Monitoring the spreading of commercial wine yeasts in the vineyard

by

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DECLARATION

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

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SUMMARY

Traditionally, wine has been produced by the spontaneous fermentation of grape juice by yeast that originate from the grapes and winery equipment. Research has shown that the population composition and dynamics of these yeasts and other microorganisms are very complex. *Kloeckera* and its anamorph, *Hanseniaspora*, dominate the yeast population found on the surfaces of grapes, although prevailing *Saccharomyces cerevisiae* strains complete the fermentation process.

The yeast *S. cerevisiae* is an important factor contributing to the quality of wines and, therefore, the improvement of wine yeasts receives considerable attention worldwide. Apart from classical yeast breeding studies, genetic engineering and recombinant DNA techniques are increasingly being used in strain development research programmes. These techniques might enable the wine yeasts to produce heterologous enzymes that degrade polysaccharides, convert malic acid to lactic acid, increase glycerol production, release roam and flavour compounds, secrete antimicrobial peptides, etc. The release of recombinant yeast strains (genetically modified organisms, GMOs) is subject to statutory approval. Therefore, it is important to answer several questions prior to the use of such genetically improved yeast in the commercial production of wine. For example, will recombinant yeast strains be able to multiply and spread in nature, and will this GMO be able to out-compete the natural microflora because of its newly acquired genetic traits. Since existing commercial wine yeasts are used in the abovementioned strain development research, it is essential to determine already at this early stage to what extent these wine yeast strains survive and spread in nature and to what extent they influence the fermentations of the following vintages.

This study is divided into two sections. The aim of the first section is to sample a representative number of yeast strains from various vineyards in different climatological areas, mainly in the Western Cape, South Africa. These yeast strains were identified mainly by electrophoretic karyotyping (contour-clamped homogenous electric field electrophoresis; CHEF).

The second part of the study summarises the results obtained when Fourier transform infrared (FT-NIR) spectroscopy was used to differentiate commercial wine yeast strains. Sets of data, containing the spectra of the mostly used commercial wine yeast strains, were constructed and used as a reference library. The spectra of the isolated yeast strains were then compared to the reference dataset with specific FT-NIR computer software using mathematical calculations.

In conclusion, the two methods used in conjunction with one another proved that the commercial wine yeast strains do not easily disperse from the cellar into the vineyard. The commercial wine yeast strains are also more likely to be found near the cellar and the places where the grape skins are dumped. Therefore, should a recombinant yeast strain be used in winemaking, it would not be dispersed into the vineyard. It therefore appears that the commercial use of genetically improved yeast

does not pose a high risk in terms of dominance of the indigenous microbial population in the environment.

OPSOMMING

Wyn is tradisioneel gemaak deur die natuurlike gisting van druiwesap deur giste wat op die druiwe en keldertoerusting voorkom. Navorsing het getoon dat die samestelling en dinamika van die gispopulasie en ander mikro-organismes baie kompleks is. *Kloeckera* en sy anamorf, *Hanseniaspora*, domineer die inheemse gispopulasie op druiwedoppe, terwyl *Saccharomyces cerevisiae* in baie klein getalle op die druiwedoppe voorkom, maar later die fermentasie oorheers en uiteindelik voltooi.

Die gis *S. cerevisiae* speel 'n baie belangrike rol in die kwaliteit van wyn en daarom geniet die verbetering van wyngiste wêreldwyd besondere aandag. Benewens die klassieke gistelingstudies, word genetiese manipuleringstegnieke toenemnd in navorsingsprojekte gebruik wat daarop gefokus is om wyngisrasse te verbeter. Hierdie tegnieke mag die giste in staat stel om heteroloë ensieme te produseer wat polisakkariedes afbreek, appelmelksuur afbreek, gliserolproduksie verhoog, smaak- en geurkomponente vrystel, antimikrobiese peptiede afskei, ens. Voordat sulke geneties gemanipuleerde giste het egter in kommersiële wynproduksie gebruik sal kan word, is daar heelwat wetlike vereistes waaraan voldoen sal moet word en vrae wat vooraf beantwoord sal moet word. Byvoorbeeld, sal die rekombinante giste in staat wees om vinniger te vermeerder as gevolg van die nuwe genetiese eienskappe en sodoende die natuurlike populasies onderdruk? Omdat kommersiële wyngiste in bogenoemde gisverbeteringprogramme gebruik word, is dit noodsaaklik om nou reeds die verspreiding van die kommersiële giste te monitor en te bepaal hoe geredelik hulle in die natuur kan versprei en oorleef, en hoe hulle wynfermentasies van die daaropvolgende jare beïnvloed.

Die studie is in twee gedeeltes verdeel. Die doel van die eerste gedeelte was om 'n verteenwoordigende aantal gisrasse uit die wingerde van 'n aantal wynplase in verskillende klimaatstreke te isoleer, spesifiek in die Wes-Kaap, Suid-Afrika. Die gisrasse was grotendeels deur elektroforetiese kariotipering (kontoer-geklampte homogene elektriese veld; CHEF) geïdentifiseer.

Die tweede deel van die navorsing was gefokus op die onderskeiding tussen die mees gebruikte kommersiële wyngiste met 'Fourier-Transform Near Infrared' (FT-NIR) spektroskopie. Eerstens is 'n stel data, bestaande uit die spektrum data oor die kommersiële wyngiste opgestel om as 'n verwysingsbiblioteek te dien. Tweedens is die spektrum van data oor die geïsoleerde giste onder presies dieselfde toestande met die verwysingsbiblioteek vergelyk. Dié tegniek maak dit moontlik om tussen die kommersiële wyngiste te onderskei.

As die twee metodes saam gebruik word vir identifikasie, kan die afleiding gemaak word dat kommersiële wyngiste nie maklik vanaf die kelder na die wingerd versprei nie. Die kommersiële wyngiste is ook meestal naby die kelder en die dopstortingsterreine gevind. Sou 'n rekombinante gisras dus gebruik word om wyn te

maak, sal dit nie maklik versprei nie. Die kommersiële gebruik van geneties gemanipuleerde wyngiste behoort dus nie 'n groot omgewingsrisiko in te hou nie.

This thesis is dedicated to my mother and family.

Hierdie tesis is aan my moeder en gesin opgedra.

BIOGRAPHICAL SKETCH

Christo Muller was born in Robertson, South Africa on 3 November 1975. He attended Dagbreek Primary School and matriculated at Langeberg Senior Secondary School, Robertson in 1993. Christo enrolled at the Stellenbosch University in 1994 and obtained a BSc degree, majoring in Microbiology and Genetics, in 1997. In 1998, he completed a HonsBSc degree in Wine Biotechnology at the Institute for Wine Biotechnology, Stellenbosch University. He then enrolled as an MSc student at the same institute.

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PREFACE

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately and is written according to the style of the journal *South African Journal of Enology and Viticulture*, to which Chapter 3 and Chapter 4 will be submitted for publication.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **LITERATURE REVIEW**
Techniques in the identification of yeasts.

Chapter 3 **Research Results**
Monitoring the spreading of commercial wine yeasts in the vineyard.

Chapter 4 **Research Results**
Identification of yeast with Fourier Transform Near Infrared (FT-NIR) spectroscopy.

Chapter 5 **General Discussion and Conclusions**

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Traditionally, wine has been produced by the spontaneous fermentation of grape juice by yeasts that originate from grapes and winery equipment. Yeasts of the genera *Kloeckera* and its anamorph, *Hanseniaspora*, grow during the first few days of fermentation and disappear during the later stages, when prevailing *Saccharomyces cerevisiae* strains complete the process (Kreger-Van Rij, 1984; Barnett, 1992; Fleet, 1993; Fleet, 1998; Pretorius *et al.*, 1999; Pretorius, 2000). However, research on the yeast ecology in vineyards shows a great deal of variation in the quantity and distribution of the relevant species (Fleet & Heard, 1993; Pretorius *et al.*, 1999; Pretorius, 2000). This could be related to differences in soil composition, climate, age of the vineyard, geographical location of the vineyard, grape variety, harvest technique, etc. (Querol *et al.*, 1992). As a result of the varying grape microflora, wine quality differs from year to year. However, it is essential to use selected pure yeast inocula of known ability in large-scale wine production, where rapid, reliable, trouble-free fermentations are essential for consistent wine flavour and predictable quality (Henschke, 1997). For this reason, modern wineries use pure yeast cultures to inoculate grape must. It is these large wineries that will be the main beneficiaries of programmes aimed at yeast strain development. These programmes will produce new strains that have even more reliable performance, thereby reducing processing inputs and consequently facilitating the production of high quality wines (Henschke, 1997).

Since the dawn of the modern biotechnology era, researchers have continuously been trying to improve the physiological and biochemical properties of wine yeasts in their quest for the "ideal" wine yeast. This is usually done by searching for wine yeasts that are naturally located in the vineyard and using these in yeast breeding programmes. Apart from the classical yeast breeding studies, a tremendous amount of research is dependent on genetic engineering to enable wine yeast to produce heterologous enzymes (pectinases, glucanases, xylanases, β-glucosidases, etc.) (Laing & Pretorius, 1993; Pérez-González *et al.*, 1993; Lambrechts & Pretorius, 2000; Lilly *et al.*, 2000; Van Rensburg & Pretorius, 2002), to convert malic acid into lactic acid (Ansanay *et al.*, 1996; Bony *et al.*, 1997; Volschenk *et al.*, 1997) or to increase glycerol production (Remize *et al.*, 1999; Eglinton *et al.*, 2002). Market-orientated wine yeast strains are currently being developed for the cost-competitive production of wine with minimised resource inputs, improved quality and low environmental impact. The emphasis is on the development of *S. cerevisiae* strains with improved fermentation, processing and biopreservation abilities, and capabilities for an increase in the wholesomeness and sensorial quality of wine (Pretorius & Bauer, 2002). However, the release of recombinant yeast strains is subject to statutory approval. Since existing commercial wine yeasts are used in the

abovementioned strain development research, it is essential to determine already at this early stage to what extent these wine yeast strains survive and spread in nature and to what extent they influence the fermentations of the following vintages.

In this study, two techniques were used for the identification of yeasts, namely electrophoretic karyotyping (contour-clamped homogenous electric field electrophoresis; CHEF) and Fourier Transform Near Infrared (FT-NIR) spectroscopy. The CHEF technique is relatively easy, but the disadvantage is that it takes about one week from culturing the yeast until the CHEF is completed (24 yeast strains can be identified per gel) and it is also very expensive. If a large number of strains have to be screened and identified, it could take several months. Due to the disadvantages associated with the above-mentioned technique, the suitability of FT-NIR spectroscopy was evaluated for the identification of commercial wine yeast strains.

1.2 PROJECT AIMS

The specific aims and approaches of this study were the following:

(i) to determine the amount of commercial wine yeasts present in nature;

(ii) to establish knowledge based on the survival rate and spreading capabilities of commercial wine yeast strains in nature;

(iii) to optimise the FT-NIR technique to differentiate between some of the commercial wine yeast strains used in the wine industry, and to screen a large number of isolated wine yeasts with this technique; and

(iv) to use this knowledge to predict the possible spreading of recombinant wine yeasts in nature and how they would influence fermentations in the cellar.

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CHAPTER 2

LITERATURE REVIEW

Techniques in the identification of yeasts.

2. LITERATURE REVIEW

2.1 INTRODUCTION

Yeasts can be defined as unicellular ascomycetous or basidiomycetous fungi whose vegetative growth predominantly results from budding or fission and which do not form their sexual states within or upon a fruiting body (Barnett, 1992; Kurtzman & Fell, 1998b; Pretorius *et al.*, 1999). Yeasts are divided into more than 700 species (De Barros Lopes *et al.*, 1998).

Originally, wine was produced by spontaneous fermentations with the natural microflora occurring on grapes. However, large-scale wine production, in which rapid, reliable and trouble-free fermentations are essential for consistent wine flavour and predictable quality, necessitates the use of selected pure yeast inocula of known ability (Henschke, 1997). It is these large wineries that will be the main beneficiaries of programmes aimed at yeast strain development. These programmes will produce new strains that have even more reliable performance, thereby reducing processing inputs and consequently facilitating the production of high quality wines (Henschke, 1997). The monitoring of induced fermentations gave an understanding of the evolution of the entire microflora during the fermentation process, making clear that wine quality is a consequence of the dynamics and composition of the microorganisms involved in its production (Querol *et al.*, 1992b; Schütz & Gafner, 1994; Pramateftaki *et al.*, 2000). Extensive ecological surveys of wine yeast strains from a number of different ecosystems (Vezinhet *et al.*, 1992; Van der Westhuizen *et al.*, 1999; 2000) have led to the introduction of more suitable and better characterised strains for commercial use (Vezinhet *et al.*, 1990; Querol *et al.*, 1992b; Lavallée *et al.*, 1994). Unambiguous identification of wine yeast strains has always been a prime concern in the wine industry, because the identification and characterisation of wine yeast strains is essential not only in technology and the extending field of oenology, but also in the production of food and other beverages.

In the past, biochemical and physiological characteristics were used as the main criteria for yeast classification and identification. Currently, more than 70 tests, such as the utilisation of more than 36 carbon sources and nitrogen compounds, can be used to identify yeasts (De Barros Lopes *et al.*, 1998). However, the unambiguous identification of wine yeast strains with the traditional morphological and physiological properties is affected by culture conditions and also requires great expertise and skill.

Due to the drawbacks of traditional classification and identification techniques, techniques based on genetic relatedness and nucleic acid methods have been developed and, in recent years, molecular taxonomy has dominated yeast classification (Pretorius *et al.*, 1999, Pretorius, 2000). The different molecular methods include DNA base composition (mol % G + C), genome reassociation, gene sequencing, chromosome karyotyping and the polymerase chain reaction (PCR).

These tests also make the classification of yeast at the strain level possible, which is not usually possible with traditional methods.

This literature study is a broad review of the current yeast identification techniques used in the wine industry. The first section of the review will briefly discuss the ecology of grape and wine yeasts to emphasise the need for fast and accurate identification techniques.

2.2 ECOLOGY OF GRAPE AND WINE YEAST

2.2.1 Occurrence of yeast on grapes

Yeasts can be isolated from terrestrial, aquatic and aerial environments, but preferred habitats are plant tissues (Pretorius *et al.*, 1999). The microflora of grapes are shown in Table 1.

TABLE 1

Classification of grape and wine sporogeneous and asporogeneous yeast genera (Kreger-Van Rij, 1984).

Saccharomycetaceae **family** (sporogeneous)		
Sub-family *Schizosaccharomycetoideae*	**Sub-family** *Nadsonioideae*	**Subfamily** *Saccharomycetoideae*
Genus *Shizosaccharomyces*	**Genus** *Saccharomycodes* *Hanseniaspora*	**Genus** *Saccharomyces* *Debaryomyces* *Dekker* *Hansenula* *Kluyveromyces* *Pichia* *Zygosaccharomyces* *Torulaspora*
	Spermophtoracae **family** (asporogeneous)	*Cryptococcaceae* **family** (asporogeneous)
	Genus *Metschnikowia*	**Genus** *Brettanomyces* *Candida* *Kloeckera* *Rhodotorula*

The microflora on grapes (Table 1) are highly variable, but consist predominantly of the low ethanol-tolerant species of *Kloeckera* and its anamorph, *Hanseniaspora*, which constitute about 50-75% of the grape microflora. Present to a lesser extent, though significant by their presence, are the species *Candida*, *Brettanomyces*, *Cryptococcus*, *Kluyveromyces*, *Pichia* and *Rhodotorula* (Pretorius *et al.*, 1999). *Saccharomyces cerevisiae*, a fermentative and high alcohol-tolerant species, occurs

in very low numbers on grape skins. The microbiota of grapes are affected by several factors, which are listed in Table 2.

TABLE 2

Factors influencing the microflora of vineyards, grapes, wineries and must (Pretorius *et al.*, 1999).

Microflora of vineyards and grapes
Climatic influences
-temperature
-rainfall
-wind
-microclimate, as affected by viticultural practices such as canopy management
Soil and viticultural practices
-soil type
-fertilisation
-irrigation
-application of fungicides
Grape
-variety
-physical damage by mould, insects or birds
Microflora of winery equipment surfaces
Nature of surfaces
-irregular, unpolished surfaces
-cracks and welds
Microflora of grape must
Method of grape harvest
-handpicked or mechanical
-grape temperature
Transport from vineyard to wine cellar
-time
-initial grape temperature
-air temperature
-sulphite addition
Condition of grapes
-time
-temperature
-sulphite addition
Must treatment
-cellar hygiene
-aeration
-sulphite addition
-clarification method
-temperature
-inoculation with yeast starter cultures

2.2.2 Occurrence of yeast in must

Although grape must is relatively complete in nutrient content, the low pH and high sugar content of the must exert a strong selective pressure on microorganisms. Fermenting must is selective once anaerobic conditions are established, since certain nutrients become depleted and increasing levels of ethanol eliminate alcohol-sensitive microbial species (Pretorius *et al.*, 1999). These conditions are highly selective for fermentative and high alcohol-tolerant species like *S. cerevisiae* and *Saccharomyces bayanus*, which occur in very low numbers on grape skins, but are abundant in the grape juice and on the must-coated surfaces of winery equipment. Therefore, *S. cerevisiae* is the most important and dominant yeast species colonising surfaces in wineries. The occurrence and extent of development of the *Saccharomyces* species and other wine yeast species, such as *Candida* and *Brettanomyces*, vary according to the degree to which the winery surfaces have been cleaned and sanitised. Irregular and unpolished surfaces, such as cracks and welds, may contain dense populations of winery yeasts (Pretorius, 2000) (Table 2). However, certain other factors, as listed in Table 2, may also affect the microflora in must.

