-3') and LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') (GC clamp sequence is underlined) (O'Donnell, 1993). The PCR reactions were performed in a total volume of 25 μl containing 1 x reaction buffer free from MgCl_2 , (Super-Therm), 3 μl (3 mM) MgCl_2 (25 mM, Super-Therm), 1 μl (0.4 mM) dNTPs (10 mM AB gene), 1.5 μl (600 nM) of each primer (10 μM), 0.25 μl (1.25 U) *Taq* DNA polymerase (5U. μl^{-1} , Super-Therm), 1 μl 99% (v/v) dimethyl sulphoxide (DMSO) (Merck) and 1 μl of DNA template. The PCR reaction consisted of an initial 5 min denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 60 s, annealing at 52°C for 45 s and elongation at 72°C for 60 s. The reaction was completed with a final elongation at 72°C for 7 min.

The PCR amplicons, together with a positive and negative control, were separated on a 1.5% (m/v) agarose gel (stained with $0.02 \mu\text{l}\cdot\text{ml}^{-1}$ ethidium bromide) in 0.5 x TBE electrophoresis buffer (100 ml 5 x TBE ($54 \text{ g}\cdot\text{l}^{-1}$ Tris (Fluka), $27 \text{ g}\cdot\text{l}^{-1}$ boric acid (Merck), $20 \text{ ml}\cdot\text{l}^{-1}$ 0.5 M EDTA (Merck)) in 900 ml distilled H_2O). The PCR fragments were visualised under an ultraviolet transilluminator (Vilber Lourmat, Marne-La-Vallée, France).

DGGE analysis

The PCR fragments obtained from the amplification using the HDA1-GC and HDA2, NL1-GC and LS2 and the WBAC1 and WBAC2-GC primers were resolved using DGGE analysis, performed with the BioRad DCode Universal Mutation Detection System (Bio-Rad Laboratories, Cape Town, South Africa). PCR products were directly applied onto 8% (m/v) polyacrylamide gels, in a 1 x TAE buffer (20 ml 50 x TAE buffer ($242 \text{ g}\cdot\text{l}^{-1}$ Tris (Fluka, supplied by Sigma, USA), $57.1 \text{ ml}\cdot\text{l}^{-1}$ acetic acid (Merck), $100 \text{ ml}\cdot\text{l}^{-1}$ 0.5 M EDTA (Merck); pH 8.0) in 980 ml distilled H_2O), with a denaturing gradient of between 45 and 70% of 7 M urea (Merck) and 40% (v/v) formamide (Merck). The electrophoresis was performed at a constant voltage of 130 mV for 5 h and a constant temperature of 60°C . The gel was stained in 1 x TAE buffer containing ethidium bromide and the fragments were visualised under an ultraviolet transilluminator (Vilber Lourmat).

Inoculation with single microbes

Sauvignon blanc white wine, produced at the Department of Viticulture and Oenology, Stellenbosch University during the 2008 season and sterilised with Velcorin (Sigma-Aldrich), and SSS were inoculated with 10^6 cfu. ml^{-1} of a specific wine microbial strain (*Lb. plantarum*, *Pd. pentosaceus*, *A. pasteurianus* or *B. bruxellensis*) to determine PCR-based DGGE detection limits for the universal, wine bacteria specific and yeast specific primer pairs.

PCR-based DGGE detection limits were not determined for *S. cerevisiae* and *O. oeni*, as these microbial species are commercially added to must and wine to commence alcoholic fermentation and MLF, respectively. These microbes are therefore commonly present in wine at concentrations exceeding 10^6 cfu. ml^{-1} .

The standard growth curves for each reference culture, as given in Fig. A1 in Appendix A, were used as a reference to determine the cell inoculation size. The selected culture inoculums were prepared by growing a single colony in the appropriate

growth medium (Table 1), where after it was inoculated into an activation medium specifically for the inoculation of the strains into the sterile white wine. The activation media, incubation temperatures and times are summarised in Table 2. The cells were harvested by centrifugation for 10 min at 10 000 x *g*, re-suspended in SSS and added to 100 ml SSS or 100 ml sterile white wine at a concentration of 10⁶ cfu.ml⁻¹. All experiments were done in triplicate.

A dilution series (10⁻¹ to 10⁻⁷) of the inoculated SSS and sterile white wine were made in SSS, and DNA was isolated from each dilution using the TZ-method, where after the DNA was amplified and resolved using the optimised PCR-based DGGE conditions. To enumerate the cells each dilution was pour plated (in duplicate) on selective growth media where after the plates were incubated and colonies were counted. A wine control was also pour plated and incubated with the dilution plates as a control for the sterility of the white wine. The selective growth media used for enumeration, as well as the incubation times and temperatures are given in Table 3.

Inoculation with mixed microbes

Lactobacillus plantarum was selected as representative wine LAB, *A. pasteurianus* as representative wine AAB and *B. bruxellensis* as the representative wine yeast. SSS and sterile white wine were inoculated with mixed microbial populations containing 10⁸ cfu.ml⁻¹ of each of the wine microbial strains in order to determine PCR-based DGGE detection limits for the universal primer pair and the wine bacteria specific primer pair with the mixtures of microbes. All experiments were done in triplicate. The standard growth curves for each reference culture, as given in Fig. A1 in Appendix A, were used as a reference to determine the cell inoculation size. The following combinations of *Lb. plantarum*, *A. pasteurianus* and *B. bruxellensis* were inoculated at concentrations of 10⁸ cfu.ml⁻¹ into SSS and sterile white wine: *Lb. plantarum*, *A. pasteurianus* and *B. bruxellensis*; *Lb. plantarum* and *B. bruxellensis*; *Lb. plantarum* and *A. pasteurianus*; or *B. bruxellensis* and *A. pasteurianus*.

A dilution series (10⁻¹ to 10⁻⁹) of the inoculated SSS and sterile white wine was made in SSS, and DNA was isolated from each dilution sample using the TZ-method, where after the DNA was amplified with the HDA1-GC and HDA2 primer pair and the WBAC1-GC and WBAC2 primer pair. The PCR fragments were then resolved using the optimised DGGE conditions. To enumerate the cells each dilution was pour plated (in duplicate) on selective growth media where after the plates were incubated and colonies were enumerated. A wine control was also pour plated and incubated with the

Table 2 Activation media, incubation times and temperatures used for the reference microbes in SSS and sterile white wine

Microbe	Activation growth media	pH^c	Incubation time (h)	Incubation temperature (°C)
<i>Pediococcus pentosaceus</i>	MRS ^a broth supplemented with 40 g.l ⁻¹ D(-) fructose (Merck), 20 g.l ⁻¹ D(+) glucose (Merck), 4 g.l ⁻¹ L(-) malic acid (Merck), 1 g.l ⁻¹ Tween 80 (Merck) and 4% (v/v) ethanol	4.6	48	30
<i>Lactobacillus plantarum</i>	MRS broth supplemented with 40 g.l ⁻¹ D(-) fructose (Merck), 20 g.l ⁻¹ D(+) glucose (Merck), 4 g.l ⁻¹ L(-) malic acid (Merck), 1 g.l ⁻¹ Tween 80 (Merck) and 4% (v/v) ethanol	4.6	48	30
<i>Acetobacter pasteurianus</i>	MRS broth supplemented with 2% (v/v) ethanol	5.5	72	30
<i>Brettanomyces bruxellensis</i>	YPD ^b broth supplemented with 6% (v/v) ethanol	6.5	96	25

^aMRS = de Man, Rogosa and Sharpe broth (Biolab Diagnostics).

^bYPD = Yeast Peptone Dextrose broth (Biolab Diagnostics).

^cpH adjusted with 1 M HCl according to The South African Wine Laboratories Association (2002).

Table 3 Growth media, incubation times and temperatures used for the enumeration of microbes inoculated in SSS and sterile white wine

Microbe	Growth media	pH^c	Incubation time (d)	Incubation temperature (°C)
<i>Pediococcus pentosaceus</i>	MRS ^a broth; 15 g.l ⁻¹ bacteriological agar (Biolab Diagnostics)	4.6	3	30
<i>Lactobacillus plantarum</i>	MRS broth; 15 g.l ⁻¹ bacteriological agar (Biolab Diagnostics)	4.6	3	30
<i>Acetobacter pasteurianus</i>	MRS broth; 15 g.l ⁻¹ bacteriological agar (Biolab Diagnostics) and supplemented with 2% (v/v) ethanol	5.5	4	30
<i>Brettanomyces bruxellensis</i>	YPD ^b broth; 15 g.l ⁻¹ bacteriological agar (Biolab Diagnostics) and supplemented with 6% (v/v) ethanol	6.5	9-11	25

^aMRS = de Man, Rogosa and Sharpe broth (Biolab Diagnostics).

^bYPD = Yeast Peptone Dextrose broth (Biolab Diagnostics).

^cpH adjusted with 1 M HCl according to The South African Wine Laboratories Association (2002).

dilution plates as a control for the sterility of the white wine. The growth media was supplemented with specific antibiotics in order to eliminate the growth of unwanted yeasts, AAB and LAB. The growth of LAB was inhibited by the addition of streptomycin sulphate (Sigma Aldrich, USA) and kanamycin sulphate (Roche, Germany) was added to the media for the elimination of AAB growth. Actistab (Gist-Brocades, France) was added for the elimination of yeast growth. A specific growth medium, *Dekkera/Brettanomyces* differential medium (DBDM) was used for the enumeration of *B. bruxellensis* (Rodrigues *et al.*, 2001). The selective growth media, supplemented antibiotics and incubation times and temperatures are summarised in Table 4.