2.2.3 Occurrence of yeast in wine

During the first and middle stages of fermentation, apiculate yeast species, such as *Kloeckera* and *Hanseniaspora* and to a lesser extent *Candida*, *Rhodotorula* and *Hansenula*, are predominant until the ethanol levels rise to about 3-4%. Later during fermentation, the prevailing *S. cerevisiae* dominates and completes the fermentation, with ethanol levels reaching 12-14%. Other yeast species, such as *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*, may also be present during the fermentation process. These microbes are reported to be capable of adversely affecting the sensorial quality of the wine, resulting in spoilage (Kreger-Van Rij, 1984; Fleet, 1993; Fleet, 1998; Pretorius *et al.*, 1999).

2.3 YEAST TAXONOMY

New taxonomic surveys and identification should not be a meaningless catalogue or inventory of yeast names, but rather be a study of yeast species in the context of their environment (Lachance & Stramer, 1998; Pretorius *et al.*, 1999). Several molecular biological taxonomic approaches are now assisting in the fundamental understanding of yeast communities in specific habitats and niches (Pretorius *et al.*, 1999). This will provide improved means of preserving and exploiting yeast biodiversity (Roberts & Wildman, 1995; Pretorius *et al.*, 1999) and monitoring the spreading of genetically modified yeasts when they are used for the production of fermented foods and beverages.

Yeast taxonomy, as a whole, is based on two main criteria, namely classification and identification. Classification is the grouping of organisms into *taxa* according to

their similarities and/or their ties to a common ancestor. Identification, on the other hand, is the comparison of unknown organisms to individuals already classed and named according to similar characteristics (Kurtzman & Fell, 1998a).

The primary level of yeast classification is based upon certain aspects of their sexuality (Ascomycotina or Basidiomycotina) or the lack of a certain sexual phase in the life cycle (Deuteromycotina) or fungi imperfecti (Kurtzman & Fell, 1998a). Secondary taxonomic subdivisions, i.e. *families, subfamilies, genera, species* and *strains*, are based upon various morphological, physiological and genetic criteria, including sexual reproduction (Kurtzman & Fell, 1998b; Pretorius *et al.*, 1999). However, many of these criteria, as well as sexual compatibility studies used for the speciation of yeasts, are derived from a small portion of the genome and are therefore often unreliable and time consuming (Pretorius *et al.*, 1999).

These phenotypic characteristics, such as microscopic appearance and ability to use certain substrates, are highly mutative, i.e. can be reversed by a mutation in a single gene. These morphological and physiological classification methods are therefore inadequate for routine yeast identification. The biological concept, which delimits species on their ability to hybridise, is also problematic in yeast systematics, i.e. a lack of fertility does not preclude conspecificity, since only a few genes can affect the ability to mate (De Barros Lopes *et al.*, 1999; Pretorius *et al.*, 1999). Although these phenotypic traits do not necessarily reflect genetic relatedness, because the phenotype might be a result of convergent evolution, they serve a purpose in yeast taxonomy since they are not all unstable and insignificant (Pretorius *et al.*, 1999).

2.4 MORPHOLOGICAL AND PHYSIOLOGICAL TESTS

Yeast identification has always been a key issue in applied microbiology. Yeasts are significant in foods and wine, as they play important roles in both production and spoilage (Török & King, 1991; Praphailong *et al.*, 1997). Due to the increasing involvement of these microorganisms in research and development, as well as in the technological and medical fields, it is very important to have accurate identification techniques which are rapid and inexpensive (Török & King, 1991).

Conventional yeast taxonomy was generally based on phenotypic traits such as morphological characteristics, sexual reproduction and certain physiological and biochemical features, which allowed for differentiation between species (Lodder & Kreger-Van Rij, 1952; Lodder, 1970; Kreger-Van Rij, 1984; Barnett *et al.*, 1990; Pretorius & Van der Westhuizen, 1991). Therefore, the first tests used in wine yeast identification had to do with cellular morphology, which is followed by the question of sexual spore-forming capabilities (Table 3).

TABLE 3

Key to the wine-related yeast genera (Kurtzman & Fell, 1998a).

I. Fission: *Schizosaccharomyces*

II. Bipolar budding (on broad base)
  A. No spores: *Kloeckera*
  B. With spores
    1. Spores spherical and smooth (conjugation in ascus): *Saccharomycodes*
    2. Spores hat or helmet shaped (no conjugation in ascus): *Hanseniaspora*

III. Multilateral budding
  A. Strong acetic acid from glucose, resistant to cycloheximide, some cells ogival, pungent odour on old plates, colonies clear $CaCO_3$
    1. Spores found: *Dekkera*
    2. Spores not found: *Brettanomyces* (may form nonseptate mycelia)
  B. Not A
    1. Spores found
      a. Nitrate assimilated: *Hansenula*
      b. Nitrate not assimilated
        i Spores needle-shaped: *Metschnikowia*
        ii. Spores hat or saturn shaped: *Pichia*
        iii. Spores reni- or crescentiform: *Kluyveromyces*
        iv Spores spherical or ellipsoidal
          (1) Asci dehiscent: *Kluyveromyces* or *Pichia*
          (2) Asci persistent
            (a) Conjugation immediately preceding ascus formation: *Torulaspora, Debaromyces, Pichia, Zygosaccharomyces*
            (b) Conjugation after ascus formation: *Saccharomyces*
    2. No spores found
      a. Colonies pink or red
        i Inositol assimilated: *Cryptococcus*
        ii. Inositol not assimilated: *Rhodotorula*
      b. Colonies not pink or red
        i. No arthrospores; inositol assimilated: (no pseudomycelium): *Cryptococcus*
        ii. Not i: *Candida*

Most of the dichotomous keys used were based on the concept of answering a series of questions regarding the properties and traits of the organisms under study. The answers to these questions are mutually exclusive, i.e. either the organism has the trait or not. The questions must, however, be asked in a proper sequence for correct identification, depending on the number and different kinds of genes required for the given property (Kreger-Van Rij, 1984). Other traits may reflect the difference in single genes. Yeasts are classified according to their spore-forming abilities as

perfect/complete yeast (those which do form spores) and imperfect/incomplete (those which do not form spores) (Table 3). It can be seen that there are other factors that must also be included in the questions being asked (Table 3), such as on the type of cell division (fission, bipolar budding or multilateral budding) and colonial morphology. The colour of the colony might be an important trait and is imperative in the identification process (Barnett, 1992). However, for these tests, the cultures need to be in a growing phase and must regularly be examined microscopically. These characteristics can be used to differentiate between the principal grape and wine yeasts. However, some of these properties might vary within the species and are even unstable for a given strain.

To facilitate the rapid identification of yeast isolates, several identification kits and automated systems have been developed, but most were initially only used for the identification of food-borne yeasts. These kits use standardised methods and previously prepared substrates; they are convenient to use and have an acceptable degree of accuracy (Török & King, 1991; Deák & Beuchat, 1993). Most commercial identification systems utilise carbohydrate assimilation tests that require 1-3 days of incubation before results can be recorded (Deák & Beuchat, 1993).

The API 20C kit is probably the most widely used manual kit and has often been considered as a reference method for evaluating other systems (Deák & Beuchat, 1993; Deák & Beuchat, 1995). The API 20C strip consists of 20 cupules, 19 with dehydrated carbohydrate substrates for assimilation reactions and one as a negative control. Adding a suspension of test yeast in API 20C basal medium constitutes the reaction. Reactions can be read at different times of incubation (i.e. 24, 48 and 72 h) at 30°C. Each isolate is identified by entering the test data into a seven-digit numerical profile that is then compared to profiles listed in the API analytical profile index for the yeast species included in the database. The profile index also indicates the likelihood of correct identification, expressed as a ratio of estimated frequency of occurrence, as well as some additional tests to distinguish between species with similar numerical profiles (Deák & Beuchat, 1993).

The extended API ID 32C assimilation kit, which can be used manually or automated, can be used in conjunction with a commercial computer program (Deák & Beuchat, 1993; Deák, 1995). However, the databases for these commercially available systems consist of only a few medically important yeast species, restricting their suitability for identifying food-borne yeast and wine yeast strains. The yeast ID 32C strip consists of 32 cupules, 30 containing dehydrated carbohydrate substrates, one containing 0.01% cycloheximide and one serving as a control. Cupules are inoculated with a yeast suspension, and reactions can be read at different times of incubation (i.e. 24, 48 and 72 h) at 30°C. Data obtained are used to construct an eight-digit numerical profile. This profile is compared to possible biocodes for species in the database of the ID 32C profile index, which also give the mathematical probability of correct identification.

Simplified identification methods (SIM) have also been developed for the identification of a predetermined group of food yeasts (Deák, 1993; Deák & Beuchat, 1993; Deák, 1995; Deák & Beuchat, 1995). The SIM method is based on a reduced number of tests selected as most appropriate and effective for the identification of a group of predetermined yeast strains. The routine identification protocol usually includes 15 tests. Supplementary tests are performed when identification needs to be confirmed. The standard identification protocol of SIM also includes microscopic examination to determine if cells form sexual spores, arthroconidia and true and/or pseudohyphae. Incubation is done at 30°C. Based on the results of the urease reaction and the assimilation of nitrate, erythritol, cellobiose and mannitol, an isolate is placed into one of seven groups in a master key. Within each group, presumptive identification is made by following a dichotomous key using the results of the other tests, or by performing a few supplementary tests. The identification of each isolate is verified by comparing test results with the SIM data matrix, which contains, in tabulated form, the probabilities of positive characteristics of each yeast species included in the database (Deák & Beuchat, 1993).

In contrast to these growth-based systems, enzyme-based systems have also been introduced. These systems are based on detecting enzyme activities using chromogenic substrates and have the advantage of generating results within 4 h of inoculation, making them very rapid. The Baxter MicroScan enzyme-based kit is frequently used in the identification of yeasts (Deák & Beuchat, 1995). The kit contains dehydrated chromogenic substrates, and reactions are visible because of the hydrolysis of the substrates. Substrates are dehydrated with a concentrated suspension of yeast and incubated at 37°C for 4 h. Reactions can be monitored visually or by an automatic apparatus (AutoScan). Results are converted into a 9-biocode number that lists species identification and a cumulative relative probability of identification (Deák & Beuchat, 1995). The successful application of the above-mentioned techniques in the food and wine industries relies heavily on the technical development of the commercialised identification systems, assisted by improved computerised databases with more food and wine yeast strains included in the databases.

2.5 FATTY ACID ANALYSIS

Differentiation with cellular fatty acid analysis is another technique used for yeast identification (Bendová *et al.*, 1991; Augustyn *et al.*, 1992; Rozes *et al.*, 1992; Botha & Kock, 1993; Van der Westhuizen *et al.*, 1999). Yeasts are cultivated (duplicate or triplicate) in a medium that consists of yeast nitrogen base and glucose (60 g/L - 80 g/L). Cells are harvested in the stationary phase, as the fatty acid profiles (CFAP) are more stable. However, cultivation parameters might vary, as all yeasts cannot be cultivated with a single set of rigid cultivation parameters (Augustyn *et al.*, 1992). Fatty acid analysis, with gas chromatographic (GC) analysis, is performed with the

terminal methyl carbon as the reference (Bendová *et al.*, 1991; Augustyn *et al.*, 1992). Computer programs with specific software that allows multiple variant analysis, and principal component analysis (PCA) to define clusters of fatty acid profiles, are used in the differentiation between the strains (Rozes *et al.*, 1992). The fatty acid profiles of each yeast strain are reproducible, but variation exists within species (Botha & Kock, 1993). In general, the fatty acid analysis technique is reliable but time consuming, which makes it unsuitable for the routine classification of yeasts (Van der Westhuizen *et al.*, 1999). The fatty acid analysis technique is not a generally applicable technique for yeasts, but was found to be a valuable chemo-taxonomical tool to distinguish between strains of certain species of certain genera (Botha & Kock, 1993).

2.6 MOLECULAR IDENTIFICATION

Morphological, physiological and biochemical techniques used in the past allowed distinction between species, but are time consuming and require great expertise and skill. Furthermore, many of the conventional criteria were derived from the analysis of a small portion of the genome. Phenotypic characteristics serve a purpose in classification, since not all of these traits are unstable and insignificant. However, these phenotypic traits do not necessarily reflect genetic relatedness, since the same phenotype may be the result of convergent evolution. Conversely, the phylogenetic relationships should be reflected in similarities at the level of the base composition of deoxyribonucleic acid (DNA) and DNA sequence homology in different yeasts (Pretorius & Van der Westhuizen, 1991). Some of the molecular techniques that have been successfully used to characterise yeast strains will be discussed briefly.

2.6.1 Protein Fingerprinting

Protein fingerprinting is a simple and rapid technique by which extracted cellular yeast and bacterial proteins create a specific fingerprint pattern when separated by sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis (Dowhanick *et al.*, 1990). The total set of proteins of a cell is constant and remains unchanged when grown under standardised conditions, because protein expression is genetically determined. Proteins of genetically related strains display similar or almost identical electropherograms (Pretorius & Van der Westhuizen, 1991). These fingerprint patterns may be analysed for relative differences or similarities and categorised on the basis of this information. A few samples can be compared visually, but the quantitative comparison and grouping of normalised densitograms of many electropherograms can only be done with computer programs. These programs compare the relative mobility, the sharpness of bands and the relative protein concentrations of the peaks and valleys (Pretorius & Van der Westhuizen, 1991). Protein profiles can also be used for the differentiation of the parental and hybrid

strains of yeasts by obtaining the genetic karyotypes, which are very important in breeding programmes.

When using this technique, it is important to take into account that yeasts consist of a plethora of differentially regulated genes that are either expressed or repressed in response to external stimuli. A particular protein fingerprint pattern results from the specific gene expression of an isolate grown under a specific set of conditions. These conditions may include the medium in which the isolates are grown, the incubation temperature, the nature of the gaseous environment and the growth phase at the time of harvesting. Due to the effect on the type of proteins and the concentrations thereof that are obtained by differences in culture conditions, pre-used controls should never be compared to freshly prepared samples (Dowhanick *et al.,* 1990). Well-characterised reference proteins should be used to compare and normalise the electropherograms of the unknown yeast strains.

2.6.2 Mt-DNA restriction analysis

Among the molecular techniques being used, mitochondrial DNA (mt-DNA) restriction analysis appears to be one of the most suitable methods to differentiate between strains (Vezinhet *et al.*, 1990; Querol *et al.*, 1992b; Vezinhet *et al.*, 1992; Ness *et al.*, 1993; Nadal *et al.*, 1996; Querol & Ramón, 1996; Pramateftaki *et al.*, 2000; Lòpez *et al.*, 2001). Yeast mitochondria contain short (20-80 kb) circular DNA molecules, which are present in many identical copies in each cell and in each mitochondrion. Growing on a non-fermentable substrate, a yeast cell contains 50 molecules of mt-DNA, representing 5-25% of the total cell DNA (Smole Možina & Raspor, 1997). With the application of restriction endonucleases, it is possible to get a few restriction fragments with a high DNA concentration, ensuring clear electrophoretic restriction patterns. In principle, mt-DNA has a low GC content when compared with the nuclear DNA of the same species. Therefore, differences in mt-DNA patterns can be revealed by digesting total genomic DNA with endonucleases that recognise GC-rich nucleotide regions. In this way, the nuclear DNA is cleaved into small fragments, allowing higher molecular weight mt-DNA bands to appear (Pramateftaki *et al.*, 2000). Mt-DNA is isolated and digested with restriction endonucleases, which recognize 4 or 5 bp sequences, depending on which restriction endonucleases are used. Restriction fragments are then separated on agarose gels (1%) by electrophoresis in TBE (Tris-Boric acid-EDTA) buffer. However, technically complicated mt-DNA isolation in the ultracentrifuge does not permit the routine industrial application of mt-DNA analysis. To avoid this problem, a simplified procedure was developed on the basis of the standard miniprep isolation of total DNA, with the use of selected endonucleases (Smole Možina & Raspor, 1997).

Querol and Ramón (1996) developed a new technique of mt-DNA restriction analysis. The technique involves the standard miniprep isolation of total DNA and digestion with specific endonucleases that recognise either four-base pair sequences (*Alu*I, *Hae*III, *Hpa*I) or five base pair sequences (*Mae*III) and also six base pair

sequences (*Eco*RI, *Pst*I, *Eco*RV) (Querol *et al.*, 1992b). Only a few sites are recognised by the restriction enzymes in mt-DNA, although a large number are recognised in yeast nuclear DNA. The length and size of the mt-DNA fragments can easily be detected using agarose gel electrophoresis. According to Querol and Ramón (1996), it is then possible to detect restriction fragment length polymorphisms (RFLP) between the yeast mt-DNAs without having to purify the mitochondria, i.e. using a sucrose gradient and ultracentrifugation. The method is rapid (3 h extraction, 2 h digestion and 2 h electrophoresis), simple, reproducible and can differentiate yeast isolates from the same must.

Lòpez *et al.* (2001) used an adaptation of the method devised by Querol and Ramón (1996). In this adapted method, the steps that were affected the most were the propagation of yeast cells and restriction analysis: cell growth was reduced to 36 h by using microfuge tubes, and the restriction analysis was carried out in 33 min using a microwave oven for DNA digestion and minigels for restriction fragment separation (Lòpez *et al.*, 2001). DNA was extracted with the same procedures as in the original protocol, but with a reduction in the duration of each step and scaling down of the volumes of the different solutions, enzymes and reagents being used. As a result, a large time reduction (52.5 h) was obtained compared to the original method (Lòpez *et al.*, 2001). The DNA obtained can be digested directly with endonucleases, displaying clear restriction patterns that are useful for *S. cerevisiae* yeast strain differentiation (Fig. 1).

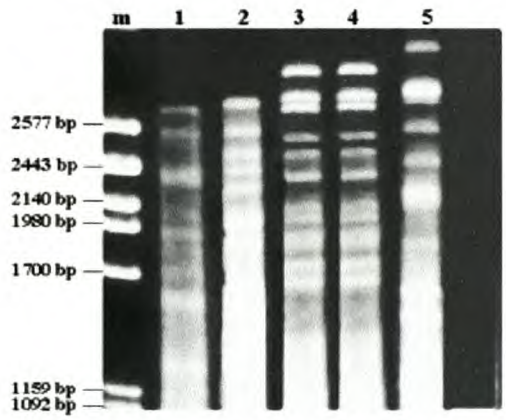

FIGURE 1

Application of the new, adapted method of mtDNA restriction analysis with *Hinf*I to *S. cerevisiae* strains. Lane 1, SK (brewer's yeast); lane 2, CECT 1833 (brewer's yeast); lane 3, P39 (baker's yeast); lane 4, CR (baker's yeast); lane 5, Uvaferm 71B (wine yeast). Lane m corresponds to a lambda DNA (digested with *Pst*I) size marker (López *et al.*, 2001).

A mitochondrial molecular marker has also been reported for the differentiation of yeast species (Smole Možina & Raspor, 1997). Diagnostic differences are based on the presence/absence of *ori-rep-tra*, a special class of mitochondrial intergenic sequences, in industrially important *Saccharomyces* yeasts and in some other yeast

genera (*Zygosaccharomyces*, *Torulaspora*, *Debaryomyces*, *Kluyveromyces*). *Ori-rep-tra* sequences play a role in mt-DNA inheritance (Smole Možina & Raspor, 1997).

2.6.3 DNA fingerprinting

Yeast chromosomes are never in a mitotically condensed form and are therefore not visible under a microscope, unlike the chromosomes of higher eukaryotes such as plants and animals (Pretorius & Van der Westhuizen, 1991). The yeast chromosomes can thus not be karyotyped by the conventional methods used for higher plants and animals, but rather need techniques based on DNA fingerprinting. DNA fingerprinting describes any procedure that provides a unique profile of DNA for a given organism (Mitrakul *et al.*, 1999). DNA fingerprinting includes various molecular methods, such as electrophoretic karyotyping, restriction fragment length polymorphism (RFLP), random amplified polymorphism analysis (RAPD-PCR) etc.

2.6.3.1 Karyotyping by chromosomal banding patterns

One approach to yeast DNA fingerprinting evolved from the development of a special type of agarose gel electrophoresis enabling separation of very large DNA fragments (200 kb-10 Mbp, i.e. the size range of intact yeast chromosomes). Chromosomal DNA fragments are reoriented in the gel due to periodic changes in direction of the electric field. The time of reorientation correlates with the molecular size. This is the basic principle for the separation of large DNA fragments by all types of pulsed field gel electrophoresis (PFGE) (Smole Možina & Raspor, 1997) (Fig. 2).

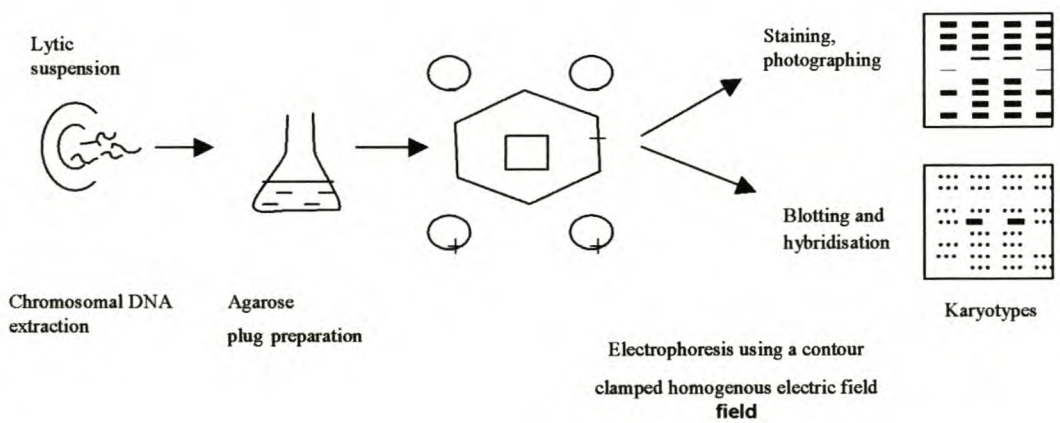

FIGURE 2

Schematic diagram of electrophoretic karyotyping (Deák, 1995).

The contour-clamped homogenous electric field gel electrophoresis (CHEF) system is probably the most commonly used electrophoretic system in the wine industry (Sheehan & Weiss, 1991; Van der Westhuizen & Pretorius, 1992; Török *et al.*, 1993; Schütz & Gafner, 1994; Van der Westhuizen *et al.*, 1999; Van der Westhuizen *et al.*, 2000). Van der Westhuizen and Pretorius (1990) reported the first

electrophoretic karyotypes of wine yeasts using the CHEF system. They showed that it was possible to differentiate between parental and hybrid strains in breeding programmes and pointed out genetic drift over a number of years in a particular yeast strain by using the CHEF method. Other applications of CHEF gel electrophoresis include characterisation of *Saccharomyces* strains (Van der Westhuizen & Pretorius, 1992; Van der Westhuizen *et al.*, 1999; Van der Westhuizen *et al.*, 2000; Khan *et al.*, 2000), identification of brewing yeasts and chromosome analysis (Sheehan & Weiss, 1991; Tornai-Lehoczki & Dlauchy, 2000), and the investigation of electrophoretic karyotypes of *Torulaspora* strains and comparison with *Zygosaccharomyces* strains (Oda & Tonomura, 1995) (Figs. 3 and 4).

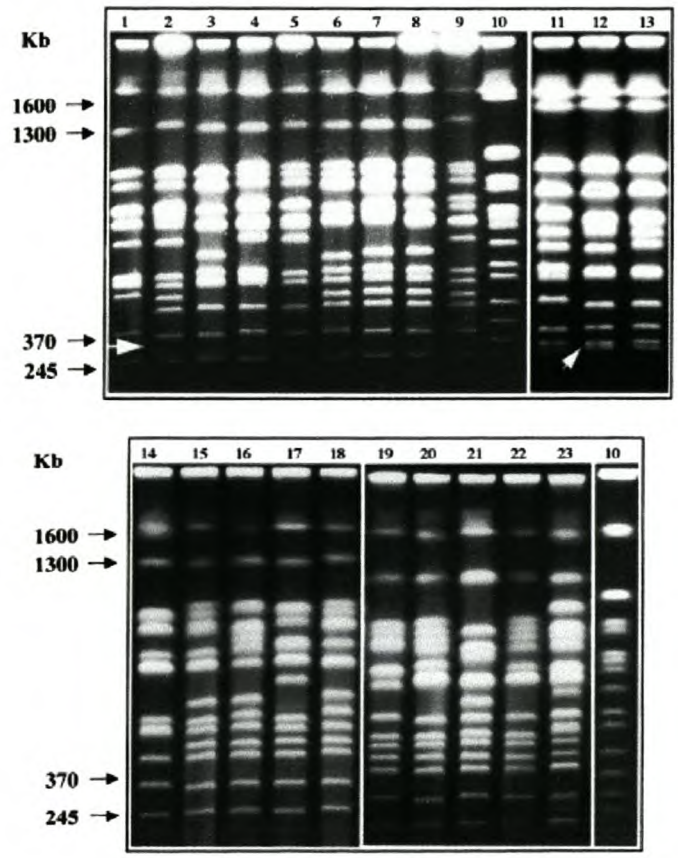

FIGURE 3

CHEF gel electrophoresis of chromosomal DNAs from cider *Saccharomyces* strains and from reference testers of *S. cerevisiae*, *S. bayanus* var. *uvarum* and *S. bayanus* var. *bayanus*. Lanes 1-9, 14-22: *S. bayanus* var. *uvarum* SRC 173, SRC 258, SRC 274, SRC 306, SRC 343, SRC 410, SRC 415, SRC 437, SRC 494, SRC 61, SRC 76, SRC 128, SRC 223, SRC 570, SRC 296, SRC 459, SRC 467 and CBS 395, respectively. Lanes 10-13: *S. cerevisiae* YNN295, SCR 120, SRC 147 and SRC 213, respectively. Lane 23: *S. bayanus* var. *bayanus* CBS 380. Arrowheads indicate two small chromosomal bands in *S. bayanus* var. *uvarum* and three in *S. cerevisiae*. The chromosome sizes refer to the chromosomes of the strain YNN 295 (Naumov *et al.*, 2001).

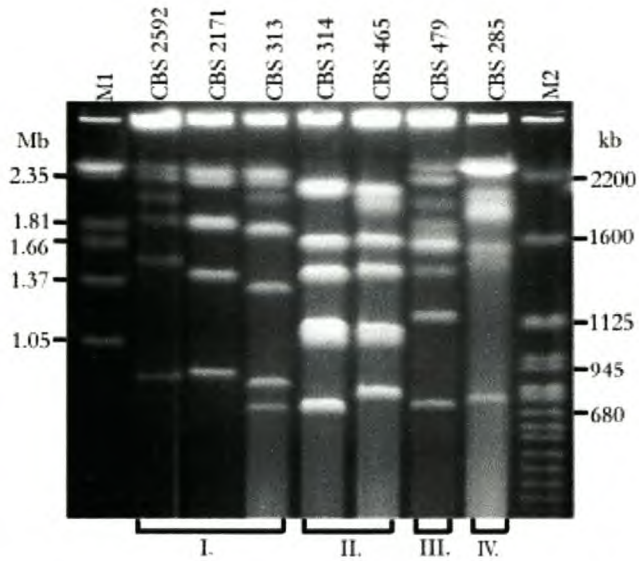

FIGURE 4

Electrophoretic karyotypes of *Hanseniaspora-Kloeckera* type strains after CHEF electrophoresis. M1, chromosomal DNA of *P. canadensis* YB-4662-VIA as size marker; M2, chromosomal DNA of *S. cerevisiae* YNN295 as size marker (both Bio-Rad) (Cadez *et al.*, 2002).

2.6.4 Polymerase chain reaction (PCR)-related techniques

The basic PCR protocol involves the use of two oligonucleotide primers, which bind to sequences flanking the target sequence on each strand of DNA to initiate DNA synthesis. Using a heat-stable DNA polymerase, 20-30 repeated cycles of denaturation, followed by primer annealing and finally by primer extension, result in the amplification of DNA by doubling the target sequence during each cycle (Deák, 1995) (Fig. 5). PCR fingerprinting seems to be adequate for both inter- and intra-species differentiation, depending on the oligonucleotide primers being used. Recent developments in molecular biology have shown that many repeated sequences, such as τ, σ and the directly repeated δ sequences of 0.3 kb that flank the TY1 retrotransposon, might be used in identification with PCR (Ness *et al.*, 1993).

2.6.4.1 Genetic tagging using δ sequences

The *TY1* element is present in approximately 35 copies dispersed throughout the genome of laboratory yeast strains. About 100 copies of the δ sequences are present in the yeast genome as a part of *TY1* or as isolated elements. Statistically, there is about one every 150 kb of nucleotides (Ness *et al.*, 1993). The δ sequences, directly or inversely repeated, are often distributed in genomic regions contiguous to the tRNA genes (Ness *et al.*, 1993) and are separated by amplifiable genomic distances.

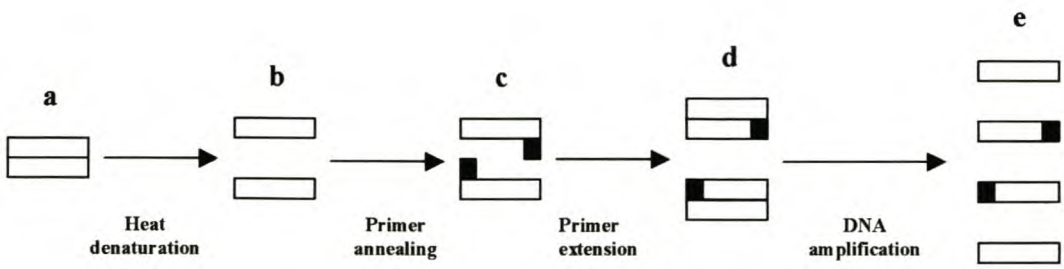

FIGURE 5

Principle of the polymerase chain reaction (PCR). A double-stranded DNA template (a) is denatured by heating; (b) oligonucleotide primers are annealed to complementary strands of DNA; (c) primers are extended by DNA polymerase; (d) repeating the cycle (a) – (d) 20-30 times results in the amplification of the DNA products (e) (Deák , 1995).

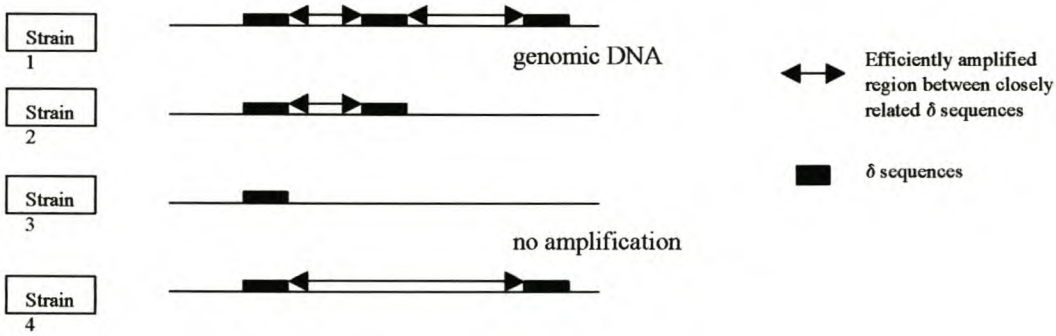

FIGURE 6

Different possible situations for the repartition of δ sequences on the same chromosome in different strains. The bands obtained on the pattern and corresponding to the amplified region of the DNA are different in size and number (Ness *et al.*, 1993).

Two primers, with consensus sequences complementary to the 5′ termini of the δ elements, are used to amplify the chromosomal sequences between the two elements with PCR (Fig. 6). The position and number of the δ elements are different for each strain; therefore the amplified products will be different for the strains. The amplification of the yeast genomic DNA δ1 and δ2 primers revealed amplified sequence polymorphism (Ness *et al.*, 1993). Even if the number of the δ elements seems less variable (Ness *et al.*, 1993), the number of the sequences associated with a difference in position are able to generate polymorphism. Although the *TY1* position varies over several generations due to transposition, reproductive patterns can still be detected (Ness *et al.*, 1993). Cells have been grown over 300 generations to see the event of transposition. It was found that, even under these conditions, the variation was low and relatively infrequent with the overall profiles obtained. Using customised oligonucleotides, some regions of the yeast genome are amplified to reveal sequence polymorphism characteristic of the strains. It is therefore possible to identify individual strains of *S. cerevisiae*.

2.6.4.2 Genetic tagging using microsatellite markers

Computer searches have been conducted for short, tandem repeat patterns on several completely sequenced small genomes, including yeast genomes (Techera *et al.*, 2001). It was concluded that trinucleotide repeats could be used to genotypically characterise yeast strains. Quantitative selective genetic assays have been conducted to study the trinucleotide repeats in *S. cerevisiae*. Expansion of these trinucleotide repeats was shown to be sequence specific and orientation dependent. Therefore, microsatellites might be used to differentiate between different yeast strains (Baleiras Couto *et al.*, 1996; Gallego *et al.*, 1998; Hennequin *et al.*, 2001). Microsatellites or simple sequence repeats (SSR) consist of direct tandem repeats of a short DNA motif, usually less than 10 bp, which have been shown to exhibit a substantial level of polymorphism ($\sim10^{-2}$ to $\sim10^{-5}$) in a number of eukaryotic genomes, because of the variation in length and repetitiveness due to DNA replication errors (Techera *et al.*, 2001). They can be used for molecular typing of cultivars such as *Vitis vinifera* and natural yeasts such as *Candida albicans* (Lunel *et al.*, 1998) and *Aspergillus fumigatus* (Bart-Delabesse *et al.*, 1998), as well as for paternity exclusion tests and forensic medicine. The complete sequence of the *S. cerevisiae* genome allows the identification of the regions because the absolute sizes of the microsatellite markers for the sequenced reference strain are known, therefore the sizes of the other DNAs can be determined according to the known sizes (Table 4).