Results and discussion

DNA isolation

Since wine is a complex medium, selecting the correct method for DNA isolation is of great importance. Three DNA isolation methods were compared in terms of the simplicity of the method, as well as the DNA concentration and purity obtained. These results are presented in Table 5 for the TZ-method (Wang & Levin, 2006), the proteinase K-method (Cocolin *et al.*, 2006) and for the phenol extraction method (Van Elsas *et al.* 1997).

The results obtained from the isolation of DNA from SSS showed that all three of the DNA isolation techniques were successful in effectively extracting DNA quantitatively and qualitatively from the inoculated samples. In SSS the phenol extraction methods produced the highest DNA concentration of the three methods, when inoculated with *A. pasteurianus* (1.641 mg.ml⁻¹), but also the lowest DNA concentration of the different methods, when inoculated with *B. bruxellensis* (0.027 mg.ml⁻¹). The proteinase K-method produced the highest DNA concentration for *B. bruxellensis* (0.196 mg.ml⁻¹) inoculated into SSS. These results indicated that the phenol extraction method was inconsistent in producing reliable and reproducible DNA templates for all the reference spoilage microbes. The TZ-method produced consistent and reproducible results for all the reference microbes, with DNA concentrations of 0.149 mg.ml⁻¹; 0.160 mg.ml⁻¹ and 0.138 mg.ml⁻¹ for *A. pasteurianus*, *Lb. plantarum* and *B. bruxellensis*, respectively.

When the reference microbes were inoculated in sterile white wine, the highest DNA concentration was produced by the phenol extraction method, with a value of 0.810 mg.ml⁻¹ for *Lb. plantarum*. The lowest DNA concentration was produced for the

Table 4 Growth media, incubation times and temperatures used for the reference microbes inoculated as mixed cultures in SSS and sterile white wine

Microbe	Growth media	pH ^c	Incubation time (d)	Incubation temperature (°C)	Antibiotics (mg.l ⁻¹)
<i>Lactobacillus plantarum</i>	MRS ^a broth; 15 g.l ⁻¹ bacteriological agar (Biolab Diagnostics)	6.5	5-7	30	Actistab; 100 Kanamycin sulphate; 25
<i>Acetobacter pasteurianus</i>	MRS broth; 15 g.l ⁻¹ bacteriological agar (Biolab Diagnostics) and supplemented with 2% (v/v) ethanol	5.5	5-7	30	Actistab; 100 Streptomycin sulphate; 25
<i>Brettanomyces bruxellensis</i>	DBDM ^b agar	5.4	11-14	25	Streptomycin sulphate; 25 Kanamycin sulphate; 25

^aMRS = de Man, Rogosa and Sharpe broth (Biolab Diagnostics).

^bDBDM = *Dekkera/Brettanomyces* differential medium (6.7 g.l⁻¹ yeast nitrogen base YNB (Difco, supplied by The Scientific Group, Cape Town, South Africa), 100 mg.l⁻¹ p-coumaric acid (Sigma-Aldrich), 22 mg.l⁻¹ bromocresol green (Merck), 6% (v/v) ethanol and 20 g.l⁻¹ bacteriological agar (Biolab Diagnostics)) (Rodrigues *et al.*, 2001).

^cpH adjusted with 1 M HCl according to The South African Wine Laboratories Association (2002).

Table 5 Determination of DNA concentration and purity using the TZ-method (Wang & Levin, 2006), the proteinase K-method (Cocolin *et al.*, 2006) and the phenol extraction method (Van Elsas *et al.*, 1997)

Inoculation medium	Microbe	TZ-method		Proteinase K-method		Phenol extraction method	
		DNA concentration (mg.ml ⁻¹)	DNA purity (A _{260/280})	DNA concentration (mg.ml ⁻¹)	DNA purity (A _{260/280})	DNA concentration (mg.ml ⁻¹)	DNA purity (A _{260/280})
Sterile saline solution	<i>A. pasteurianus</i>	0.149	0.357	0.118	1.523	1.641	1.852
	<i>Lb. plantarum</i>	0.160	0.770	0.101	1.533	1.467	1.581
	<i>B. bruxellensis</i>	0.138	1.704	0.196	1.578	0.027	0.55
Sterile white wine	<i>A. pasteurianus</i>	0.530	0.453	0.041	2.382	0.508	2.016
	<i>Lb. plantarum</i>	0.160	0.116	0.109	2.565	0.810	1.300
	<i>B. bruxellensis</i>	0.187	0.471	0.051	3.091	0.202	1.403
Sterile red wine	<i>A. pasteurianus</i>	0	0	0	0	0	0
	<i>Lb. plantarum</i>	0	0	0	0	0	0
	<i>B. bruxellensis</i>	0	0	0	0	0	0

inoculation of *A. pasteurianus* (0.041 mg.ml^{-1}) using the proteinase K-method. The proteinase K-method generally produced very low DNA yields for all three of the reference microbes when inoculated into sterile white wine, while the TZ-method produced higher DNA yields.

The results obtained when the reference microbes were inoculated into sterile red wine showed that the three DNA isolation methods could not be used to isolate DNA from sterile red wine, and it was not possible to determine a value for the DNA concentration and purity for the inoculated reference microbes. This result can be explained by the fact that polyphenolic compounds, which are present in red wine, co-purify with DNA and strongly inhibit successful DNA isolation (Ibeas *et al.*, 1996). The phenol extraction method was supplemented with polyvinylpyrrolidone (PVP) to eliminate the interference of possible polyphenols present in the wine (Lodhi *et al.*, 1994). The integration of PVP in the phenol extraction method, however did not improve the efficacy of this method.

The purity of the extracted DNA can have a significant influence on the outcome of the PCR amplification and subsequent DGGE analysis of the reference microbes. In terms of DNA purity all three of the DNA isolation methods produced DNA of low purity. Using the phenol extraction method, only the DNA isolated from *A. pasteurianus* could be considered pure with values of 1.852 and 2.016 when inoculated into SSS and sterile white wine, respectively. No DNA templates produced from the reference microbes by the TZ-method could be considered pure. The proteinase K-method produced better results in terms of DNA purity, with pure DNA produced for the reference microbes that were inoculated into sterile white wine. This could be due to the fact that this method is the only method that uses proteinase K in the extraction protocol, which removes proteins and other possible contaminants of plant origin present in the wine during extraction. It has also been found that with the use of proteinase K it is possible to degrade proteins enzymatically into sub-tetrameric fragments, which would improve the efficiency of PCR-based applications by eliminating DNases and RNases (Wiegers & Hilz, 1971). The use of phenol extraction to separate the extracted DNA from RNA and other contaminants appears to be less efficient.

In terms of simplicity of the DNA isolation methods, the TZ-method and proteinase K-method were superior when compared to the phenol extraction method. The phenol extraction method is time-consuming and uses toxic compounds, including phenol. The proteinase K-method produced satisfactory results in terms of DNA purity, but the DNA templates could not be stored as DNA degradation was observed. This

degradation is possibly due to incomplete inactivation of the proteinase K used in the protocol or due to the presence of enzymes and contaminants other than RNA present in the DNA template which could cause the degradation of the DNA. The TZ-method produced better results in terms of reproducibility between the samples when compared to the other two methods. Due to the consistency and simplicity, the TZ-method was selected as the preferred method for the isolation of DNA from the reference microbes and for the determination of the detection limits of the respective reference microbes inoculated into SSS and sterile white wine.

PCR optimisation

The universal primer pair, HDA1-GC and HDA2, the wine bacteria specific primer pair, WBAC1-GC and WBAC2, as well as the yeast specific primer pair NL1-GC and LS2 were selected for the amplification of DNA isolated using the TZ-method (Wang & Levin, 2006) from the reference microbes *Pd. pentosaceus*, *Lb. plantarum*, *O. oeni*, *A. pasteurianus*, *B. bruxellensis* and *S. cerevisiae*. The three primer pairs, the primer sequences and microbial species amplified with the respective primers are presented in Table 6. The primers successfully amplified the specific yeasts and bacterial species evaluated in this study. Approximately 250 base pairs (bp) of the 5' end of the 16S rRNA gene (bacterial DNA), as well as 250 bp of the 26S rRNA (yeast DNA), was successfully amplified using the HDA1-GC and HDA2 primers (Fig. 1). The WBAC1-GC and WBAC2 primers successfully amplified approximately 320 bp of the 5' end of the 16S rRNA gene of *Pd. pentosaceus*, *Lb. plantarum*, *O. oeni* and *A. pasteurianus* (Fig. 2). Using the NL1-GC and LS2 primers approximately 250 bp amplicons were successfully produced with the PCR amplification of *B. bruxellensis* and *S. cerevisiae* (Fig. 3).

DGGE optimisation

Amplicons obtained after PCR amplification were successfully resolved using DGGE analysis. Approximately 250 bp amplicons, amplified with the HDA1-GC and HDA2 primers were successfully resolved using DGGE analysis (Fig. 4). It was observed that *Pd. pentosaceus* and the two yeast species, *S. cerevisiae* and *B. bruxellensis*, had the same migration distances in the DGGE gel. This means that it would not be possible to distinguish these three microbial species from each other on a DGGE gel when amplified using this primer pair.