An important advantage of the method is its portability. Since all results can be expressed as a number of repeats, computer translation is easy, offering the opportunity to compare results via the Internet (Hennequin *et al.*, 2001). This is an important issue for further comparisons of larger sets of strains isolated in very different settings, e.g. in human medicine, environmental studies and wine production.

TABLE 4

Different sizes of microsatellites markers (in bp) used in the study of Techera *et al.* (2001).

	Microsatellite loci		
	SCYOR267C	**SC8132X**	**SCPTSY7**
Yeast strain			
AB972	409	207	332
AWRI 796	427	195	311
CHP	421-427	186-197	N.D.*
M522	458	210-218	300
CP 863	392	156	352
CP 873	433	165	303
CP 874	433	210	303
CP 881	433-458	156-165	303
CP 882	433	210-218	303-350
CP KU1	392	156-210	352

For confirmation that the genetic differences and the microsatellite lengths are not due to mutations, independent experiments of DNA isolation from glycerol freeze cultures were performed (Techera *et al.*, 2001). It was confirmed that a different allele size for a unique microsatellite marker is appropriate to distinguish yeast strains. Techera *et al.* (2001) further concluded that microsatellite polymorphism analysis is a reproducible method, because specific primers and high annealing temperatures are used for DNA amplification of short fragments. It can also be done for the analysis of multiple samples and thus multiple microsatellite loci.

2.6.4.3 Genetic tagging using introns

De Barros Lopes *et al.* (1996; 1998) used a PCR-based method in which oligonucleotide primers complementary to yeast intron splice sites are used to differentiate between yeast at both the species and intraspecies levels (Fig. 7). Yeast introns are highly mutable, but the splice site sequences seem to be conserved in yeasts. Introns are not essential for gene function and therefore evolve with minimal constraint between yeast generations. Introns consist of certain conserved sequences (TACTAAC) or motifs that may be apparent for their removal during mRNA synthesis. If mutations occur at this sequence, they will prevent spliceosome assembly and cleavage of the 5′ splice site (De Barros Lopes *et al.*, 1996). The GTATGT sequence is primarily utilised at the 5′ splice site, whereas the (C/T)AG sequences occurs at the 3′ site of the intron. Polymorphisms can be screened with PCR by targeting these variable sequences in yeast genomes. De Barros Lopes *et al.* (1996) synthesised oligonucleotide primers complementary to yeast intron splice sites to target mutative regions of the genome. The primers E11, E12, LA1 and LA2, with sequences CTGGCTTGGTGTAGT, CTGGCTTGCTACATAC, GCGACGGTGTACTAAC and CGTGCAGGTGTTAGTA, respectively, were used to differentiate between commercial wine yeasts used in the Australian wine industry. Primers were used in pairs or singularly.

A new method, based on the variation in the number and position of introns in the mitochondrial gene *COX1*, has been developed to monitor inoculated wine fermentations (López *et al.*, 2002). This gene encodes the largest subunit of the cytochrome c oxidase, which has been reported to be the most intron-rich gene (López *et al.*, 2002). A variable number of introns has been reported in species of yeasts. The *COX1* intron variation exists between strains within a species. This is the case in *Kluyveromyces lactis* and *S. cerevisiae* that possess strains with one, three or four introns (Skelly *et al.*, 1991) and strains with six or seven introns (Foury *et al.*, 1998) respectively. Oligonucleotide primers homologous to the regions flanking the *S. cerevisiae COX1* introns have been designed and tested for wine yeast strain differentiation. Four primers were used in a multiple PCR reaction and proved very effective in uncovering polymorphism in natural and commercial yeast strains (López *et al.*, 2002). The advantages of the technique include speed, no

isolation of DNA and its simplicity. The main advantage is that the must sample can be used directly for the PCR reaction, which means that results can be obtained fast.

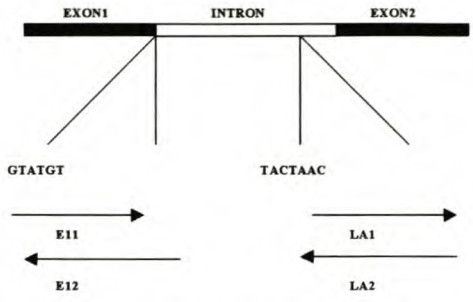

FIGURE 7

Design of intron primers. The sequences of primers E11 and E12 were based on the consensus sequence GTATGT at the end 5′ exon-intron splice site. The sequences of primers LA1 and LA2 were based on the lariat branch point consensus sequence TACTAAC (De Barros Lopes *et al.*, 1996).

According to De Barros Lopes *et al.* (1998), primer E11 used in their PCR reactions was effective in identifying yeasts on grape surfaces as well as those found on winery equipment. De Barros Lopes *et al.* (1996) showed that several yeasts produced related amplification products. Although polymorphisms may exist between different *Saccharomyces* strains, the strains may also share common amplified products. A characteristic fingerprint for each strain will then be produced. The amplification products of unknown isolates of the vineyard and nature can also be compared to those of the known laboratory and commercially-used wine yeast strains. By doing this, an effective identification system can be produced (De Barros Lopes *et al.*, 1996).

2.6.4.4 Ribotyping

Ribotyping can be defined as a molecular differentiation method that combines PCR-amplified rDNA fragments and the use of restriction enzymes (in different combinations) to verify the specificity of restriction patterns for a number of yeasts. 18S rDNA + ITS1/2 and 25S rDNA cover more than 95% of the nuclear ribosomal DNA repeat unit of *Saccharomyces* sensu stricto and *Torulaspora* yeast and their anamorph forms (Smole Možina *et al.*, 1997). The ITS product length appears to be a useful characteristic for delineation within *Saccharomyces* (Valente *et al.*, 1996). As a result of these highly conserved and variable regions, Molina *et al.* (1992a & b), Kurtzman (1993) and Messner and Prillinger (1995) showed by means of sequencing and restriction fragment length polymorphisms (RFLPs) of yeast ribosomal DNA (rDNA) repeats that there are both inter- and intraspecific relationships of different yeast taxa. Although random amplified polymorphic DNA (RAPD) analysis can be used to show tight relationships at the species level, it is not reproducible and patterns of the fragments even differ within the same laboratory (Messner &

Prillinger, 1995; Molnár *et al.*, 1995). In contrast to molecular differentiation methods with the highest resolution, such as RAPD-PCR or fingerprinting, ribotyping can be used to bridge the gap between the resolution power of the RAPD analysis and the database compatibility of DNA sequencing because the results are highly reproducible (Messner & Prillinger, 1995).

With ribotyping, the full length of the rDNA repeats is divided into a section comprising the 18S rDNA + the internal ITS regions and the 5.8S rDNA gene (2605 bp), and a section that consists of the 25S rDNA fragment (3333 bp). The amplification reaction is then performed with different primers. After amplification, the fragments (aplicons) are cut with different restriction enzymes in various combinations to obtain specific and characteristic patterns (Fig. 8).

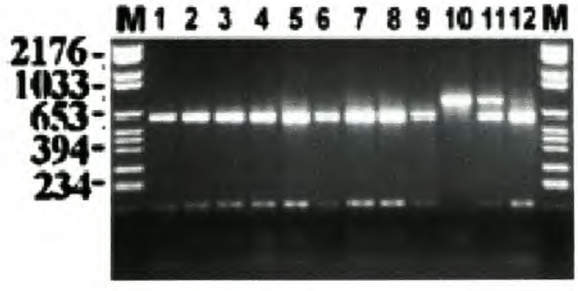

FIGURE 8

Photograph of the ethidium bromide-stained, UV-transilluminated, *Mae*I-digested PCR products after electrophoresis in a 3% agarose gel. The DNA from the PCR was first purified by the Wizard PCR Preps purification system prior to overnight digestion with the 10 U of restriction endonuclease *Mae*I. Molecular size markers are in lanes M, and their corresponding sizes (in base pairs) are given on the left of the figure. Lanes: 1, *S. boulardii* Sb 48; 2, *S. boulardii* Sb 49; 3, *S. boulardii* Sb It; 4, *S. cerevisiae* ATCC 52530; 5, *S. cerevisiae* ATCC 26108; 6, *S. cerevisiae* YJM128, a clinical isolate; 7, *S. cerevisiae* ItB9, a clinical isolate; 8, *S. cerevisiae* ItB8, a clinical isolate; 9, *S. bayanus* ATCC 76515; 10, *S. paradoxus* ATCC 76856; 11, *S. paradoxus/S. cerevisiae* hybrid YJM508; 12, *S. bayanus/S. cerevisiae* hybrid YJM334 (McCullough *et al.*, 1998).

Due to the high reproducibility of this technique, a database similar to the DNA sequence database can be constructed (Smole Možina *et al.*, 1997). The correctness of the ribotyping patterns can be checked easily, since the fragment staining intensity has to be proportional to its length upon complete digestion (Messner & Prillinger, 1995).

Because ribotyping reveals information across the full length of the rDNA repeat unit, it is possible to check which kind of phylogenetic information could be obtained from the fragment patterns. Phylogenetic computation data for type strains can be obtained from computerised databases (similar to DNA sequence databases) as soon as the respective DNA sequences are available. Ribotyping is well suited for screening large arrays of isolates prior to sequence analysis (Messner & Prillinger, 1995).

2.6.4.5 Restriction fragment length polymorphism (RFLP)

With RFLP, specific structural genes or other evolutionary conserved sequences are isolated, used as hybridisation probes and rapidly amplified with PCR. For characterisation, the amplified genes are treated with restriction enzymes for a possible identification of yeast at species or strain level, which can be followed by electrophoresis, blotting and probing (Fig. 9).

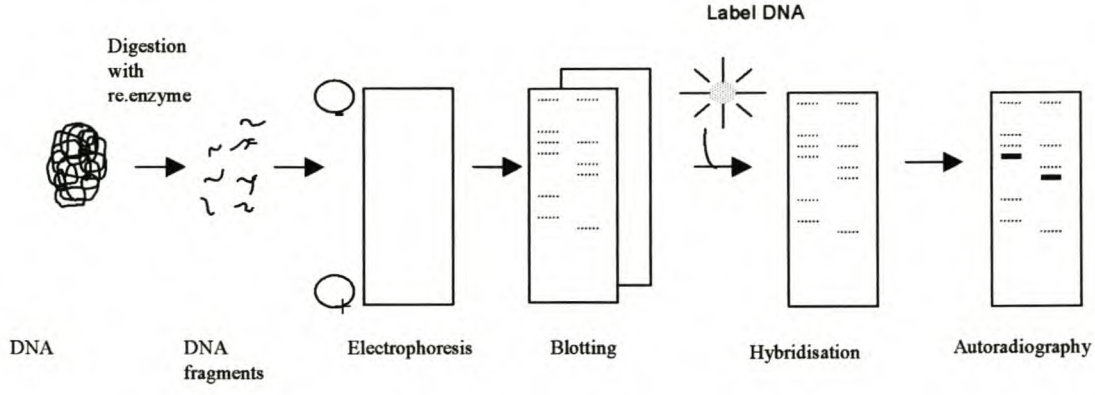

FIGURE 9

Schematic diagram of restriction fragment length polymorphism (RFLP) analysis (Deák, 1995).

Restriction enzymes cleave different sizes of base pair palindromes. The eight base pair palindromes have been used in RFLP and produce larger but fewer fragments. The restriction fragments are then separated with PFGE according to their size. It is important to know the sequence of the gene in order to know which restriction enzymes to use in the RFLP technique. RFLP analysis involves a considerable expenditure of time and effort on DNA extraction, restriction digestion, Southern blotting and hybridisation with probes (Deák, 1995).The banding patterns of the fragments on the agarose gels are stained with ethidium bromide and visualised under ultra-violet light (Fig. 10).

Molina *et al.* (1992a & b) used RFLP-PCR of the External Transcribed Spacer (ETS) and the Intergenic Spacer (IGS) of ribosomal DNA as a method to differentiate between *S. cerevisiae*, *Saccharomyces carlsbensis* and *Saccharomyces pastorianus*.

Nguyen and Gaillardin (1997) used the NTS2-ETS sequence of rDNA as target and the amplification achieved by primers annealing to the end and beginning of the 5S and 18S conserved sequences, respectively. They produced a 5' primer of 18 nucleotides: (upper primer) 5' AAC GGT GCT TTC TGG TAG 3', and a 3' primer of 16 nucleotides: (lower primer) 5' TGT CTT CAA CTG CTT T 3' after analysis of the sequence between 5' and 3', i.e. from nucleotide 1220 to nucleotide 2554 (Fig. 11).

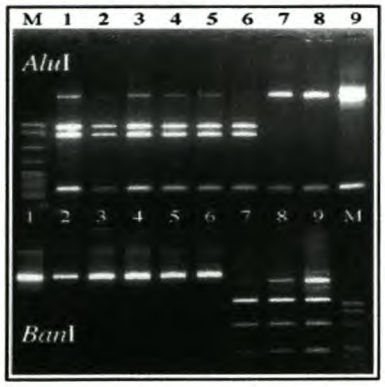

FIGURE 10

*Alu*I and *Ban*I restriction profiles of the NTS2 of cider *Saccharomyces* strains compared to the tester strains of *S. bayanus* var. *uvarum* MCYC 623 and *S. cerevisiae* VKM Y-502. Lanes 1–6: *S. bayanus* var. *uvarum* MCYC 623, SRC 258, SCR 274, SCR 306, SRC 410 and SRC 437, respectively. Lanes 7–9: *S. cerevisiae* SRC 120, SRC 147 and VKM Y-502. M, size marker pBR 322 digested with *Msp*I (Naumov *et al.*, 2001).

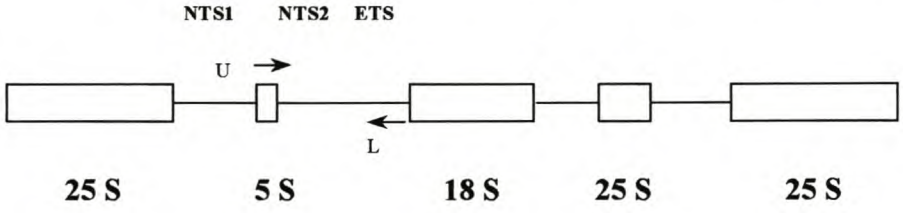

FIGURE 11

Structure of rDNA in *S. cerevisiae* showing the region amplified using the upper (U) and lower (L) primers (Nguyen & Gaillardin, 1997).

The primers amplify the variable Non-Transcribed Spacer sequence between the 5S and 18S conserved sequences of rDNA. An analysis of the RFLP patterns exhibited by amplified products appeared both species specific and variable from species to species (Nguyen & Gaillardin, 1997). After PCR amplification, the amplified products were digested with *Ban*I, *Alu*I, *Hph*I, *Nsp*I, *Sma*I and *Taq*I.

Two *Ban*I sites were obtained in the PCR product of *S. cerevisiae* (three bands of approximately 740, 420 and 220 bp), but none for *S. bayanus S. paradoxus* and *S. pastorianus*. Two types of *Alu*I patterns, *S. bayanus* and *S. pastorianus* with four sites (five bands of approximately 470, 370, 240,170 and 76 bp) and *S. cerevisiae* with one site, were shown. Three *Taq*I sites were obtained for *S. bayanus* and *S. cerevisiae* (one band at 680 bp, one doublet at 200 bp, one band at 160 bp).

Masneuf *et al.* (1996) used PCR/RFLP analysis of the *MET2* gene to differentiate between *S. cerevisiae* and *S. bayanus*. The synthetic oligonucleotide primers used for *MET2* amplification were 5′-CGAAAACGCTCCAAGAGCTGG and 5′-GACCACGATATGCACCAGGCAG, respectively, and the PCR products were digested with restriction enzymes. Restriction enzyme *Pst*I was used with *Eco*RI, *Nco*I and *Mae*III for *S. cerevisiae*, because there is no *Pst*I site in the *MET2*

sequence of *S. cerevisiae*, while a *Pst*I site exists in the corresponding genomic region of *S. bayanus*. The restriction fragments were separated on agarose gels with gel electrophoresis, stained with ethidium bromide and visualised under UV light. It was shown that *Eco*RI cleaved the *MET2* fragment of *S. cerevisiae*, but not that of *S. bayanus*. *Mae*III cleaved both the *MET2* fragments of *S. cerevisiae* and *S. bayanus*. *Nco*I cleaved the *MET2* fragment of *S. cerevisiae*, but not that of *S. bayanus*. DNA/DNA hybridisation was done to prove that the DNA consisted of intact chromosomes and not fragments.

Fernández *et al.* (1999) extracted the region between the 18S rRNA and 28S rRNA genes and amplified this with the specific internally transcribed spacers ITS1 and ITS4 primers. This region contains a highly conserved region of ribosomal 5-8S and a variable zone (Fernández *et al.*, 1999). The fact that both zones are combined in the same fragment makes it a useful tool for the differentiation of yeast strains with RFLP analysis.

Loureiro (2000) used RFLP-PCR to detect spoilage organisms in food and beverages. The small subunit rRNA-encoding region was amplified and digested with different four base pair-recognising restriction endonucleases. The simple repeat primers (GAC)5, (GTG)5 and M13 were used. However, discrimination between two related species is not always possible with RFLP-PCR. On one occasion, *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* could not be differentiated with the RFLP-PCR approach on single stranded (ss) rDNA (Loureiro, 2000). This similarity is in agreement with the DNA sequence data of the ss rDNA of both species. The two species could, however, be differentiated with the PCR fingerprinting approach (Baleiras Couto *et al.*, 1996; Loureiro, 2000). Loureiro (2000) used the data from PCR fingerprinting with the primer (GTG)5 to build a database for the rapid identification of newly typed yeast isolates. The database contains several fingerprints of *S. cerevisiae*, *S. bayanus*, *S. pastorianus*, *Z. bailii*, *Zygosaccharomyces* rouxii, *Z. bisporus*, *Pichia membranifaciens*, *Candida valida*, *Candida krusei, Candida lipolytica*, *Candida pelliculosa* and *C. lambica*. Loureiro (2000) extracted the internal transcribed spacer (ITS) and the external non-transcribed (NTS) spacer. Both spacers are present in and around the operon coding for rRNA.

2.6.4.6 Random amplified polymorphic DNA (RAPD) analysis

RAPD analysis allows for the direct analysis of amplified sequences without digestion with restriction enzymes, Southern blotting and hybridisation. The technique uses short oligonucleotide (5-15 mer) primers of arbitrary sequence at low annealing temperatures to hybridise at loci distributed randomly throughout the genome, thus allowing the amplification of polymorphic DNA fragments. These amplification products can be used as genetic markers and in the separation of species and strains of many organisms (Paffetti *et al.*, 1995; Quesada & Cenis, 1995; Cadez *et al.*, 2002) (Fig. 12). With the RAPD technique, it is not necessary to know the

sequence of the genome to amplify the gene/fragment. A high degree of polymorphism is also revealed, enabling the distinction between strains belonging to different or to the same species. The RAPD technique, however, has poor reproducibility, even when the same primers are used in different reactions. This can be attributed to many factors, such as the thermal cycler, the DNA polymerase, the concentration of the DNA and primers and the composition and concentration of the reaction buffer. If the reaction variables are standardised, the difficulty, i.e. poor reproducibility of bands, can be minimised. Quesada and Cenis (1995) used a single reaction to separate as many as four strains, but when the strains appeared identical to one primer, they could be separated using different primers. Thus, by combining the information from several reactions, the strains investigated in that study could be separated. However, the combination of several RAPD reactions to differentiate between strains can be problematic when monitoring yeasts in fermentations. It is therefore very important to standardise the parameters and the amplification and replication reactions each time.

Van der Westhuizen *et al.* (1999) and Khan *et al.* (2000) have used the identification of yeast strains with RAPD-PCR in a number of studies. Khan *et al.* (2000) used RAPD-PCR to establish the geographic distribution of *S. cerevisiae* isolated from vineyards in the warmer, inland regions of the Western Cape in South Africa. Decamer oligonucleotides of random sequence were purchased. Five primers (OPC-02, OPC-07, OPC-08, OPC-09 and OPC-13) from the Operon Kit C were used to determine their differentiating ability. PCR products were separated using CHEF electrophoresis.

Van der Westhuizen *et al.* (1999, 2000) used RAPD-PCR for the characterisation of yeasts in studies that included the seasonal variation of indigenous *S. cerevisiae* strains isolated from vineyards of the Western Cape in South Africa. PCR reactions were performed using different primers from the Operon Kit C. In the above-mentioned studies, RAPD-PCR analysis was only carried out to verify that those yeast isolates that displayed identical electrophoretic karyotypes were indeed the same.

Loureiro (2000) used RAPD-PCR, selecting 10-mer oligonucleotides, to detect spoilage organisms in food and beverages. In addition to the RAPD typing method, the principal component analysis (PCA) was used to analyse the complex multivariate data. The statistical PCA approach enabled RAPD data analysis without any reduction of the resolution, in such a way that the strains belonging to one species could also be observed between different strains that belonged to the same species, thus differentiating to a subspecies level.

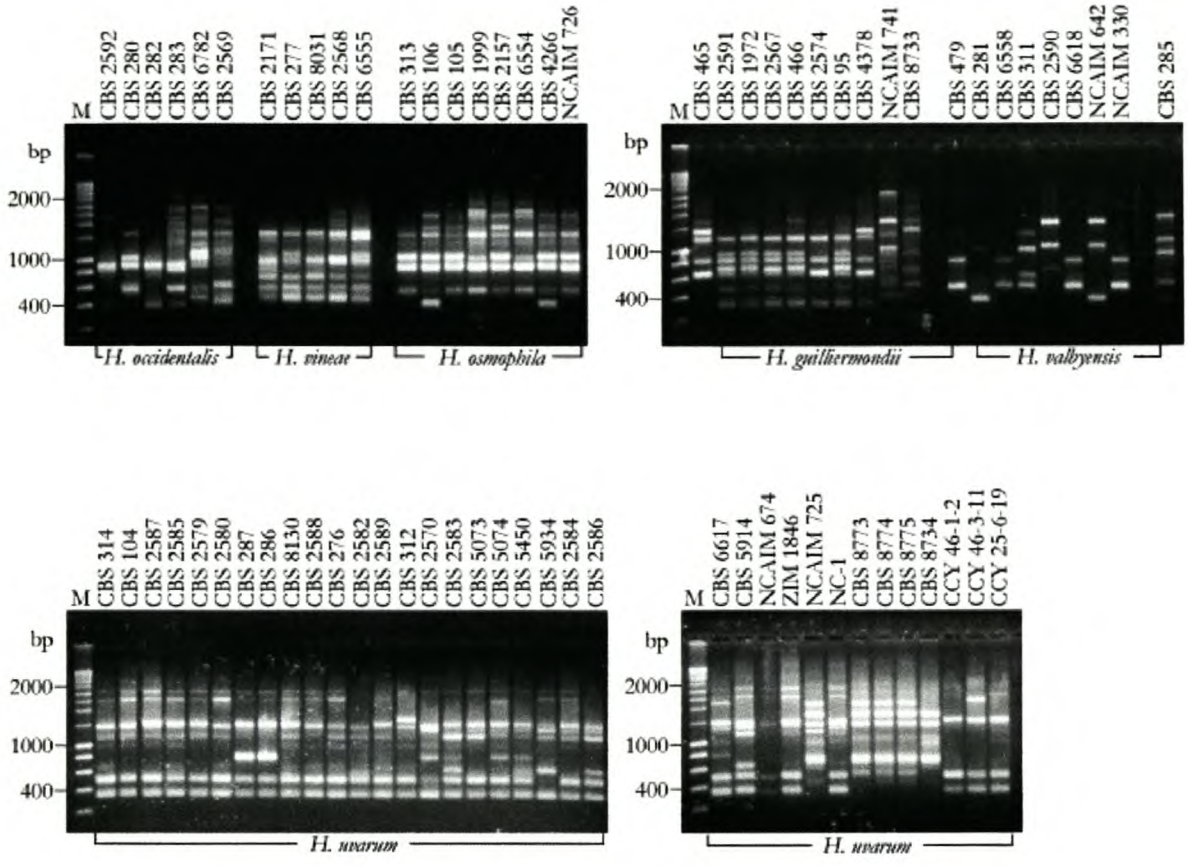

FIGURE 12

RAPD fingerprints of *Hanseniaspora–Kloeckera* strains generated with Opa-13 primer. M, SmartLadder 200 bp (Eurogentec) (Cadez *et al.*, 2002).

2.6.5 Fourier transform near infrared spectroscopy (FT-NIR)

Fourier transform near infrared spectroscopy (FT-NIR) is a rapid, non-destructive, analytical technique that can be used to quantitatively measure a constituent of interest. Near infrared (NIR) spectroscopy has been demonstrated to have potential for effective discrimination between various types of biological materials (Baumgarten, 1987; Downey *et al.*, 1994; Downey, 1996; Downey & Spengler, 1996; Downey & Beauchêne, 1997; Downey *et al.*, 1997; Dambergs *et al.*, 2001; Manley *et al.*, 2001; Downey *et al.*, 2002). Most biological material has characteristic chemical compositions and will also have a characteristic spectrum (NIR absorption properties), since this is a result of the absorption of radiation by many (or all) of its chemical constituents (Downey, 1996). Figure 13 shows typical spectra obtained with FT-NIR. Such information can be extracted by the appropriate mathematical treatment of the data (Willard *et al.*, 1988).

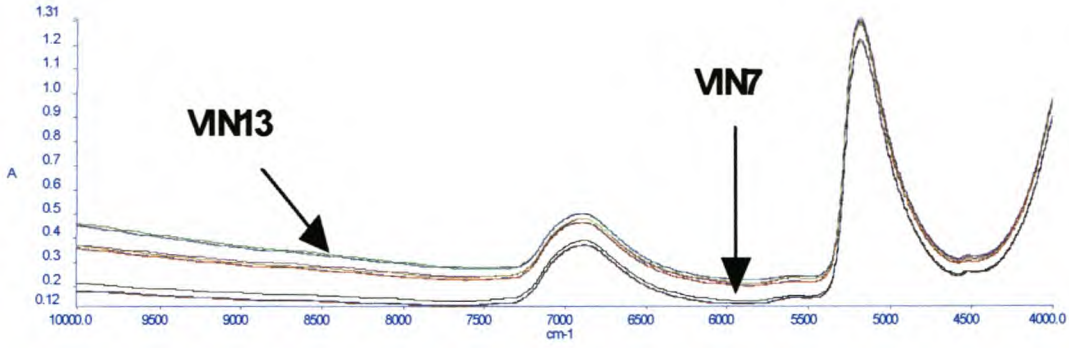

FIGURE 13

Spectra obtained by scanning different samples. The spectra of VIN13 and VIN7 are shown to illustrate the difference between the spectra.

In wine analysis, one is concerned with hydrocarbons, protein (amides), carbohydrates, lipids and moisture. Therefore, mainly the CH, OH and NH bands must be considered in wine because the three main components of wine are ethanol, water and natural sugars. According to the literature, there are specific regions in the spectra that are obtained in which overtones of these fundamental vibrations of the CH, OH and NH bands are visible and thus are reflected in the spectra. With reference to the above-mentioned regions, it is clear that there are four regions in the spectra, going from the highest to the lowest wavelength, in which the most prominent CH combination bands of hydrocarbons are found (Wetzel, 1998). The near infrared spectrum region lies between 850 and 2500 nm (11 000 to 4000 cm^{-1}) and the mid-infrared spectrum region lies between 2500 and 15 000 nm (4000 to 600 cm^{-1}) (Wetzel, 1998). The sensitivity of an optical absorption spectroscopic analytical method is dependent on the probability of a transition from the ground state to an elevated state within the molecule (Wetzel, 1998). Mid-infrared has a high sensitivity and the absorptivity coefficients are high. In contrast, the sensitivity of near infrared is much lower and the absorptions are weak. This is actually an advantage, as absorption bands that have sufficient intensity to be observed in the near infrared region arise primarily from functional groups that have a hydrogen atom attached to a carbon, nitrogen or oxygen, which are common groups in the major constituents of food (Wetzel, 1998).

With soft independent modelling by class analogy (SIMCA), the data collected are separated into different classes or groups. SIMCA first centres and compresses the raw data by means of PCA (Downey & Beauchêne, 1997). Through mathematical calculations, each cluster model treats new samples separately and an assessment of cluster membership is made on the basis of the distance to the cluster centroid.

An F-test is employed to measure the degree of similarity of an unknown sample spectrum to sample spectra in each modelling cluster, allowing an estimate of confidence to be attached to any identification decision (Downey, 1996; Downey &

Beauchêne, 1997). The SIMCA diagnostic procedure validates every standard spectrum to ensure that the spectra from a single class fit that class (recognition) and that those selected from other selected classes are rejected (rejection). The recognition rate, also known as the sensitivity, is the number of spectra that are assigned to the class as a percentage of the number of spectra that should have been assigned to the class. The rejection rate, also known as the specificity, is the number of spectra that are rejected, thus not assigned to the class, as a percentage of the number of spectra that should have been rejected (Perkin Elmer, 1997; Van Zyl, 2000).

Mid-infrared spectroscopy has been used in conjunction with pyrolysis mass spectroscopy (PyMS) for the differentiation of 22 production brewery *S. cerevisiae* strains. Multivariate discriminant analysis of the spectral data was then performed to observe the relationships between the 22 isolates (Timmins *et al.*, 1998). Mid-infrared spectroscopy was used to analyse 53 *Lactobacillus* strains found in breweries. The identification was correct for 94% of the strains and the strain level was correct for 91% of the strains (Curk *et al.*, 1994). Selected strains of the taxa *Staphylococcus*, *Streptococcus*, *Clostridium*, *Legionella* and *Eschericia coli* were also identified with FT-IR. Twenty-six clinical isolates of *Staphylococcus areus* and 24 of *Streptococcus faecalis* were tested and all were assigned to the correct species cluster (Helm *et al.*, 1991). Kümmerle *et al* (1998) assembled a reference spectrum library of 332 defined food-borne yeast strains from international yeast collections. In order to assess identification quality, another 722 unknown yeast isolates, not included in the reference spectrum library, were identified both by classical methods and comparison of their FT-IR spectra with those of the reference spectrum library. Of these isolates, 99.5% were identified correctly by FT-IR (Kümmerle *et al.,* 1998).

The most common application of FT-NIR spectroscopy in the wine industry has been in the determination of the alcohol content of wine (Baumgarten, 1987; Van den Berg *et al.*, 1997; Dambergs *et al.*, 2001; Manley *et al.*, 2001). It has also been applied to routine analyses that include methanol, glycerol, fructose, glucose and total residual sugars present during the production of alcoholic beverages (Van den Berg *et al.*, 1997). Other applications include the determination of the malolactic fermentation status in rebate wine and the determination of the free amino nitrogen (FAN) and °Brix values of must samples (Manley *et al.*, 2001). NIR has also been used for the identification of yeast strains (Halász *et al.*, 1997). This study tested the potential of FT-NIR spectroscopy to identify and differentiate between well-known commercial yeast strains (Chapter 3).

2.7 CONCLUSION

Traditionally, the identification of yeast species and strains was based on morphological and physiological abilities (Kreger-Van Rij, 1984; Barnett *et al.,* 1990; Guillamón *et al.*, 1998), but these techniques are expensive, time consuming and

require great expertise and skill. As a result, several identification kits and automated systems that use standardised methods, prepared substrates and are convenient to use with an acceptable degree of accuracy have been developed (Török & King, 1991; Deák & Beuchat, 1993; Deák & Beuchat, 1995). These systems include the API 20C, API ID 32C and SIM identification methods, of which the API 20C system is the most widely used. These methods have been used as reference methods to evaluate other systems. Other methods, based on the detection of enzyme activities, have also been developed. These methods, e.g. Baxter Microscan, have the advantage of generating results within 4 h after inoculation, making them very rapid (Deák & Beuchat, 1995). The fatty acid analysis technique has proven to be a valuable chemo-taxonomical tool to distinguish between strains of certain species of certain genera (Botha & Kock, 1993). This technique is time consuming, which makes it unsuitable for the routine classification of yeasts (Van der Westhuizen *et al.*, 1999). In addition, methods employing morphological, biochemical and physiological properties are highly influenced by culture conditions, including the growth media, incubation time and incubation temperature, and can therefore produce uncertain results (Yamamoto *et al.*, 1991).

As a result, identification and differentiation methods shifted towards developing and using molecular techniques. These methods are based on similarities at the level of the base composition of deoxyribonucleic acid (DNA) and DNA sequence homology in different yeasts (Pretorius & Van der Westhuizen, 1991). Karoytyping was selected as the starting point for genetic identification because of its use to differentiate strains of *S. cerevisiae* (Vezinhet *et al.*, 1990; Yamamoto *et al.*, 1991; Shütz & Gafner, 1994). Although karyotyping showed differentiated chromosome patterns for every strain, it did not provide any information on species level.

Among the molecular techniques described in the literature, RFLP analysis of mtDNA appears to be one of the most suitable methods to differentiate between strains. MtDNA analysis is rapid, simple, reproducible and can differentiate yeast isolates from the same must and to strain level. The method yields results within three days (Lopéz *et al.*, 2001). However, in the industry, a much shorter time would be required for routine controls. Lopéz *et al.* (2001) have used an adaptation of the method described by Querol and Ramón (1996), resulting in a large time reduction of 52.5 h in comparison to the original method.