Table 6 Primers used for PCR amplification of reference potential wine spoilage microbes

Primer	Primer sequence ^a	Target	Fragment size	Microbes amplified	Reference
Universal		D1/D2 26 rRNA gene;	250 bp	<i>Lb. plantarum</i>	Lopez <i>et al.</i> ,
HDA1-GC	5'- <u>CGC CCG CCG CGC CCC GCG CCC GTC CCG</u> <u>CCG CCC CCG CCC G</u> ACT CCT ACG GGA GGC AGC AGT-3'	V3 16S rRNA gene		<i>Pd. pentosaceus</i> <i>O. oeni</i> <i>A. pasteurianus</i> <i>B. bruxellensis</i> <i>S. cerevisiae</i>	2003
HDA2	5'- GTA TTA CCG CGG CTG CTG GCA C -3'				
Wine-bacteria specific		V7 to V8 16S rRNA	320 bp	<i>Lb. plantarum</i>	Lopez <i>et al.</i> ,
WBAC1-GC	5'- <u>CGC CCG CCG CGC CCC GCG CCC GGC CCG</u> <u>CCG CCC CCC</u> CCC GGTC GTC AGC TCG TGT CGT GAG A -3'	gene		<i>Pd. pentosaceus</i> <i>O. oeni</i> <i>A. pasteurianus</i>	2003
WBAC2	5'-CCC GGG AAC GTA TTC ACC GCG-3'				
Yeast specific		D1/D2 26 rRNA gene	250 bp	<i>B. bruxellensis</i> <i>S. cerevisiae</i>	O'Donnell, 1993
NL1-GC	5'- <u>CGC CCG CCG CGC GCG GCG GGC GGG</u> <u>GCG GGG</u> GCC ATA TCA ATA AGC GGA GGA AAA G-3'				
LS2	5'-ATT CCC AAA CAA CTC GAC TC-3'				

^aGC clamp sequence is underlined.

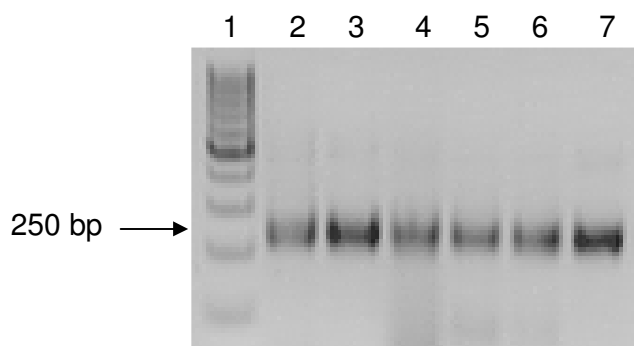


Figure 1 PCR amplification products (250 bp in size) using the primers HDA1-GC and HDA2 separated on a 1% (m/v) agarose gel. Lane 1: 100 bp DNA ladder (Fermentas, supplied by Inqaba Biotech); Lane 2: *Lb. plantarum*; Lane 3: *O. oeni*; Lane 4: *Pd. pentosaceus*; Lane 5: *A. pasteurianus*; Lane 6: *S. cerevisiae*; Lane 7: *B. bruxellensis*.

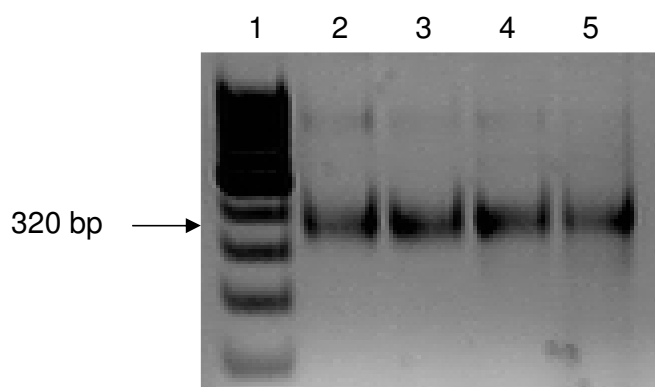


Figure 2 PCR amplification products (320 bp in size) using the primers WABC1-GC and WBAC2 separated on a 1% (m/v) agarose gel. Lane 1: 100 bp DNA ladder (Fermentas); Lane 2: *Lb. plantarum*; Lane 3: *O. oeni*; Lane 4: *Pd. pentosaceus*; Lane 5: *A. pasteurianus*.

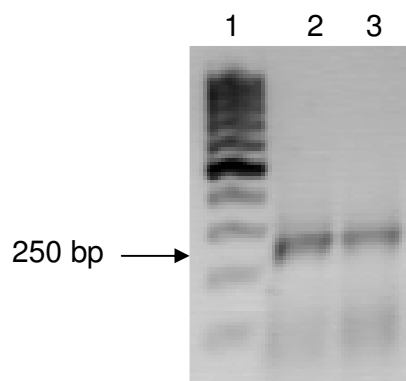


Figure 3 PCR amplification products (250 bp in size) using the primers NL1-GC and LS2 separated on a 1% (m/v) agarose gel. Lane 1: 100 bp DNA ladder (Fermentas); Lane 2: *S. cerevisiae*; Lane 3: *B. bruxellensis*.

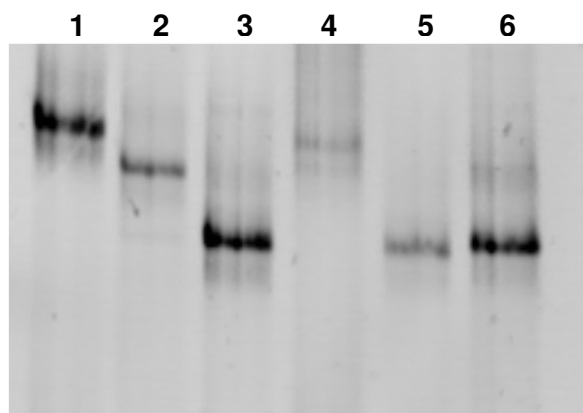


Figure 4 DGGE profile for the reference microbes amplified with HDA1-GC and HDA2 and resolved on a polyacrylamide gel. Lane 1: *Lb. plantarum*; Lane 2: *O. oeni*; Lane 3: *Pd. pentosaceus*; Lane 4: *A. pasteurianus*; Lane 5: *S. cerevisiae*; Lane 6: *B. bruxellensis*.

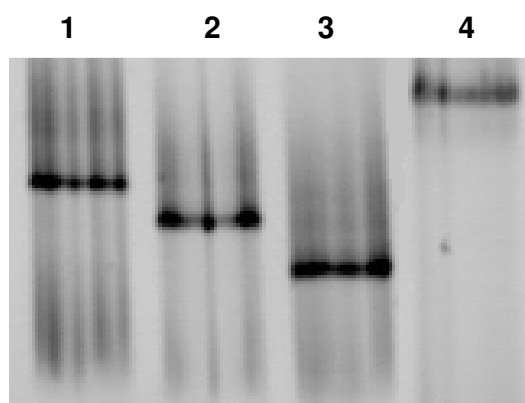


Figure 5 DGGE profile for the reference microbes amplified with WBAC1-GC and WBAC2 and resolved on a polyacrylamide gel. Lane 1: *Lb. plantarum*; Lane 2: *O. oeni*; Lane 3: *Pd. pentosaceus*; Lane 4: *A. pasteurianus*.

Approximately 320 bp amplicons, amplified with the WBAC1-GC and WBAC2 primers were successfully resolved using DGGE analysis (Fig. 5). All of the amplicons showed to have different migration distances in the DGGE gel, and it would be possible to distinguish these bacterial species when separated on a DGGE polyacrylamide gel. The reference bacteria would also have different positions in a reference ladder that could be used for species identification.

Approximately 250 bp amplicons, amplified with the NL1-GC and LS2 primers were successfully resolved using DGGE analysis (Fig. 6). The two amplicons obtained for *B. bruxellensis* and *S. cerevisiae* indicated to have different migration distances in the DGGE gel and would thus have different positions in a reference ladder. The yeast specific primer pair gave better results than the universal primer pair for the differentiation between the two yeast species, *S. cerevisiae* and *B. bruxellensis*, since it is possible to distinguish between these bands.

The optimised DGGE conditions can be used for reference ladders as an alternative to the sequencing of DGGE bands to presumptively identify the microbial species (Ercolini, 2004) inoculated into SSS and sterile white wine. The identification of the microbial species are achieved by comparing the PCR fragments migration distances in the DGGE polyacrylamide gels with those of the reference species present (Ercolini, 2004).

Detection limits for single microbes

The performance of PCR-based DGGE analysis for the detection and identification of wine spoilage yeasts and bacteria was evaluated and the results were confirmed with culture-dependent methods of pour plating for enumeration. After PCR and DGGE optimisation, the limit of microbial detection by PCR-based DGGE analysis was determined for *Pd. pentosaceus*, *Lb. plantarum*, *A. pasteurianus* and *B. bruxellensis* when each microbial species was separately inoculated at 10^6 cfu.ml⁻¹ into SSS and sterile white wine using the appropriate standard growth curves (Fig. A1).

Detection limits for Acetobacter pasteurianus

PCR and DGGE detection limits were determined for *A. pasteurianus* and the detection limits are given in Table 7. PCR amplicons were successfully obtained for the dilution samples when *A. pasteurianus* was inoculated into SSS and sterile white wine and when amplified with the HDA1-GC and HDA2 primer pair and the WBAC1-GC and WBAC2 primer pair. The PCR detection limits were determined as 10^1 cfu.ml⁻¹ when

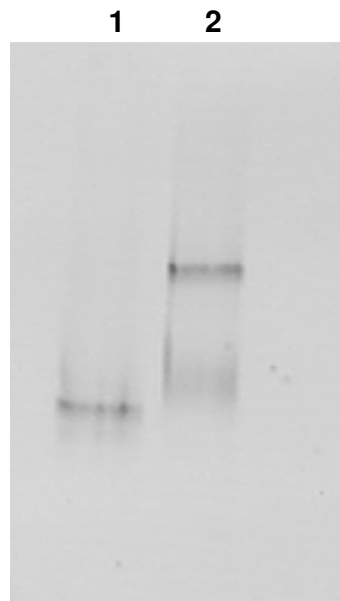


Figure 6 DGGE profile for the reference microbes amplified with NL1-GC and LS2 and resolved on a polyacrylamide gel. Lane 1: *S. cerevisiae*; Lane 2: *B. bruxellensis*.

Table 7 PCR and DGGE detection limits for reference microbial strains inoculated singly (10^6 cfu.ml⁻¹)

Microbe	Inoculation medium	Primer pair	PCR detection limit (cfu.ml⁻¹)	DGGE detection limit (cfu.ml⁻¹)
<i>A. pasteurianus</i>	sterile saline solution	HDA1-GC and HDA2	10^1	10^2
		WBAC1-GC and WBAC2	10^1	10^2
	sterile white wine	HDA1-GC and HDA2	10^1	10^2
		WBAC1-GC and WBAC2	10^1	10^3
<i>Lb. plantarum</i>	sterile saline solution	HDA1-GC and HDA2	10^1	10^1
		WBAC1-GC and WBAC2	10^1	10^2
	sterile white wine	HDA1-GC and HDA2	10^1	10^2
		WBAC1-GC and WBAC2	10^2	10^3
<i>Pd. pentosaceus</i>	sterile saline solution	HDA1-GC and HDA2	10^1	10^1
		WBAC1-GC and WBAC2	10^1	10^2
	sterile white wine	HDA1-GC and HDA2	10^2	10^2
		WBAC1-GC and WBAC2	10^2	10^4
<i>B. bruxellensis</i>	sterile saline solution	HDA1-GC and HDA2	10^1	10^4
		NL1-GC and LS2	10^4	10^5
	sterile white wine	HDA1-GC and HDA2	10^3	10^3
		NL1-GC and LS2	10^4	10^5

A. pasteurianus was inoculated into SSS. When inoculated into sterile white wine the same PCR detection limit (10^1 cfu.ml⁻¹) was observed. The PCR fragments were successfully resolved using DGGE analysis and DGGE detection limits of 10^2 cfu.ml⁻¹ were determined for *A. pasteurianus* when inoculated into SSS using both the primer pairs.

When the inoculation was done in sterile white wine and the fragments amplified with the WBAC1-GC and WBAC2 primers, a higher DGGE detection limit was determined than when the inoculation was done in SSS. The DGGE amplicons for the PCR fragments amplified with the primers HDA1-GC and HDA2 showed intense bands for the dilutions containing $10^4 - 10^6$ cfu.ml⁻¹ and lighter but visible bands for the dilutions $10^2 - 10^3$ cfu.ml⁻¹. No bands were observed for dilutions less than 10^2 cfu.ml⁻¹ and this dilution thus represented the detection limit when analysed under the conditions used in this study. When PCR fragments were amplified with the primers WBAC1-GC and WBAC2 a DGGE detection limit of 10^3 cfu.ml⁻¹ was observed for *A. pasteurianus* in sterile white wine.

Detection limits for Lactobacillus plantarum

When *Lb. plantarum* was inoculated into SSS and sterile white wine, PCR detection limits of 10^1 cfu.ml⁻¹ was determined when amplified with the HDA1-GC and HDA2 primers (Table 7). When amplified with the wine bacteria specific primers WBAC1-GC and WBAC2 a PCR detection of 10^1 cfu.ml⁻¹ was determined when the inoculation was done in SSS, but a higher PCR detection limit of 10^2 cfu.ml⁻¹ was determined when the inoculation was done in sterile white wine. The PCR fragments were successfully resolved with DGGE analysis and a DGGE detection limit of 10^1 cfu.ml⁻¹ was determined when *Lb. plantarum* was inoculated into SSS and amplified with the HDA1-GC and HDA2 primers, and a DGGE detection limit of 10^2 cfu.ml⁻¹ when amplified with the WBAC1-GC and WBAC2 primers.

The DGGE results obtained for the PCR fragments amplified using the primers HDA1-GC and HDA2 when sterile white wine was inoculated with 10^6 cfu.ml⁻¹ of *Lb. plantarum* is shown in Fig. 7. Intense bands were visible for the dilutions $10^4 - 10^6$ cfu.ml⁻¹ and lighter bands were visible for the $10^2 - 10^3$ cfu.ml⁻¹ dilution. The detection limit was determined as 10^2 cfu.ml⁻¹, since no band was visible for the 10^1 cfu.ml⁻¹ dilution. When the PCR fragments were amplified with the WBAC1-GC and WBAC2 primers and resolved with DGGE analysis, DGGE amplicons was obtained

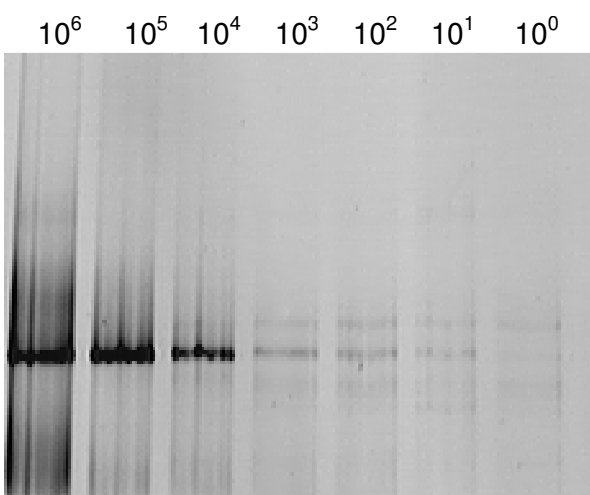


Figure 7 DGGE analysis of the different concentrations of *Lb. plantarum* ($10^6 - 10^0$ cfu.ml⁻¹) inoculated into sterile white wine and amplified with the primers HDA1-GC and HDA2.

which indicated a detection limit of 10^3 cfu.ml⁻¹ for *Lb. plantarum*. Intense bands were visible for the dilutions 10^4 – 10^6 cfu.ml⁻¹, and a lighter band for the 10^3 cfu.ml⁻¹ dilution.

Detection limits for Pediococcus pentosaceus

With the inoculation of *Pd. pentosaceus* into SSS, PCR detection limits of 10^1 cfu.ml⁻¹ were determined when the inoculation was performed in SSS for both the universal and the wine bacteria specific primers pairs (Table 7). When the inoculation was performed in sterile white wine, a PCR detection limit of 10^2 cfu.ml⁻¹ was determined for both the primer pairs. The PCR fragments were resolved using DGGE analysis and when *Pd. pentosaceus* was inoculated into SSS, a DGGE detection limit of 10^1 cfu.ml⁻¹ were determined for the PCR fragments amplified with the HDA1-GC and HDA2 primers. A DGGE detection limit of 10^2 cfu.ml⁻¹ was determined for *Pd. pentosaceus* inoculated into SSS when amplified with the WBAC1-GC and WBAC2 primers.

DGGE detection limits were also determined when *Pd. pentosaceus* was inoculated into sterile white wine. The DGGE detection limit was determined as 10^2 cfu.ml⁻¹ when amplified with the HDA1-GC and HDA2 primer pair as a distinct band was visible for the 10^2 cfu.ml⁻¹ dilution. When the fragments, amplified with the WBAC1-GC and WBAC2 primers were resolved with DGGE, a detection limit of 10^4 cfu.ml⁻¹ was determined. No bands were visible for the 10^0 – 10^3 cfu.ml⁻¹ dilutions and intense bands were visible for the 10^5 – 10^6 cfu.ml⁻¹ dilutions. It was observed that it was possible to detect lower cell concentrations of *Pd. pentosaceus* with the HDA1-GC and HDA2 primer pair in SSS and sterile white wine than with the WBAC1-GC and WBAC2 primer pair.

Detection limits for Brettanomyces bruxellensis

PCR detection limits of 10^1 cfu.ml⁻¹ and 10^3 cfu.ml⁻¹ were determined when *B. bruxellensis* was amplified with the HDA1-GC and HDA2 primers in SSS and sterile white wine, respectively (Table 7). When amplified with the yeast specific primers NL1-GC and LS2, a PCR detection limit of 10^4 cfu.ml⁻¹ was determined when *B. bruxellensis* was inoculated into SSS and sterile white wine. The PCR detection limits were higher than expected for the yeast, *B. bruxellensis* and generally higher than the detection limits determined for the reference wine bacteria. A DGGE detection limit of 10^4 cfu.ml⁻¹ was determined for the inoculation into SSS when the fragments were

amplified with the HDA1-GC and HDA2 primers and when amplified with the NL1-GC and LS2 primers a DGGE detection limit of 10^5 cfu.ml⁻¹ was observed.