Genetic tagging of sequences, genes and dimeric repeats in the genome also seems to be useful in the classification of *S. cerevisiae* strains (Ness *et al.*, 1993; De Barros Lopes *et al.*, 1998; Gallego *et al.*, 1998; Techera *et al.*, 2001). Tagging of microsatellites, introns and the δ repeat sequences seems extremely helpful in the differentiation between yeast strains. These PCR-based techniques can be performed with different primers and under different PCR conditions, but numerous type strains must be selected to identify the unknown strains correctly. Another important advantage of these methods is their portability. Since all the results can be

expressed as a number of repeats, computer translation is easy, offering the opportunity to compare results from different groups via the Internet.

Restriction patterns generated from the DNA region comprising the 25S rRNA and 18S rRNA, including the internal transcribed regions (ITS1 and ITS2), have made it possible to differentiate among species using a pattern of bands characteristic for each species observed (Messner & Prillinger, 1995; Nguyen & Gaillardin, 1997; Guillamón *et al.*, 1998; Smole Možina *et al.*, 1997). The 25S rDNA fragment seems to be more conserved than the 18S rDNA fragment because of the variable ITS1 and ITS2 regions included (Messner & Prillinger, 1995; Smole Možina *et al.*, 1997).

RAPD-PCR is a reliable and rapid technique for the routine identification of wine yeast (Khan *et al.*, 2000). The technique also provides information on species level. With RAPD-PCR, as well as with the other PCR-based techniques, the level of differentiation depends largely on the primers used (Baleiras Couto *et al.*, 1996). With the RAPD technique it is not necessary to know the sequence of the genome to amplify the gene/fragment. This technique also reveals a high degree of polymorphism, enabling the distinction between strains belonging to different or to the same species. The RAPD technique has poor reproducibility, however, even when the same primers are used in different reactions.

Ribotyping is a convenient tool for differentiation at the species level. The restriction patterns of reference strains can be computed instead of having to make use of real experiments, making it a rapid and reliable technique.

Other techniques employing computer technology can also be used for the differentiation of large numbers of yeasts. FT-NIR is one of these techniques. If optimally calibrated, FT-NIR can be of great value, as it is rapid, economical, non-destructive and simple for analytical purposes. With FT-NIR spectroscopy, a large amount of yeast strains can be screened within a short period of time.

Since yeast is now being used commercially on a large scale, its identification and classification can be of great economical significance. Fast, rapid, cost-effective and accurate identification methods are required. Large numbers of yeasts can be isolated from the vineyards, characterised and even be commercialised and used as starter cultures in wine fermentations. Methods are also required to determine if GMOs spread in nature and to monitor this spreading, if it should occur. Yeast producers could also implement and perform good quality control practices and identify their yeast cultures and strains faster. Nowadays, the trend is definitely towards rapid molecular techniques with little cost involved.

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CHAPTER 3

RESEARCH RESULTS

Monitoring the spreading of commercial wine yeasts in the vineyard.

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3. RESEARCH RESULTS

Monitoring the spreading of commercial wine yeasts in the vineyard

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The yeast *Saccharomyces cerevisiae* is an important factor contributing to the quality of wines and the improvement of wine yeasts therefore receives considerable attention worldwide. Apart from classical yeast breeding studies, a significant amount of research aimed at the improvement of wine yeasts is dependent on recombinant DNA techniques. These techniques are used to genetically modify wine yeasts in an attempt to improve the fermentation, processing, biopreservation of wine, thereby enhancing the quality/price ratio, wholesomeness and sensory aspects of the end product. The release of such genetically improved yeast strains is still subject to statutory approval and general public acceptance. Important concerns regarding their release include whether these yeast strains will be able to multiply and spread in nature in an uncontrollable way, or whether they will be able to out compete the natural microflora due to their newly acquired genetic traits. Since existing commercial wine yeasts are used in current strain development programmes, the aim of this study was to determine to what extent these commercial wine yeast strains survive and spread in nature, and whether they influence the fermentations of the following year. Yeasts were isolated from vineyards on six wine farms in different climatological regions in the Western Cape, South Africa during the 1998 and 1999 harvesting seasons. Six sampling sites, identified according to their position relative to the cellar, the dumping sites for grape skins and the prevailing wind direction, were selected on each farm. The yeast isolates were compared to commercial strains by electrophoretic karyotyping. Of 1 600 isolates, only seven seemed to be identical to commercial wine yeast strains. Six of them were identical to VIN13 and the other one N96. Both these strains are widely used starter strains in the South African wine industry. These findings indicate that commercial wine strains are not easily dispersed from the cellar to the vineyard. The low recovery rate of commercial wine yeast strains in vineyards therefore reiterates the fact that the likelihood that commercial yeasts will eventually replace the natural microflora in vineyards seems remote.

Key Words: Dissemination, yeast identification, wine yeasts, GMOs

3.1 INTRODUCTION

At first, wine has been produced by the natural fermentation of grape juice by yeast that originates from the grapes and winery equipment. Current literature shows that *Kloeckera* and *Hanseniaspora* dominate the indigenous yeast population on the surfaces of grapes, but that prevailing *Saccharomyces cerevisiae* strains complete the fermentation (Pretorius *et al.*, 1999; Pretorius, 2000). As a whole, the non-*Saccharomyces* strains produce a broader spectrum of aroma compounds, which is desirable in regions with well-established oenological features. These indigenous yeasts might be responsible for the spontaneous fermentation of grape juice to wine. However, research on the yeast ecology of the vineyard shows a great deal of variation in the quantity and distribution of the relevant species (Fleet & Heard, 1993; Pretorius *et al.*, 1999; Pretorius, 2000). This could be related to differences in the soil composition, climate, age of the vineyard, the geographical location of the vineyard, grape variety, harvest technique, etc. (Querol *et al.*, 1992). Therefore, as a result of the varying grape microflora, wine quality may differ from year to year. For this reason, modern wineries use pure yeast cultures to inoculate the grape must.

Only a few wine yeasts were commercially available in the 1970s, but today more than 100 wine yeasts are readily available for commercial use. This increase can be ascribed to the ever-changing demands made on wine yeasts. Winemakers are looking for custom-tailored yeasts that are suitable for their every need. Some of the more popular characteristics that are demanded include the ability to ferment at low temperatures, to ferment high sugar musts to dryness, to produce low amounts of SO_2, and yeast with a low nitrogen demand. These custom-tailored wine yeasts were obtained by isolating and screening naturally occurring yeasts from vineyards and spontaneous fermentations, as well as through yeast breeding programmes (Pretorius *et al.*, 1999; Van der Westhuizen *et al.*, 1999; Khan *et al.*, 2000; Van der Westhuizen *et al.*, 2000a, b).

Classical techniques are limited in their possibilities, as they are affected by the combined effect of several factors. Due to the complexity of the vinification process and the wide-spread practice of seeding must with dry commercial wine yeast cultures with favourable characteristics, it has become increasingly important to use an easily identifiable strain to ensure consistency of wine type, style and quality (Pretorius & Van der Westhuizen, 1991). In an effort to achieve this, much attention is paid to the genetic improvement of wine yeast strains. Wine yeast strains are tailored to, amongst others, produce colour- and aroma-liberating enzymes (pectinases, glucanases, arabinofuranosidases and glycosidases) (Laing & Pretorius, 1993; Pérez-González *et al.*, 1993; Lambrechts & Pretorius, 2000; Lilly *et al.*, 2000; Van Rensburg & Pretorius, 2000), convert malic acid into lactic acid (malolactic fermentation) (Ansanay *et al.*, 1996; Bony *et al.*, 1997; Volschenk *et al.*, 1997), and increase glycerol production (Remize *et al.*, 1999; Eglinton *et al.*, 2002). Existing commercial wine yeast strains are generally used for these manipulations. The release of recombinant yeast strains (genetically modified organisms, GMOs) is,

however, subject to statutory approval, since the GMOs might be able to multiply and spread in nature and be in an advantageous position to out compete the natural microflora due to their newly acquired genetic traits. In order to establish a foundation from which knowledgeable decisions regarding the release of recombinant yeast can be made, research needs to be done on their behaviour in nature. The aim of this study was therefore to investigate the ability of current commercial wine yeast strains to survive and spread in nature. This study can be used as a stepping stone from which future studies on the monitoring of recombinant wine yeast in nature can be done.

3.2 MATERIALS AND METHODS

3.2.1 Areas sampled

Six wine estates in different climatological areas in the Western Cape, South Africa were identified for sampling (Fig. 1). These cellars are located at Stellenbosch, Rawsonville, Robertson, Botrivier, Constantia and Riebeeck Kasteel. Constantia, Botrivier and Stellenbosch are situated in the cooler coastal regions, while Robertson, Rawsonville and Riebeeck Kasteel are part of the warmer inland regions. The grapes used for the study were harvested in the 1998 and 1999 seasons.

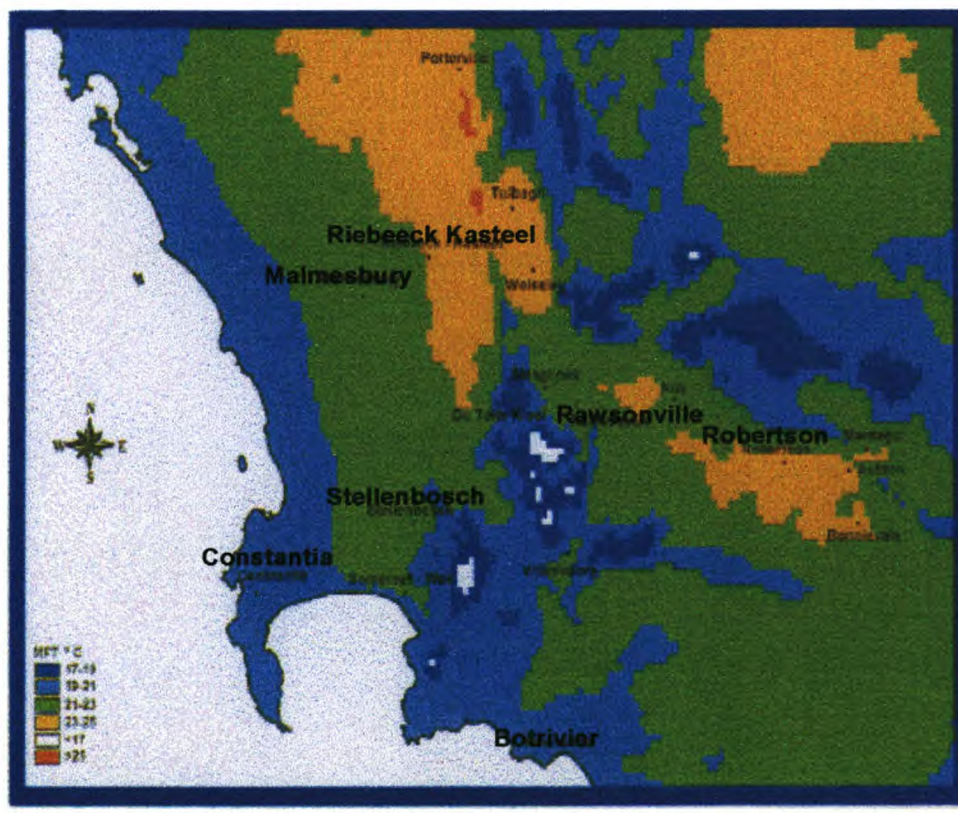

FIGURE 1

The areas sampled in different climatological regions in the Western Cape, South Africa.

3.2.2 Yeast strain isolation

Six sampling sites were identified on each farm. These sites were selected according to their position relative to that of the cellar and the general dumping site for grape skins. Two sites were selected close to the dumping site, another two close to the cellar and the last two were selected far away from both the cellar and the dumping site. The prevailing wind directions were also taken into consideration in determining the sampling sites.

Grape clusters of approximately 1 kg were aseptically harvested in plastic bags and transported back to the laboratory, where the grapes were crushed. After 4 h, the grapes and must were thoroughly mixed before the must was decanted into a sterile bottle and incubated at 25°C until fermentation stopped. Samples were taken at the end of fermentation and streaked onto YPD plates (1% yeast extract, 2% peptone, 2% glucose and agar) to isolate single colonies. Twenty colonies were randomly selected from each plate, cultured at 30°C and used to prepare glycerol freeze cultures (20% YPD and 80% glycerol). The glycerol stocks were stored at -80°C in an Ultralow Freezer and used for further identification of the unknown isolates.

3.2.3 Separation of intact chromosomal DNA by pulsed field gel electrophoresis

Chromosomal DNA samples were prepared according to the embedded-agarose procedure of Carle and Oslen (1985). Samples were run on 2% agarose gels in the pulse field gel electrophoresis system CHEF-DR11 (Bio-Rad Laboratories, Richmond, USA) and electrophoretic karyotypes were determined for 20 isolates per site. The temperature of the 0.5 x TBE electrophoresis buffer was maintained at 13.5°C. Gels were run for a total of 42 h, consisting of 14 h with a switching interval of 60 s and 28 h with a switching interval of 90 s. The commercial yeast strains VIN13, VIN7, WE14, WE372, WE228, N96 (Anchor Yeast), D254, CY3079, Bordeaux Red, EC1118, D47, L2056, QA23 and L2226 (Lallemand) were used as standard reference strains. Visual data were used as the primary criterion when comparing strains and the final results were confirmed by running additional gels.

3.3 RESULTS AND DISCUSSION

3.3.1 Climatic conditions at sample areas

As indicated in Table 1, there are notable differences in the climate of the different sampling areas. Rawsonville, Riebeeck Kasteel and Robertson are warmer, inland areas, while Constantia, Botrivier and Stellenbosch are cooler coastal areas. The differences in the climate (i.e. temperature and rainfall) may affect the yeast population on the grapes (Pretorius, 2000).

TABLE 1

Differences in climate at the sampling sites.

Warmer, inland regions					Cooler, coastal regions				
Malmesbury					**Constantia**				
Monthly Averages for Minimum Temperature(°C)					Monthly Averages for Minimum Temperature(°C)				
YEAR	JAN	FEB	MRT	APR	YEAR	JAN	FEB	MRT	APR
1998	13.7	16.8	13.4	11.8	1998	15.7	18	15.7	14.6
1999	15.1	15.7	14.4	12.1	1999	17.3	16.8	16.5	14.2
Monthly Averages for maximum temperature(°C)					Monthly Averages for maximum temperature(°C)				
YEAR	JAN	FEB	MRT	APR	YEAR	JAN	FEB	MRT	APR
1998	28.6	31.4	27.7	25.7	1998	24.2	26.6	23.5	22.5
1999	30.3	30.9	30.5	26.5	1999	25.9	25.5	26	23
Monthly Totals for Rain(mm)					Monthly Totals for Rain(mm)				
YEAR	JAN	FEB	MRT	APR	YEAR	JAN	FEB	MRT	APR
1998	6.5	0	7	32.5	1998	19.5	0.8	15.5	71
1999	0	14	0	47	1999	8.8	2.8	2.1	111.6
Robertson					**Wildekrans, Botrivier**				
Monthly Averages for Minimum Temperature(°C)					Monthly Averages for Minimum Temperature(°C)				
YEAR	JAN	FEB	MRT	APR	YEAR	JAN	FEB	MRT	APR
1998	14.8	18.1	15.1	11.9	1998	14.2	*****	*****	*****
1999	17.1	17.7	16.2	12.4	1999	17.4	18.1	17.2	13.6
Monthly Averages for maximum temperature(°C)					Monthly Averages for maximum temperature(°C)				
YEAR	JAN	FEB	MRT	APR	YEAR	JAN	FEB	MRT	APR
1998	30.8	31.8	28.5	27.1	1998	26.4	*****	*****	*****
1999	32	30.7	30.8	26.5	1999	28.7	29.1	28.3	24
Monthly Totals for Rain(mm)					Monthly Totals for Rain(mm)				
YEAR	JAN	FEB	MRT	APR	YEAR	JAN	FEB	MRT	APR
1998	28	2.6	18.4	35.4	1998	8.6	*****	*****	*****
1999	5.2	17.4	0.8	0.4	1999	4.5	3	0	38.5
Rawsonville					**Stellenbosch**				
Monthly Averages for Minimum Temperature(°C)					Monthly Averages for Minimum Temperature(°C)				
YEAR	JAN	FEB	MRT	APR	YEAR	JAN	FEB	MRT	APR
1998	14.6	17.7	14.7	11.6	1998	14.3	17.3	14.4	12.5
1999	16.7	17.4	16	12.6	1999	15.8	16.3	15.2	12.7
Monthly Averages for maximum temperature(°C)					Monthly Averages for maximum temperature(°C)				
YEAR	JAN	FEB	MRT	APR	YEAR	JAN	FEB	MRT	APR
1998	29.9	31.6	28	26.3	1998	26.8	29.9	25.9	24.8
1999	32.1	30.7	31.1	26.2	1999	29	29.8	29.4	25.2
Monthly Totals for Rain(mm)					Monthly Totals for Rain(mm)				
YEAR	JAN	FEB	MRT	APR	YEAR	JAN	FEB	MRT	APR
1998	18	0	2	12.9	1998	22.4	0	27.2	40.6
1999	0	1	3.5	22.5	1999	1.5	0.3	0	67.4

*****Data not available due to a dysfunctional weather station during the periods.

3.3.2 Identification of isolated yeasts

The chromosomal banding patterns of 20 colonies randomly selected from the yeasts isolated from a particular sampling site on a wine farm have been determined. The results in Figure 2 are typical of the results obtained. Lanes 6 to 9 and 13 to 17; lanes 2 to 4; lanes 5 and 11; and lanes 10 and 18 in Figure 2a contain similar sets of banding patterns. Unique strains are shown in lanes 11 and 19. The chromosomal banding patterns in lane 1 represent those of a non-*Saccharomyces* strain. In Figure 2b, similar sets of bands can be seen in lanes 1, 7, 13, 16 and 21; lanes 2, 8, 9 and 19; lanes 3 and 6; lanes 4 and 12; lanes 5 and 15; lanes 11 and 14; and lanes 17 and 18. Unique banding patterns are shown in lane 10. The yeast strain in lane 20 cannot be compared due to degradation. No particular yeast strain dominated this site. In Figure 2c, identical sets of bands can be seen in lanes 1 to 7, 9 to 17, and 19 and 20, indicating complete dominance of this site. Similar strains are shown in lanes 8 and 18. Similar sets of bands were found in lanes 1, 4, 5, 7, 14 and 18;

lanes 12, 13, 19 and 20; lanes 3 and 17; and lanes 2, 6, 8, 9, 15 and 16 in Figure 2d. A different set of bands is shown in lane 10. Figure 2e shows identical sets of bands for lanes 3, 4, 6, 7, 10, 13 to 17 and 19, indicating the dominance of this strain at the site. Similar sets of bands were found in lanes 1, 2, 9, 18 and 20; lanes 5 and 8. A unique set of bands is shown in lane 11, while the bands in lane 12 are not comparable due to degradation. The chromosomal banding patterns of the strains in Figure 2e differ from the sites shown in Figure 2a to d. In Figure 2f, similar sets of bands are seen in lanes 1, 5 to 7, 9 and 12; lanes 2 and 10; and lanes 3 and 4. The yeast strains found here differ from the strains shown in Figure 2a to e.

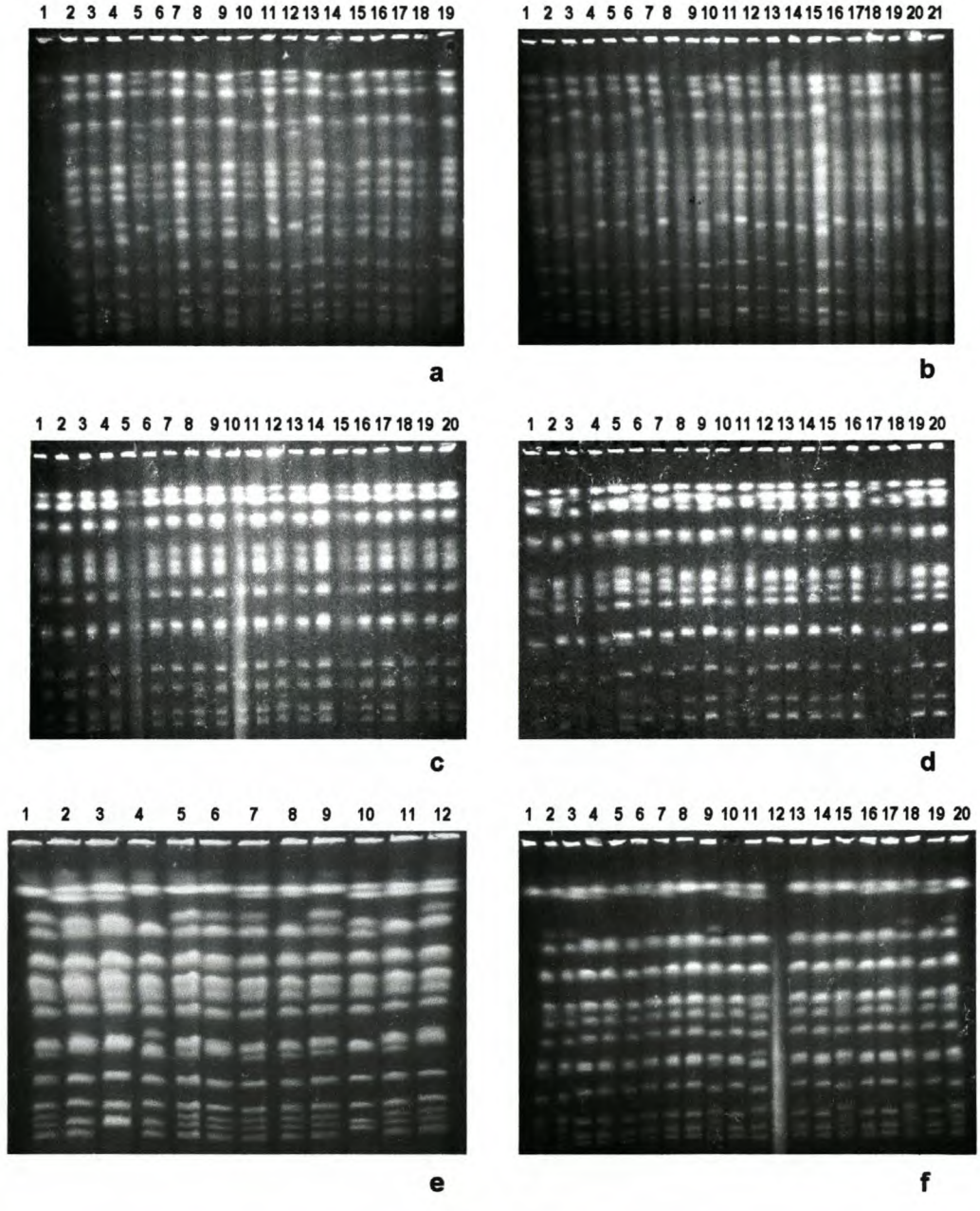

FIGURE 2

CHEF of yeast strains isolated from various sampling points. (**a**) 1998: Rawsonville cellar (**b**) 1999: Rawsonville cellar (**c**) 1998: Rawsonville grape skin dumping site (**d**) 1999: Rawsonville grape skin dumping site (**e**) 1999: Stellenbosch cellar (**f**) 1999: Constantia cellar.

During the 1998 harvesting season, 34 different *Saccharomyces* strains were isolated from the designated sites. The yeast strains differed between the sites on each farm and also varied between the farms. Only three commercial wine yeast strains were found during the 1998 harvesting season (Table 2).

During the 1999 harvesting season, 55 different *Saccharomyces* strains were found. The yeast strains again varied from site to site and from farm to farm. The yeast strains also differed between the 1998 and 1999 harvesting seasons. Only four commercial wine yeast strains were found during the 1999 harvesting season (Table 2).

TABLE 2

Commercial yeast strains found during the 1998 & 1999 season.

Isolate	Yeast strain	Site on farm
ST2-2(1)[1]	N96	Near cellar
LR2-1(5)	VIN13	Near cellar
ST2-2(4)	VIN13	Near cellar
WK1-5(3)	VIN13	Near dumping site
WK1-5(4)	VIN13	Near dumping site
WK1-5(7)	VIN13	Near dumping site
WK1-5(8)	VIN13	Near dumping site

1 - ST2-2(1): Stellenzicht wine estate, sampled at site 2, first out of 20 isolates sampled at that specific site.

Of the 1 600 samples isolated from the vineyards over two harvesting seasons, only seven isolates showed identical chromosomal banding patterns to commercial yeast strains. Six of the eight strains were VIN13 and one was N96. Not surprisingly, these yeast strains are widely used in the South African wine industry. However, if one takes into account the number of yeast isolates investigated, it seems that commercial yeast strains are not easily dispersed from the cellar into the vineyard. This may be ascribed to the fact that every strain has certain environmental preferences in which it flourishes. It can be presumed that the yeast strains do not always adapt well to the different conditions to which they are subjected, such as climate and vineyard maintenance practices, which may include insect control (Schütz & Gafner, 1994; Pretorius, 2000). Furthermore, all the commercial strains were found either near the cellar or the place where the grape skins are dumped (Table 2). This is to be expected, since some of the wines were fermented on the grape skins and yeasts are therefore dispersed to the dumping sites. It is important to note that the commercial yeasts were isolated from the cooler coastal regions in 1998, but from the warmer inland regions in 1999. The identity of the isolated commercial yeast strains was confirmed by running additional gels (Figures 3 & 4).

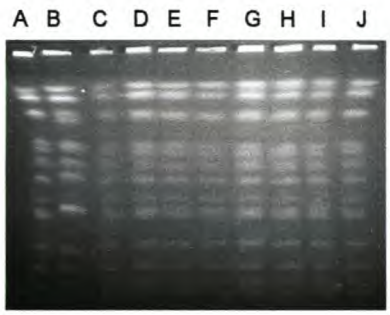

FIGURE 3

Confirming gel of the commercial strains found during the 1999 season. **A** – VIN13, **B** – WK1-5(2), **C** – WK1-5(3), **D** – WK1-5(4), **E** – WK1-5(5), **F** – WK1-5(6), **G** – WK1-5(7), **H** – WK1-5(8), **I** – WK1-5(9), **J** – (VIN13).

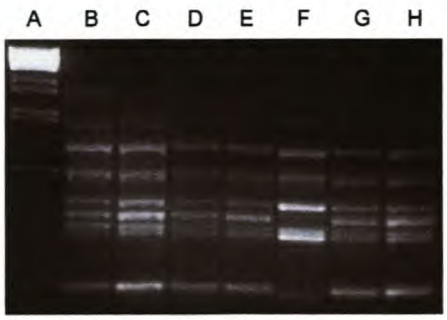

FIGURE 4

Confirmation by PCR of isolated yeast strains from Wildekranz. (**A**) – molecular size marker; (**B**) – VIN13 [control]; (**C**) – VIN13 [control]; (**D**) – WK1-5(3); (**E**) – WK1-5(4); (**F**) – WK1-5(6); (**G**) – WK1-5(7); (**H**) – WK1-5(8). All the strains are VIN13, except for (**F**) – WK1-5(6).

The yeast strain in lanes B, E, F and I did not show the typical chromosomal banding patterns obtained with VIN13, therefore these strains are not considered to be VIN13 (Fig. 3). In Figure 4, the yeast strain in lane F do not seem to be VIN13.

3.4 CONCLUSION

In South Africa, several research programmes have been undertaken to isolate natural yeast strains from the vineyard. DNA fingerprinting techniques have been used with great success in biogeographical surveys of yeast strains isolated from grapes and musts, as well as in wine yeast breeding programmes (Van der Westhuizen & Pretorius, 1992; Pretorius *et al.*, 1999; Van der Westhuizen *et al.*, 1999; 2000a, b; Khan *et al.*, 2000). In these studies, very few commercial wine yeast strains were isolated, but a diversity of *Saccharomyces* strains were found. This study focussed specifically on commercial wine yeast strains to determine whether commercial wine yeast strains are spreading to the vineyards. For improved risk assessment, the sampling sites were selected near the cellar and grape skin dumping sites.

Most of the isolated strains were identified as *Saccharomyces*, but the untypical chromosomal banding patterns suggest that some of the strains could have been non-*Saccharomyces* strains. According to the literature, low alcohol-tolerant species of *Kloeckera* and its anamorph, *Hanseniaspora*, account for 50 to 75% of the total yeast population on grapes, while species of *Candida*, *Brettanomyces*, *Cryptococcus*, *Kluyveromyces*, *Pichia* and *Rhodotorula* are present to a lesser extent (Fleet & Heard, 1993; Pretorius, 2000). Although fermentative species of *Saccharomyces* occur at very low concentrations on grapes, they predominate and conclude fermentation. Vaughan-Martini and Martini (1995) reported that *Saccharomyces* species are present on various surfaces in the winery, but Török *et al.* (1996) proved

that the vineyard is indeed the primary source of these yeasts. Furthermore, Török *et al.* (1996) stated that each grape cluster differs in terms of the presence/absence of *S. cerevisiae*. The micro-population of yeasts on grapes may be affected by various factors, such as climate, age of the vineyard, pesticides, etc. The microflora on the grapes, as well as on the winery equipment and surfaces, could affect the wine quality during spontaneous fermentations. However, great emphasis is placed on the use of selected inoculations and the manipulation of commercial wine yeast strains with recombinant technology and yeast breeding methods (Pretorius, 2000).

In this study, seven commercial yeast strains were found out of 1 600 yeast strains isolated from the vineyards over two harvesting seasons. Six of these were VIN13 and the other one was N96. Not surprisingly, these strains are widely used in the South-African wine industry. It seems as if commercial wine yeasts are not easily dispersed from the cellar to the vineyard, and are also more likely to be found near the cellar and the places where the grape skins are dumped. The fears that GMOs might pose a risk to the natural yeast population in the vineyard seemed to be well-overrated.

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CHAPTER 4

RESEARCH RESULTS

Identification of wine yeast strains with Fourier transform near infrared (FT-NIR) spectroscopy.

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4. RESEARCH RESULTS

Identification of wine yeast strains with Fourier transform near infrared (FT-NIR) spectroscopy.

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This study evaluated the suitability of Fourier transform near infrared (FT-NIR) spectroscopy for the rapid identification of commercial wine yeast strains. A database of FT-NIR spectra was constructed for the commercial wine yeast strains VIN13, VIN7, WE228, WE14, WE372, N96, D254, CY3079, Bordeaux Red, EC1118, D47, L2056, QA23 and L2226. The FT-NIR technique was then further evaluated to determine if it could be used to determine whether any of 1 600 unknown yeast strains isolated from South African vineyards might be one of the commercial wine yeast strains in the database. Recognition rates exceeding 70% were obtained by applying soft independent modelling by class analogy (SIMCA) as a discriminative method to identify the respective commercial wine yeast strains. FT-NIR spectroscopy could clearly distinguish between the commercial wine yeast strains and the isolated strains, although no commercial wine yeast strains were found among the isolated strains. Principal component analysis (PCA) could clearly distinguish between yeast strains isolated from different wine estates.

Keywords: FT-NIR, electrophoretic karyotyping, yeast identification

4.1 INTRODUCTION

Yeasts are used in the industrial production of beer, wine, various spirits, food, bread and other fermented products. However, these yeasts, including *Saccharomyces* and non-*Saccharomyces* species, may also cause economical losses due to spoilage by unwanted strains. The unambiguous identification of yeast strains is of paramount importance because of the increasing numbers of strains and the use of these

microorganisms in research and development in technological and medical fields (Török & King, 1991). The conventional identification and classification of yeasts are based mainly on certain morphological and physiological properties, as well as on the mode of sexual reproduction (Barnett, 1992). Currently, more than 70 tests, including the utilisation of carbon and nitrogen compounds, are being used for identification. The ability of the yeast strains to assimilise carbon sources are tested with more than 36 carbon sources. (De Barros Lopes *et al.*, 1998). Although these methods provide valuable information for the classification and differentiation of yeasts, they are not completely reliable, as they are influenced by culture conditions (Yamamoto *et al.*, 1991). These methods employ morphological, physiological and biochemical properties and therefore are laborious, time consuming and require sophisticated equipment and highly trained staff (Barnett *et al.*, 1990; Deák & Beuchat, 1993; Deák & Beuchat, 1995). Capillary gas chromatograpy (GC) analysis of long-chain fatty acids also seems to be less efficient as the profiles of each yeast strain are reproducible, but variation exists within species (Botha & Kock, 1993) and the cultivation parameters might vary, as all yeasts cannot be cultivated with a single set of rigid cultivation parameters (Bendová *et al.*, 1991; Augustyn *et al.*, 1992).

As a result of the various shortcomings of the abovementioned identification techniques, a number of molecular methods for inter- and intraspecific identification have been developed over the past decade. The molecular techniques that are most often used in the identification and classification of wine yeast strains are polyacrylamide electrophoresis or protein fingerprinting (Dowhanick *et al.*, 1990; Van der Westhuizen & Pretorius, 1992), mt-DNA restriction analysis (Ness *et al.*, 1993; Guillamón *et al.*, 1996; Querol & Ramón, 1996), karyotyping by chromosomal banding patterns (Pretorius & Van der Westhuizen, 1991; Yamamoto *et al.*, 1991; Deák & Beuchat, 1995) – including the CHEF method and genetic tagging with amplification of repeated δ sequences (Ness *et al.*, 1993), microsatellites (Techera *et al.*, 2001) and introns (De Barros Lopes *et al.*, 1996). Ribotyping that combines the use of PCR-amplified rDNA fragments and restriction enzymes in different combinations to check the specificity of the restriction patterns of 18S rDNA (including the ITS regions) and 25S rDNA fragments of a number of yeasts (Molina *et al.*, 1992a & b; Messner & Prillinger, 1995) has also been used successfully to identify yeast species. Of the above-mentioned classification techniques, the CHEF method is used most often for the identification of commercial wine yeast strains. The technique is costly and time consuming and the results, which can only be obtained after a week, could take up to a few months to compile if a large number of strains must be screened.

Near infrared (NIR) spectroscopy [850 to 2500 nm (11 000 to 4000 cm^{-1})] has been demonstrated to have potential for the effective discrimination between various types of biological materials (Downey *et al.*, 1994; Downey, 1996; Downey & Spengler, 1996; Downey & Beauchêne, 1997; Downey *et al.*, 1997; Wetzel, 1998; Dambergs *et al.*, 2001; Manley *et al.*, 2001; Downey *et al.*, 2002). Most biological

material has a characteristic chemical compositions and will also have a characteristic NIR absorption spectrum. This spectrum is a result of the absorption of radiation by its chemical constituents (Downey, 1996), leading to the formation of FT-NIR diffuse signals.