When *B. bruxellensis* was inoculated into sterile white wine, bands could be observed when the fragments were amplified with both the primer pairs, HDA1-GC and HDA2 and NL1-GC and LS2, and when resolved using DGGE analysis. When amplified with the universal primers HDA1-GC and HDA2 a DGGE detection limit of 10^3 cfu.ml⁻¹ was determined. When amplified with the NL1-GC and LS2 primers a DGGE detection limit of 10^5 cfu.ml⁻¹ was determined. The $10^5 - 10^6$ cfu.ml⁻¹ dilutions gave visible bands, and no bands were visible for the $10^0 - 10^4$ cfu.ml⁻¹ dilutions. The NL1-GC and LS2 primers could only resolve high concentrations of *B. bruxellensis* of greater than 10^5 cfu.ml⁻¹ in sterile white wine and SSS with DGGE and did not give reproducible and reliable results for the determination of PCR and DGGE detection limits in white wine. This could possibly mean that the yeast specific primer pair is less sensitive in comparison to the universal primer pair, thus more DNA is required for PCR amplification with the NL1-GC and LS2 primer pair. This would suggest that NL1-GC and LS2 would not be a suitable primer pair for the detection of *B. bruxellensis* using PCR-based DGGE analysis.

General discussion of detection limits for single microbes

The results obtained from the determination of PCR and DGGE detection limits when 10^6 cfu.ml⁻¹ of the wine reference microbial strains were separately inoculated into SSS and sterile white wine, illustrated that the universal, the wine bacteria and the yeast specific primer pairs used in this study could successfully be used to detect and identify spoilage microbes that are present in white wine. When the inoculations were done in sterile white wine, higher detection limits were determined for the reference microbes than when the inoculations were done in SSS. This may be due to the presence of many inhibitors that are present in wine. Many plant materials, such as polysaccharides, plant lipids and polyphenols are known to inhibit PCR reactions, which will also ultimately influence the outcome of the DGGE detection limit results (Lodhi *et al.*, 1994). The plant material and inhibitory substances that were extracted during DNA isolation could also have had an influence on PCR amplification of the DNA template and can cause a decrease in the sensitivity of this detection method (Prakitchaiwattana *et al.*, 2004). The sensitivity of the primer pairs used in this study differed in terms of PCR, as well as DGGE detection limits. The universal primer pair, HDA1-GC and

HDA2, and the wine bacteria specific WBAC1-GC and WBAC2 primers had similar sensitivity for the PCR amplification of the DNA templates from the inoculated samples.

Detection limits for mixed microbes

Several bacterial and yeast species are present in wine during alcoholic fermentation and MLF (Prakitchaiwattana *et al.*, 2004). Detection limits of the reference microbes inoculated into SSS and sterile white wine were, therefore, determined as part of a mixed population with the universal and wine bacteria primer pairs. Due to the high detection limits obtained with the NL1-GC and LS2 primer pair, it was decided not to use this primer pair for the detection of *B. bruxellensis* in mixed microbial populations.

The performance of PCR-based DGGE analysis was thus evaluated in detecting individual wine microbial strains in cell suspensions containing a variety of microbial populations. The different reference microbial strains, *A. pasteurianus*, *Lb. plantarum* and *B. bruxellensis* were inoculated into SSS and sterile white wine at a concentration of 10^8 cfu.ml⁻¹ using the appropriate standard growth curves (Fig. A1). The microbes showed to have different base pair compositions within the variable regions of the 16S and 26S rRNA gene, which makes it possible to distinguish them using PCR-based DGGE analysis (Ercolini, 2004).

Detection limits for Acetobacter pasteurianus and Lactobacillus plantarum

When *A. pasteurianus* and *Lb. plantarum* were inoculated into SSS and sterile white wine, a PCR detection limit of 10^1 cfu.ml⁻¹ was determined for both these bacterial species when amplified with HDA1-GC and HDA2 and WBAC1-GC and WBAC2 (Table 8). The PCR detection limits determined for these two bacteria compared well with the results for the detection limits of the single reference microbial strains inoculated in SSS and sterile white wine. The PCR detection limits were observed as 10^1 cfu.ml⁻¹ for both bacterial species, except for *Lb. plantarum* that has a detection limit of 10^2 cfu.ml⁻¹ when inoculated as a single strain in sterile white wine and amplified with the WBAC1-GC and WBAC2 primer pair (Table 7).

The DGGE detection limits were determined as 10^3 cfu.ml⁻¹ for both *A. pasteurianus* and *Lb. plantarum* when amplified with the HDA1-GC and HDA2 primers and a DGGE detection limit of 10^1 cfu.ml⁻¹ when amplified with the wine bacteria specific WBAC1-GC and WBAC2 primers. When *A. pasteurianus* and *Lb. plantarum* were inoculated in sterile white wine, a DGGE detection limit of 10^1 cfu.ml⁻¹ was determined for *A. pasteurianus* and a higher detection limit of

Table 8 PCR and DGGE detection limits for the inoculation of *A. pasteurianus* and *Lb. plantarum* in sterile saline solution and sterile white wine

Inoculation medium	Detection limit	Primer pair	<i>A. pasteurianus</i> (cfu.ml ⁻¹)	<i>Lb. plantarum</i> (cfu.ml ⁻¹)
Sterile saline solution	PCR detection limit	HDA1-GC and HDA2	10 ¹	10 ¹
		WBAC1 and WBAC2-GC	10 ¹	10 ¹
	DGGE detection limit	HDA1-GC and HDA2	10 ³	10 ³
		WBAC1 and WBAC2-GC	10 ¹	10 ¹
Sterile white wine	PCR detection limit	HDA1-GC and HDA2	10 ¹	10 ¹
		WBAC1 and WBAC2-GC	10 ¹	10 ¹
	DGGE detection limit	HDA1-GC and HDA2	10 ¹	10 ⁴
		WBAC1 and WBAC2-GC	10 ¹	10 ¹

10^4 cfu.ml⁻¹ for *Lb. plantarum* when amplified with the HDA1-GC and HDA2 primers (Table 8). When the PCR amplicons that were amplified with the WBAC1-GC and WBAC2 primers were resolved using DGGE, a detection limit 10^1 cfu.ml⁻¹ was determined for both *A. pasteurianus* and *Lb. plantarum* (Table 8). The primer pair was capable of amplifying a lower amount of cells and was thus more sensitive than the HDA1-GC and HDA2 primers in amplifying a mixed population of these wine bacteria. When compared to the DGGE detection limits of the reference microbes inoculated as single strains, it was observed that HDA1-GC and HDA2 primer pair was more sensitive than the WBAC1-GC and WBAC2 primer pair in the detection of the single microbial strains, but as part of mixed populations it was observed that the WBAC1-GC and WBAC2 primer pair was more sensitive.

Detection limits for Acetobacter pasteurianus and Brettanomyces bruxellensis

When the wine AAB, *A. pasteurianus* and the wine yeast, *B. bruxellensis*, were inoculated in SSS and sterile white wine, a PCR detection limit of 10^1 cfu.ml⁻¹ was determined for both these microbes when amplified with both the HDA1-GC and HDA2 and WBAC1-GC and WBAC2 primer pairs (Table 9). When compared to the PCR detection limits of the single reference microbial strains, it was observed that the same PCR detection limit was determined using the HDA1-GC and HDA2 primers, but a higher detection limit of 10^3 cfu.ml⁻¹ was observed for *B. bruxellensis* inoculated in sterile white wine when amplified with the HDA1-GC and HDA2 primers.

The PCR amplicons were successfully resolved on a DGGE gel and a DGGE detection limit of 10^1 cfu.ml⁻¹ was determined for *A. pasteurianus* and *B. bruxellensis* when inoculated into SSS and when amplified with the HDA1-GC and HDA2 primers. When amplified with the WBAC1-GC and WBAC2 primers, a DGGE detection limit of 10^5 cfu.ml⁻¹ was determined for *A. pasteurianus*. When the inoculation was done in sterile white wine, a DGGE detection limit of 10^5 cfu.ml⁻¹ was determined for *A. pasteurianus* and a DGGE detection limit of 10^6 cfu.ml⁻¹ was determined for *B. bruxellensis* using HDA1-GC and HDA2 (Table 9, Fig. 8). When *A. pasteurianus* and *B. bruxellensis* were inoculated in sterile white wine, the same DGGE detection limit of 10^5 cfu.ml⁻¹ was observed (Table 9). The two primer pairs had a similar sensitivity for the amplification of this wine bacterium when inoculated with a wine yeast. When compared to the results obtained from the inoculation of the single microbial reference strains (Table 7), it was observed that lower detection limits was obtained for the inoculation of the single microbial strains, than when inoculated as part of a mixed

Table 9 PCR and DGGE detection limits for the inoculation of *A. pasteurianus* and *B. bruxellensis* in sterile saline solution and sterile white wine

Inoculation medium	Detection limit	Primer pair	<i>A. pasteurianus</i> (cfu.ml ⁻¹)	<i>B. bruxellensis</i> (cfu.ml ⁻¹)
Sterile saline solution	PCR detection limit	HDA1-GC and HDA2	10 ¹	10 ¹
		WBAC1 and WBAC2-GC	10 ¹	not expected
	DGGE detection limit	HDA1-GC and HDA2	10 ¹	10 ¹
		WBAC1 and WBAC2-GC	10 ⁵	not expected
Sterile white wine	PCR detection limit	HDA1-GC and HDA2	10 ¹	10 ¹
		WBAC1 and WBAC2-GC	10 ¹	not expected
	DGGE detection limit	HDA1-GC and HDA2	10 ⁵	10 ⁶
		WBAC1 and WBAC2-GC	10 ⁵	not expected

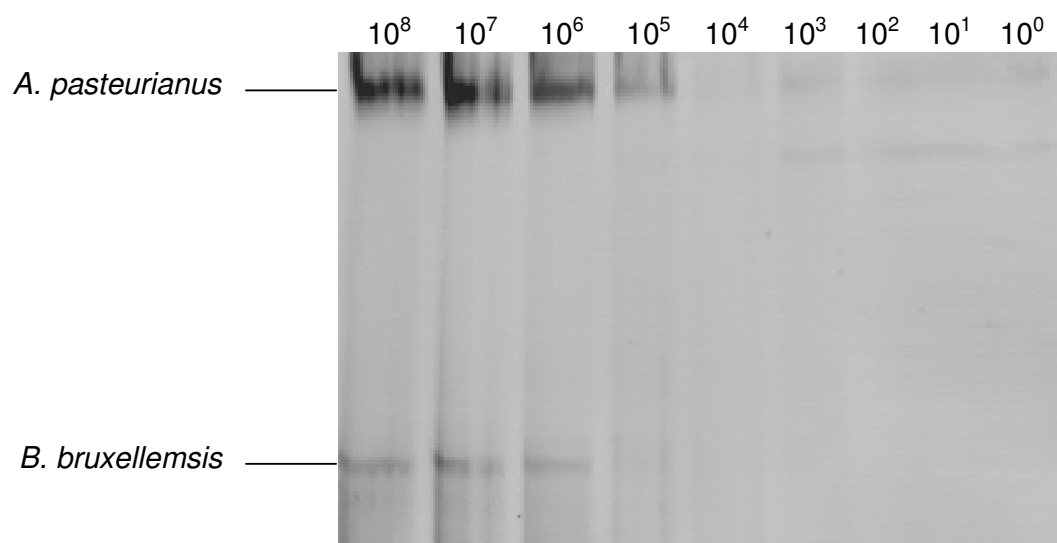


Figure 8 DGGE analysis of the different concentration of *A. pasteurianus* and *B. bruxellemsis* ($10^8 - 10^1$ cfu.ml⁻¹) inoculated into sterile white wine and amplified with the primers HDA1-GC and HDA2.

microbial population. When inoculated at a concentration of 10^8 cfu.ml⁻¹, *B. bruxellensis* and *A. pasteurianus* have lower DGGE detection limits of 10^1 cfu.ml⁻¹ than when inoculated as a single strain in SSS (10^2 cfu.ml⁻¹ and 10^4 cfu.ml⁻¹ for *A. pasteurianus* and *B. bruxellensis*, respectively). The reason for these findings is uncertain, and no similar studies on these microbes have been reported in the literature. Therefore, further research is needed to explain why a lower detection limit was observed for these microbial species in mixed population than as single microbial strains.

Detection limits for Lactobacillus plantarum and Brettanomyces bruxellensis

When *Lb. plantarum* and *B. bruxellensis* were inoculated into SSS and sterile white wine, and when amplified with the HDA1-GC and HDA2 primers, as well as WBAC1-GC and WBAC2, a PCR detection limit of 10^1 cfu.ml⁻¹ was determined for both the wine bacterium and the yeast species (Table 10). When *Lb. plantarum* and *B. bruxellensis* were inoculated into SSS and when amplified with the HDA1-GC and HDA2, a DGGE detection limit of 10^1 cfu.ml⁻¹ was determined for both these microbes. When amplified with the WBAC1-GC and WBAC2 primers a higher DGGE detection limit of 10^4 cfu.ml⁻¹ was determined for *Lb. plantarum* when compared to amplification with the universal primer pair. When *Lb. plantarum* and *B. bruxellensis* were inoculated in sterile white wine a DGGE detection limit of 10^1 cfu.ml⁻¹ was determined for both the microbial species, although the bands for the dilutions of *Lb. plantarum* were observed to be much more intense than the bands for the *B. bruxellensis* dilutions. When the PCR amplicons that were amplified with the WBAC1-GC and WBAC2 primers were resolved with DGGE analysis, a detection limit of 10^5 cfu.ml⁻¹ was determined for *Lb. plantarum*.

When compared to the results obtained for the inoculation of the single microbial strains (Table 7), it was observed that higher DGGE detection limits of 10^4 cfu.ml⁻¹ and 10^3 cfu.ml⁻¹ was obtained for *B. bruxellensis* inoculated as a single microbial strain and when amplified with HDA1-GC and HDA2 in SSS and sterile white wine, respectively. With the DGGE detection limits, it was also observed that the WBAC1-GC and WBAC2 primer pair was more sensitive when *Lb. plantarum* was inoculated as a single strain than when inoculated with *B. bruxellensis*. The results with the HDA1-GC and HDA2 primer pair compared well when *Lb. plantarum* were inoculated as a single strain and when inoculated as part of a mixed population. A DGGE detection of 10^1 cfu.ml⁻¹ was obtained for *Lb. plantarum* when inoculated as a single microbial strain and as part of a mixed microbial strain, and DGGE detection limits of 10^1 cfu.ml⁻¹ and 10^2 cfu.ml⁻¹ was

Table 10 PCR and DGGE detection limits for the inoculation of *Lb. plantarum* and *B. bruxellensis* in sterile saline solution and sterile white wine

Inoculation medium	Detection limit	Primer pair	<i>Lb. plantarum</i> (cfu.ml ⁻¹)	<i>B. bruxellensis</i> (cfu.ml ⁻¹)
Sterile saline solution	PCR detection limit	HDA1-GC and HDA2	10 ¹	10 ¹
		WBAC1 and WBAC2-GC	10 ¹	not expected
	DGGE detection limit	HDA1-GC and HDA2	10 ¹	10 ¹
		WBAC1 and WBAC2-GC	10 ⁴	not expected
Sterile white wine	PCR detection limit	HDA1-GC and HDA2	10 ¹	10 ¹
		WBAC1 and WBAC2-GC	10 ¹	not expected
	DGGE detection limit	HDA1-GC and HDA2	10 ¹	10 ¹
		WBAC1 and WBAC2-GC	10 ⁵	not expected

determined when inoculated as a single microbial strain and as part of a mixed population, respectively.

Detection limits for Acetobacter pasteurianus, Lactobacillus plantarum and Brettanomyces bruxellensis

The PCR and DGGE detection limits determined for the inoculation of the mixed population of *A. pasteurianus*, *Lb. plantarum* and *B. bruxellensis* into SSS and sterile white wine is given in Table 11. The PCR detection limit for all three of the microbes were determined as 10^1 cfu.ml⁻¹ when inoculated into SSS and sterile white wine and when amplified with both the HDA1-GC and HDA2 primer pair and the WBAC1-GC and WBAC2 primer pair. Since, the wine bacteria specific primer pair WBAC1-GC and WBAC2 is not specific for the amplification of yeast species, it was not expected that this primer pair would amplify *B. bruxellensis*, and thus, there is no detection limit for *B. bruxellensis* when amplified with the WBAC1-GC and WBAC primers. The DGGE detection limits for the reference microbes inoculated into SSS as part of mixed population were determined as 10^1 cfu.ml⁻¹ when amplified with both primer pairs (Table 11).

When inoculated in sterile white wine a DGGE detection limit of 10^1 cfu.ml⁻¹ was determined for *Lb. plantarum* and *B. bruxellensis* and a DGGE detection limit of 10^7 cfu.ml⁻¹ for *A. pasteurianus* when amplified with the HDA1-GC and HDA2 primers. When the two bacterial species and yeast species were inoculated into sterile white wine and amplified with the WBAC1-GC and WBAC2 primers a DGGE detection limit of 10^2 cfu.ml⁻¹ was determined for both the bacterial species. It could also possibly be explained by the fact that DNA isolation, PCR amplification and DGGE analysis were done on a 48 h old culture of *Lb. plantarum* and on a 72 h old culture of *A. pasteurianus*. Although these cultures contained the same viable populations of 10^8 cfu.ml⁻¹, the older culture of *A. pasteurianus* could possibly contain more amounts of dead cells that could interfere with the PCR amplification reaction. This result also indicated that the WBAC1-GC and WBAC2 primer pair was more sensitive and specific in the amplification of the wine bacteria than the HDA1-GC and HDA2 primers and could detect low concentrations of the bacterial species.

When compared to the inoculation of single microbial strains in SSS and sterile white wine (Table 7), it was observed that the DGGE detection limits was lower, 10^1 cfu.ml⁻¹ for the three microbial species, than when inoculated as single strains in SSS. It was also observed that the DGGE detection limits was lower for the reference

Table 11 PCR and DGGE detection limits for the inoculation of *A. pasteurianus*, *Lb. plantarum* and *B. bruxellensis* in sterile saline solution and sterile white wine

Inoculation medium	Detection limit	Primer pair	<i>A. pasteurianus</i> (cfu.ml ⁻¹)	<i>Lb. plantarum</i> (cfu.ml ⁻¹)	<i>B. bruxellensis</i> (cfu.ml ⁻¹)
Sterile saline solution	PCR detection limit	HDA1-GC and HDA2	10 ¹	10 ¹	10 ¹
		WBAC1 and WBAC2-GC	10 ¹	10 ¹	not expected
	DGGE detection limit	HDA1-GC and HDA2	10 ¹	10 ¹	10 ¹
		WBAC1 and WBAC2-GC	10 ¹	10 ¹	not expected
Sterile white wine	PCR detection limit	HDA1-GC and HDA2	10 ¹	10 ¹	10 ¹
		WBAC1 and WBAC2-GC	10 ¹	10 ¹	not expected
	DGGE detection limit	HDA1-GC and HDA2	10 ⁷	10 ¹	10 ¹
		WBAC1 and WBAC2-GC	10 ²	10 ²	not expected

microbial species when inoculated as part of a mixed population in sterile white wine, with the exception of *A. pasteurianus* with a DGGE detection limit of 10^7 cfu.ml⁻¹ when inoculated in sterile white wine as a single microbial strain.

Conclusions

An important aspect of culture-independent techniques that are frequently overlooked is the method of DNA isolation. In this study, three methods of DNA isolation were investigated and DNA could only be consistently and reliably isolated from SSS and sterile white wine when using the TZ-method. It was also found that it is of great importance to ensure that the correct DNA isolation method is selected in order to obtain a DNA template that can successfully be used for amplification with PCR and analysis with DGGE. None of the methods used in this study was found to be suitable for the isolation of DNA from red wine. The results obtained in this study indicated that PCR-based DGGE analysis can successfully be used for the detection of the potential wine spoilage microbes *Pd. pentosaceus*, *Lb. plantarum*, *A. pasteurianus* and *B. bruxellensis* as single microbes and as part of mixed populations inoculated into SSS and sterile white wine. It is, however, extremely important to optimise the PCR and DGGE conditions that will be used in the assay. PCR and DGGE conditions were successfully optimised for *Pd. pentosaceus*, *Lb. plantarum*, *O. oeni*, *A. pasteurianus*, *S. cerevisiae* and *B. bruxellensis* with a universal, a wine bacteria specific and a yeast specific primer pair. These optimised DGGE profiles can be used to presumptively identify microbial species present in wine samples by constructing a reference ladder. It was found that the yeast specific primer pair could not amplify low concentrations of microbial cells, but that it was the only one of the primer pairs used in the study that could discriminate between *S. cerevisiae* and *B. bruxellensis*.

References

- Andorrà, I., Landi, S., Mas, A., Guillamón, J.M. & Esteve-Zarzoso, B. (2008). Effect of oenological practices on microbial populations using culture-independent techniques. *Food Microbiology*, **25**, 849-856.
- Cocolin, L., Bisson, L.F. & Mills, D.A. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiology Letters*, **189**, 81-87.

- Cocolin, L., Diez, A., Urso, R., Rantsiou, K., Comi, G., Bergmaier, I. & Beimfohr, C. (2006). Profiling of bacterial populations in food by culture-independent methods, *FoodMicro 2006 International Journal of Food Microbiology Special Issue*.
- Di Maro, E., Ercolini, D. & Coppola, S. (2007). Yeast dynamics during spontaneous wine fermentation of the Catalanesca grape. *International Journal of Food Microbiology*, **117**, 201-210.
- Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, **56**, 297-314.
- Fleet, G.H. (1993). The microorganisms of winemaking – isolation, enumeration and identification. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 1-26. New York: Taylor & Francis.
- García-Beneytez, E., Moreno-Arribas, M.V., Borrego, J., Polo, M.C. & Ibez, J. (2002). Application of a DNA analysis method for the cultivar identification of grape musts and experimental and commercial wines of *Vitis vinifera* L. using microsatellite markers. *Journal of Agricultural Food Chemistry*, **50**, 6090-6096.
- Giraffa, G. & Neviani, E. (2001). DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. *International Journal of Food Microbiology*, **67**, 19-34.
- Heard, G.M. & Fleet, G.H. (1986). Occurrence and growth of yeast species during the fermentation of some Australian wines. *Food Technology in Australia*, **38**, 22-25.
- Hernán-Gómez, G., Espinosa, J.C. & Ubeda, J.F. (2000). Characterization of wine yeasts by temperature gradient gel electrophoresis (TGGE). *FEMS Microbiology Letters*, **193**, 45-50.
- Hugenholtz, P. & Pace, N.R. (1996). Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. *Trends in Biotechnology*, **14**, 90-97.
- Ibeas, J.I., Lozano, I., Perdigones, F. & Jimenez, J. (1996). Detection of *Dekkera-Brettanomyces* strains in sherry by a nested PCR method. *Applied and Environmental Microbiology*, **62**, 998-1003.
- Johnson, J.L. (1994). Similarity analysis of DNAs. In: *Methods for General and Molecular Bacteriology* (edited by P. Gerhardt, R.G.E. Murray, W.A. Wood & N.R. Krieg). Pp. 656-682. Washington: ASM.

- Kopke, C., Cristovão, A., Prata, A.M., Silva Pereira, C., Figueiredo Marques, J.J. & San Romão, M.V. (2000). Microbiological control of wine. The application of epifluorescence microscopy method as a rapid technique. *Food Microbiology*, **17**, 257-260.
- Lodhi, M.A., Ye, G.N., Weeden, N.F. & Reisch, B.I. (1994). A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and *Ampelopsis*. *Plant Molecular Biology Reporter*, **12**, 6-13.
- Lopez, I., Ruiz-Larrea, F., Cocolin, L., Orr, E., Phister, T., Marshall, M., VanderGheynst, J. & Mills, D.A. (2003). Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, **69**, 6801-6807.
- Mills, D.A., Johannsen, E.A. & Cocolin, L. (2002). Yeast diversity and persistence in Botrytis-affected wine fermentation. *Applied and Environmental Microbiology*, **68**, 4884-4893.
- Muyzer, G. (1999). DGGE/TGGE a method for identifying genes from natural ecosystems. *Current Opinion in Microbiology*, **2**, 317-322.
- O'Donnell, K. (1993). *Fusarium* and its near relatives, In: *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics* (edited by D.R. Reynolds & J.W. Taylor). Pp. 225-233. Wallingford: CAB International.
- Prakitchaiwattana, C.J., Fleet, G.H. & Heard, G.M. (2004). Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Research*, **4**, 865-877.
- Querol, A. & Ramón, D. (1996). The application of molecular techniques in wine microbiology. *Trends in Food Science & Technology*, **7**, 73-78.
- Rankine, B. (1995). Microbiology and fermentation. In: *Making Good Wine – A Manual of Winemaking Practice for Australia and New Zealand*. Pp. 118-130. Sydney: Pan Macmillan.
- Renouf, V., Strehaiano, P. & Lonvaud-Funel, A. (2007). Yeast and bacteria analysis of grape, wine and cellar equipments by PCR-DGGE. *Journal International Vigne Vin*, **41**, 51-61.
- Rodrigues, N., Gonçalves, G., Pereira-da-Silva, S., Malfeito-Ferreira, M. & Loureiro, V. (2001). Development and use of a new medium to detect yeast of the genera *Dekkera/Brettanomyces*. *Journal of Applied Microbiology*, **90**, 588-599.

- Siret, R., Boursiquot, J.M., Merle, M.H., Cabanis, J.C. & This, P. (2000). Toward the authentication of varietal wines by the analysis of grape (*Vitis vinifera* L.) residual DNA in must and wine using microsatellite markers. *Journal of Agricultural Food Chemistry*, **48**, 5035-5040.
- Stahl, D.A. & Chapman, W.C. (1994). Application of molecular genetics to the study of microbial communities, *NATO ASI Series*, **G35**, 193-206.
- The South African Wine Laboratories Association (2002). *Methods of Analysis for Wine Laboratories*. South Africa: South African Society for Enology and Viticulture.
- Van Elsas, J.D., Matynen, V. & Wolters, A. (1997). Soil DNA extraction and assessment of the fate of *Mycobacterium chlorophenolicum* strain PCR-1 in different soils by 16S ribosomal RNA gene sequence based most-probable-number PCR immunofluorescence. *Biological Fertilization*, **24**, 188-195.
- Wang, S. & Levin, R.E. (2006). Rapid quantification of *Vibrio vulnificus* in clams (*Protochaca staminea*) using real-time PCR. *Food Microbiology*, **23**, 757-761.
- Wiegers, U. & Hilz, H. (1971). A new method using 'proteinase K' to prevent mRNA degradation during isolation from HeLa cells. *Biochemistry Biophysics Research Communication*, **44**, 513-519.

APPENDIX A

To Chapter 3

To simplify the discussion of the results, the data for the standard growth curves illustrated in Fig. A1 have been included in this Appendix.

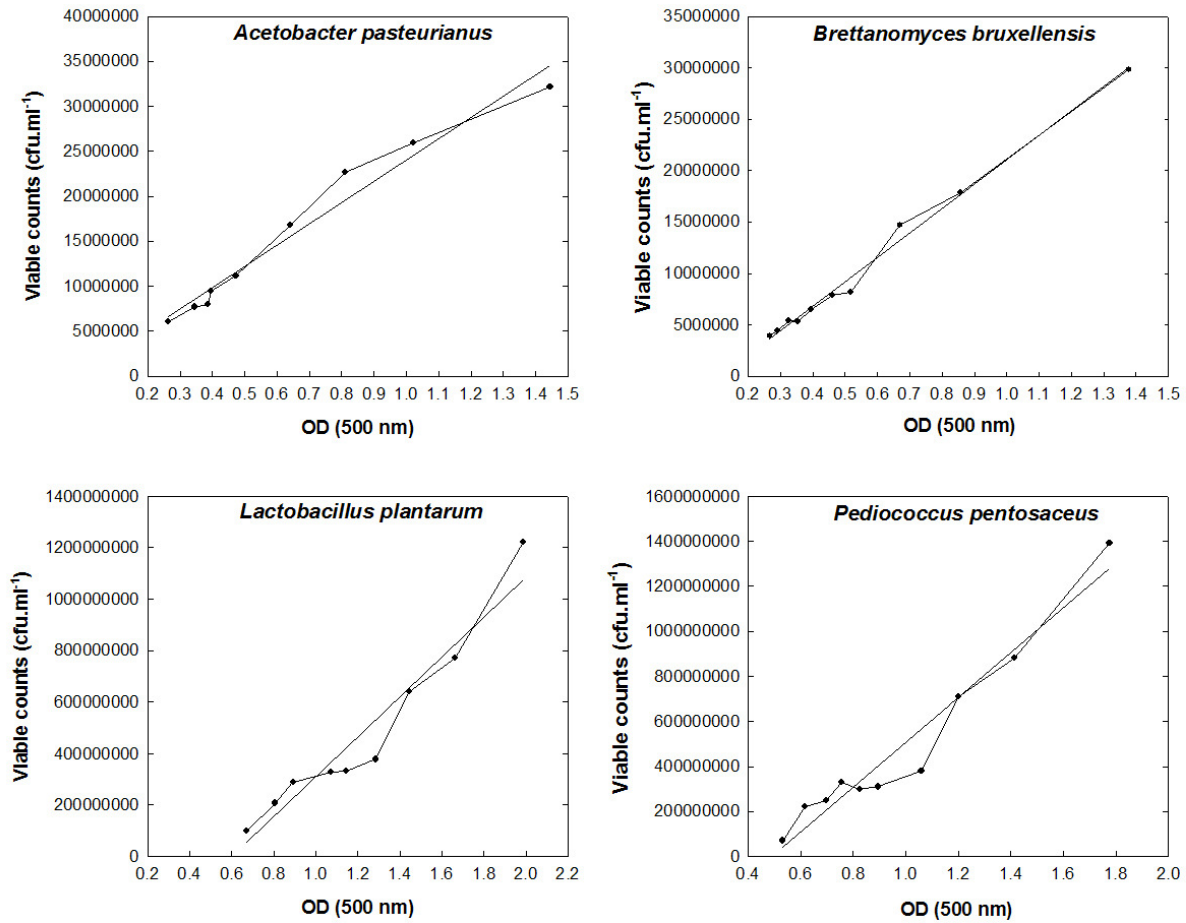


Figure A1 Standard growth curves of a 72 h pure culture of *A. pasteurianus*, a 96 h pure culture of *B. bruxellensis* and 48 h pure cultures of *Lb. plantarum* and *Pd. pentosaceus*.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

Various microbes, including lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeasts plays an essential role in the production of wine through their microbiological and biochemical interactions (Fleet, 1993). These microbes are adapted to survive and grow in the wine environment and are present during alcoholic fermentation and malolactic fermentation (MLF). Yeasts, such as *Saccharomyces cerevisiae* is commonly present during alcoholic fermentation, while LAB, such as *Oenococcus oeni* are present during MLF. The microbes that are present plays an essential role in ensuring a high quality end-product, but it is essential to control the growth of possible spoilage microbes that may be present in the wine during the fermentation process (Rankine, 1995).

The spoilage of wine by microbes such as *Brettanomyces bruxellensis*, *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Acetobacter pasteurianus* leads to the formation of off-flavours and odours, uncharacteristic sensorial properties and other wine spoilage, which leads to significant annual financial losses to the South African wine industry. It is possible to detect spoilage microbes early in the fermentation process with the use of culture-independent techniques, such as PCR-based DGGE analysis (Cocolin *et al.*, 2000; 2001; Mills *et al.*, 2002). Early detection of possible spoilage microbes is necessary for the timely prevention of wine spoilage.

The aim of this study was to determine PCR and DGGE detection limits for possible spoilage microbes inoculated into sterile saline solution (SSS) and white wine, using PCR-based DGGE analysis. In order to ensure the outcome of reliable results, different DNA isolation methods were compared in terms of simplicity of the technique, as well as in terms of DNA concentration and purity and PCR primers were optimised for the specific microbial species used in the study.

It was found that the method of DNA isolation plays an important role in the successful PCR amplification and subsequent DGGE analysis of the PCR fragments. There are many possible inhibitors present in wine, such as polysaccharides, tannins and polyphenols that may interfere during DNA isolation (Siret *et al.*, 2000; Garcia-Beneytez *et al.*, 2002). DNA was isolated from SSS, sterile white wine and sterile red wine inoculated with the reference microbial strains, using the TZ-method, the proteinase K-method and the phenol extraction method. When DNA was isolated from

red wine that was inoculated with the reference microbial strains, it was not possible to obtain a DNA template with any of the DNA isolation methods used. The TZ-method gave the best results in SSS and sterile white wine in terms of simplicity of the technique and it is also a rapid technique, and could easily be implemented in any quality control laboratory.

Three primer pairs, including a universal primer pair, a wine bacteria specific primer pair and a yeast specific primer pair were optimised. With the universal primer pair it was not possible to distinguish between *Pd. pentosaceus* and the two yeast species, *B. bruxellensis* and *S. cerevisiae*. The yeast specific primer pair was used to distinguish between these two yeast species and the wine bacteria specific primer pair was used to distinguish between the bacterial reference strains.

With the inoculation of the single reference microbial strains into SSS and white wine, it was found that the universal and wine bacteria specific primer pairs gave excellent results in detecting low concentrations of microbial cells. PCR and DGGE detection limits as low as 10^1 cfu.ml⁻¹ was determined with these two primer pairs. With the inoculation of the reference microbial strains into SSS and white wine as part of a mixed population, it was observed that the universal primer pair and the wine bacteria primer pair could successfully amplify the microbial strains inoculated. When resolved using DGGE analysis, it was possible to distinguish between these microbial species.

Concluding remarks

The results obtained from this study indicated that PCR-based DGGE analysis could successfully be used for the detection and identification of microbes present in wine. This technique allows the early detection of possible spoilage microbes present in wine. When compared to the current techniques of culture-dependent microbiology used in the industry, PCR-based DGGE analysis proved to be an efficient technique, since the technique is rapid, reliable, reproducible and inexpensive (Muyzer, 1999) and also allows the detection of viable, but non-culturable microbial cells (Giraffa & Neviani, 2001).

The main limitations of this culture-independent technique are that the population fingerprint or profile obtained from DGGE analysis does not directly generate information on the taxonomy of the microbial species present in the sample (Giraffa & Neviani, 2001). A possible solution to this limitation is the use of a reference ladder as an alternative to sequencing of DGGE bands to identify the microbial species. The identification of the microbial species are achieved by comparing the PCR fragments

migration distances in the DGGE polyacrylamide gels with those of the reference species present (Ercolini, 2004).

The results obtained from the comparison of different DNA isolation methods, indicated that the TZ-method could successfully be used for the isolation of DNA from white wine as a routine and standardised method. It was also indicated that none of the three methods could be used for the isolation of DNA from red wine. In future studies, the DNA isolation methods could be modified specifically for the isolation of DNA from red wine, with the possible integration of polyvinylpyrrolidone into the protocol of other possible DNA isolation methods to eliminate the presence of possible polyphenols present in the wine during DNA isolation and purification (Lodhi *et al.*, 1994).

With the results obtained from the determination of PCR and DGGE detection limits for the reference microbial strains, it was concluded that the universal primer pair and the wine bacteria specific primer pair could successfully be used to amplify the reference microbial strains with PCR and to resolve the PCR fragments with DGGE analysis. It was also concluded that the yeast specific primer pair could not be recommended for use with PCR-based DGGE analysis of wine samples contaminated with *B. bruxellensis*. Future studies could include the determination of PCR and DGGE detection limits of other possible spoilage wine yeasts, such as species of *Candida*, *Metschnikowia*, *Pichia* and *Hansenula* that are responsible for the spoilage of wine due to biofilm formation (Fugelsang & Edwards, 2007).

References

- Cocolin, L., Bisson, L.F. & Mills, D.A. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiology Letters*, **189**, 81-87.
- Cocolin, L., Heisey, A. & Mills, D.A. (2001). Direct identification of the indigenous yeasts in commercial wine fermentations. *American Journal of Enology and Viticulture*, **52**, 49-53.
- Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, **56**, 297-314.
- Fleet, G.H. (1993). The microorganisms of winemaking – isolation, enumeration and identification. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 1-26. New York: Taylor & Francis.
- Fugelsang, K.C. & Edwards, C.G. (2007). *Wine Microbiology, Practical Applications and Procedures*, 2nd ed. Pp. 3-19. New York: Springer Science and Business Media.

- García-Beneytez, E., Moreno-Arribas, M.V., Borrego, J., Polo, M.C. & Ibez, J. (2002). Application of a DNA analysis method for the cultivar identification of grape musts and experimental and commercial wines of *Vitis vinifera* L. using microsatellite markers. *Journal of Agricultural Food Chemistry*, **50**, 6090-6096.
- Giraffa, G. & Neviani, E. (2001). DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. *International Journal of Food Microbiology*, **67**, 19-34.
- Mills, D.A., Johannsen, E.A. & Cocolin, L. (2002). Yeast diversity and persistence in Botrytis-affected wine fermentation. *Applied and Environmental Microbiology*, **68**, 4884-4893.
- Muyzer, G. (1999). DGGE/TGGE a method for identifying genes from natural ecosystems. *Current Opinion in Microbiology*, **2**, 317-322.
- Lodhi, M.A., Ye, G.N., Weeden, N.F. & Reisch, B.I. (1994). A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and *Ampelopsis*. *Plant Molecular Biology Reporter*, **12**, 6-13.
- Rankine, B. (1995). Microbiology and fermentation. In: *Making Good Wine – A Manual of Winemaking Practice for Australia and New Zealand*. Pp. 118-130. Sydney: Pan Macmillan.
- Siret, R., Boursiquot, J.M., Merle, M.H., Cabanis, J.C. & This, P. (2000). Toward the authentication of varietal wines by the analysis of grape (*Vitis vinifera* L.) residual DNA in must and wine using microsatellite markers. *Journal of Agricultural Food Chemistry*, **48**, 5035-5040.