The most common application of FT-NIR spectroscopy in the wine industry has been the determination of the alcohol content of wine (Baumgarten, 1987; Van den Berg *et al.*, 1997; Dambergs *et al.*, 2001; Manley *et al.*, 2001). It has also been applied to routine analyses of the methanol, glycerol, fructose, glucose and total residual sugars present during the production of alcoholic beverages (Van den Berg *et al.*, 1997). Other applications include the determination of the malolactic fermentation status in rebate wine and the determination of the free amino nitrogen (FAN) and °Brix values of must samples (Manley *et al.*, 2001). NIR has also been used for the identification of yeast strains (Halász *et al.*, 1997).

Mid-infrared [2500 to 15 000 nm (4000 to 600 cm^{-1})] has a high sensitivity and its absorptivity coefficients are high. In contrast, the sensitivity of near infrared is much lower and the absorptions are weak. This is actually an advantage, as absorption bands that have sufficient intensity to be observed in the near infrared region arise primarily from functional groups that have a hydrogen atom attached to a carbon, nitrogen or oxygen, which are common groups in the major constituents of food (Wetzel, 1998). The sensitivity of an optical absorption spectroscopic analytical method is dependent on the probability of a transition from the ground state to an elevated state within the molecule (Wetzel, 1998). Chemical property can now be extracted by the appropriate mathematical treatment of the data (Willard *et al.*, 1988).

With soft independent modelling by class analogy (SIMCA), the spectral data that are collected are divided into the respective classes or groups. SIMCA first centres and compresses raw data by means of principle component analysis (PCA) (Downey & Beauchêne, 1997). Through mathematical calculations, each cluster model treats new samples separately and an assessment of cluster membership is made on the basis of the distance to the cluster centroid. An F-test is employed to measure the degree of similarity of an unknown sample spectrum to sample spectra in each modelling cluster, allowing an estimate of confidence to be attached to any identification decision (Downey, 1996; Downey & Beauchêne, 1997). The SIMCA diagnostic procedure validates every standard spectrum to ensure that the spectra from a single class fit that class (recognition) and that those selected from other selected classes are rejected (rejection). The recognition rate, also known as the sensitivity, is the number of spectra that are assigned to the class as a percentage of the number of spectra that should have been assigned to the class. The rejection rate, also known as the specificity, is the number of spectra that are rejected, thus not assigned to the class, as a percentage of the number of spectra that should have been rejected (Perkin Elmer, 1997; Van Zyl, 2000).

Mid-infrared spectroscopy has been used for the differentiation of brewing yeast strains (Timmins *et al.*, 1998), the identification of bacteria, including *Lactobacillus*

(Curk *et al.*, 1994), *Staphylococcus*, *Streptococcus*, *Clostridium*, *Legionella* and *Escherichia coli* (Helm *et al.*, 1991), and the identification of food-borne yeasts (Kümmerle *et al.*, 1998). The aim of this study was to assess the potential of FT-NIR spectroscopy to identify and differentiate between well-known commercial yeast strains and unknown isolates.

4.2 MATERIALS AND METHODS

4.2.1 Yeast strains and media

The commercial wine yeast strains VIN13, VIN7, WE14, WE228, WE372 and N96 from Anchor Yeast, and the Lallemand yeasts strains D254, CY3079, Bordeaux Red, EC1118, D47, L2056, QA23 and L2226 were used to construct the FT-NIR database. A total of 1 600 unknown yeast strains were isolated, following the same procedures and from the same regions as described in Muller *et al.* (2003). These strains were also used to evaluate the FT-NIR technique.

4.2.2 Yeast cultivation for FT-NIR spectroscopy

The glycerol freeze cultures were streaked out on YPD agar medium (1% yeast extract, 2% peptone and 2% dextrose) to isolate single yeast colonies. The plates were cultured at 30°C for 2 days and treated in a number of ways to optimise the FT-NIR spectroscopy analysis: a single colony was taken directly from the YPD plate; a single colony was taken and cultured overnight in 10 ml of YPD at 30°C; and the 10 ml YPD culture was centrifuged at 12 000 rpm for 5 minutes and the pellet dissolved in 2 ml of sterile distilled water.

4.2.2.1 FT-NIR measurements

FT-NIR spectroscopy analyses of the cultured yeast samples were carried out in transmission mode. The spectra were recorded in a 0.5 mm path length quartz cuvette at 4 cm^{-1} intervals with a 16-scan sequence, using a Spectrum Identicheck™ 2.0 FT-NIR System (Perkin Elmer, 1997). The wavelength region for all calibrations was 10 000 to 4 000 cm^{-1} (1 000 to 2 500 nm), resulting in a total of 1 501 data points per spectrum. The yeast strains evaluated were cultured weekly from glycerol freeze cultures, with 25 repacks of each strain analysed. Approximately 250 repacks of each strain were available for the chemometrical data analysis after a period of five weeks.

4.2.2.2 Data analysis

Spectral pretreatment and calibration model development was performed using QUANT+™ 4.1 software (Perkin Elmer, 1997). Multiplicative scatter correction (MSC) was applied to the spectra to eliminate interference and they were then transformed with second derivative processing. Second derivatives were calculated with a

segment of nine and a gap of 13. PCA models were derived from the pretreated data for the development of SIMCA models to allow differentiation between the spectra of the different yeast strains.

4.2.2.3 SIMCA analysis

The spectra were divided into classes, i.e. VIN13, VIN7, WE228, N96, WE14, WE372 and the Lallemand yeasts D254, CY3079, Bordeaux Red, EC1118, D47, L2056, QA23 and L2226 respectively. SIMCA models were created for the different classes and validated on the validation set, which consisted of 100 samples, after diagnostics had been performed on the models.

4.3 RESULTS AND DISCUSSION

4.3.1 Yeast cultivation for FT-NIR

The FT-NIR analysis of a single yeast colony or yeast cultured in liquid YPD resulted in unsatisfactory spectra. The colony formed a smear in the cuvette, while the liquid YPD culture gave additional peaks in the spectra due to the presence of the YPD. However, with the pellet dissolved in sterile distilled water, no additional peaks were observed in the spectra. This method of yeast cultivation was used for the subsequent experiments. The sensitivity of FT-NIR spectroscopy is dependent on the composition of the biological material. The growth conditions must therefore be identical for all the yeast strains to ensure that they do not affect the chemical structure, i.e. proteins, etc., that may be formed.

4.3.2 Identification of commercial wine yeasts

The FT-NIR spectra obtained for some of the commercial wine yeast strains that were analysed are shown in Figure 1. A database was constructed that included all the spectra of the wine yeast strains analysed, i.e. VIN13, VIN7, WE228, N96, WE14, WE372, D254, CY3079, Bordeaux Red, EC1118, D47, DV10, L2056, QA23 and L2226. Although a large proportion of the samples consist of moisture, FT-NIR could still differentiate between the different classes (Fig. 1). By applying SIMCA models, it was possible to differentiate between the commercial wine yeast strains (Fig. 2). The SIMCA models of the different commercial yeast strains recognised similar yeast strains and rejected strains that were not similar, i.e. the VIN13 model recognised all other VIN13 spectra with a 72% recognition rate and rejected all the other spectra with a rejection rate of 14%. In Figure 2, similar tendencies can be seen for all the other yeast strains used in the library. For example, the VIN7 model recognised all the spectra in the same class with a rate of 80%, but rejected all the other spectra with a rate of 27%.

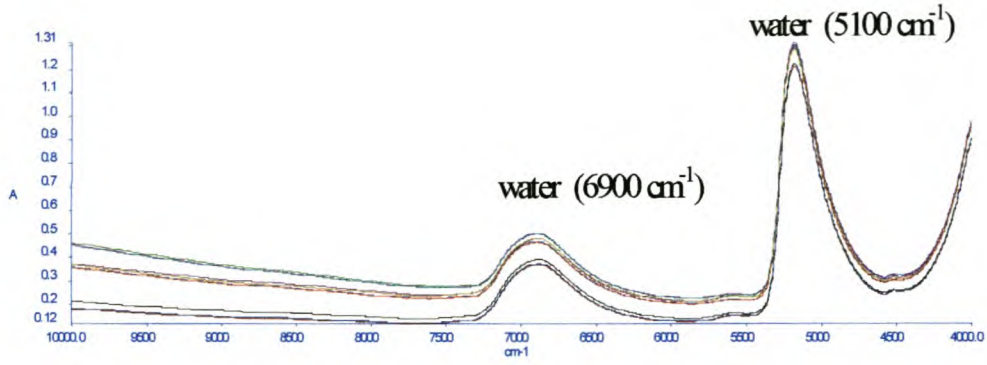

FIGURE 1

Examples of some of the spectra of the commercial wine yeast strains, viz. VIN13, WE372, WE228, WE14, N96 and VIN7, from the top to the bottom, respectively. A total of 1501 data points were compared between 10 000 cm^{-1} and 4 000 cm^{-1}.

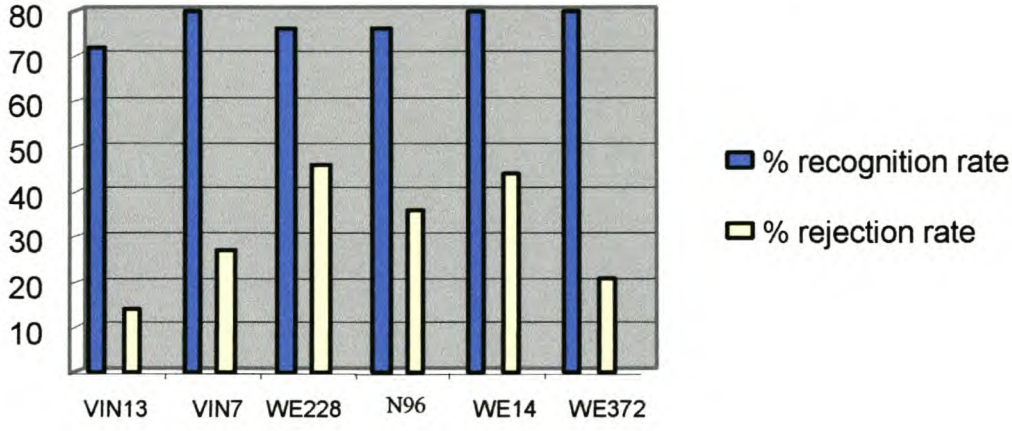

FIGURE 2

Validation results of some of the commercial wine yeast strains. The recognition and rejection rates for some of the commercial wine yeast strains are shown.

4.3.3 Identification of commercial wine yeast strains among 1 600 isolated yeast strains

This study focussed specifically on the possibility of commercial wine yeast strains spreading to vineyards. Six sampling sites were selected on each farm and 1 600 yeast strains were isolated. The isolated yeast strains were identified with pulsed-field gel electrophoresis (CHEF) (Muller *et al.*, 2003). Since the CHEF electrophoretic karyotyping is expensive and time consuming, FT-NIR spectroscopy was used to identify the isolated yeast strains with a single scan.

Characteristic spectra were obtained for the unknown yeast strains. However, when these unknown yeast strains were tested with the FT-NIR SIMCA models developed for the commercial wine yeast strains, no commercial wine yeast strains could be found among the isolated strains (Fig. 3). The database rejected the unknown samples with high rejection rates, i.e. VIN13 with 90%, VIN7 with 94%,

WE228 with 96%, N96 with 95%, WE14 with 95% and WE372 with 92%. The average rejection rate was 94%, with a recognition rate of 0% for all the classes, indicating that virtually none of the unknown isolates were commercial wine yeast strains.

When confirming the results with electrophoretic karyotyping, a limited number of commercial wine yeast strains were found among the isolated strains. Only 0.375% (3/800) was found during the 1998 harvesting season and 0.5% (4/800) during the 1999 harvesting season.

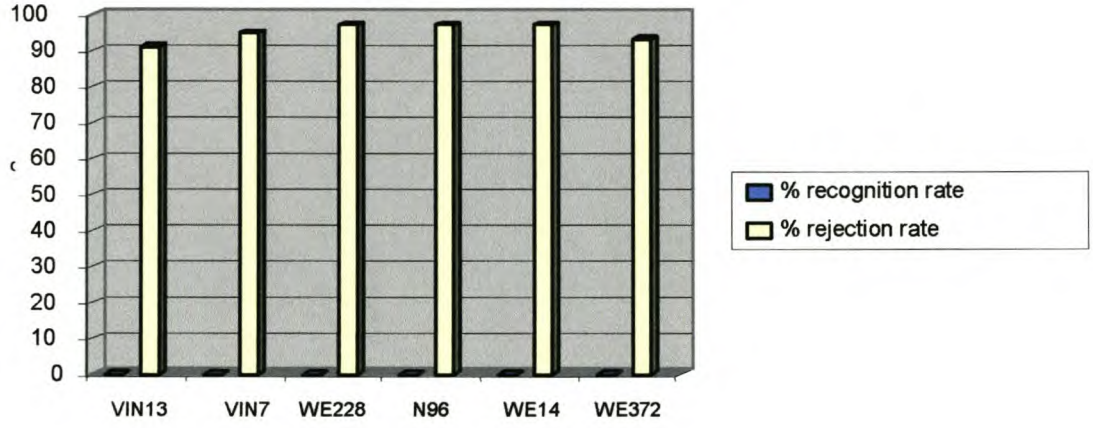

FIGURE 3

SIMCA validations of some of the unknown isolates. Recognition and rejection rates indicated that the isolated yeast strains were not commercial wine yeast strains.

With PCA algorithms, it was found that yeasts from specific wine farms clustered together. This could specifically be seen for the strains from the Buitenverwachting wine estate (Fig. 4). These results were confirmed by electrophoretic karyotyping (Muller *et al.*, 2003).

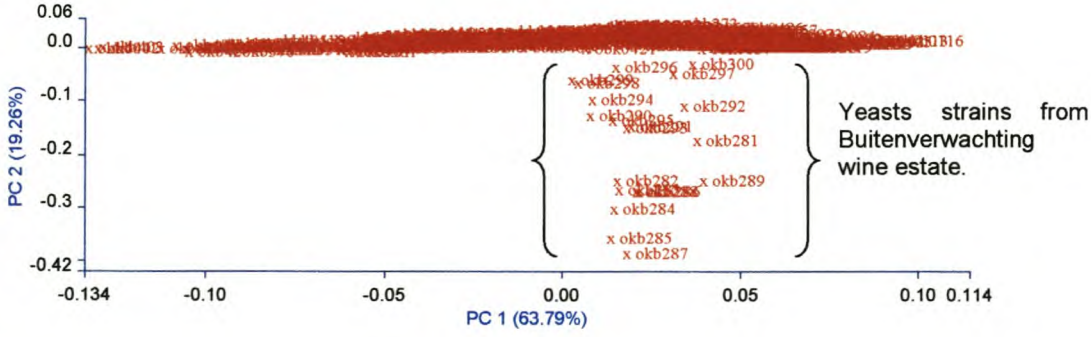

FIGURE 4

PCA plot showing the Buitenverwachting wine yeast strain cluster. PC1 represents 63.79% of the variance in the data, while PC2 represents 19.26%.

In spectral comparisons, the strains from the same farm always recognised each other, before picking up similarities with strains from other farms (Table 1). In Table

1, the average of 25 spectra of the yeast strain Okb288 from Buitenverwachting was compared to the averages of the rest of the yeast strain spectra in the database. It was observed that strain Okb288 always recognised itself first, and then the spectra from the same farm and particularly from the same site on the farm where the yeast strains were isolated originally. The same tendency can be seen in the second part of Table 1, where strain Okb0033 from Lebensraum was compared to the averages of the spectra in the database. The yeast strain always recognised itself first, and then the spectra of the yeast strains from the same site on the farm.

TABLE 1

Summary of the comparison of yeast strain Okb288 from Buitenverwachting wine estate after spectral comparison with the other isolated wine yeast strains. Samples were taken after fermentation had stopped, assuming that the strains were mostly *S. cerevisiae* (Muller *et al.*, 2003).

File	Correlation	Comments
okb288.sp[1]	1.0000	BV1-3(8)[2]
okb282.sp	1.0000	BV1-3(2)
okb286.sp	0.9999	BV1-3(6)
okb283.sp	0.9999	BV1-3(3)
okb284.sp	0.9998	BV1-3(4)
okb289.sp	0.9998	BV1-3(9)
okb281.sp	0.9996	BV1-3(1)
okb285.sp	0.9995	BV1-3(5)
okb293.sp	0.9995	BV1-3(13)
okb291.sp	0.9994	BV1-3(11)
File	**Correlation**	**Comments**
obk0033.sp	1.0000	LR1-2(13)
obk0032.sp	1.0000	LR1-2(12)
obk0027.sp	1.0000	LR1-2(7)
obk0028.sp	1.0000	LR1-2(8)
obk0038.sp	1.0000	LR1-2(18)
obk0051.sp	1.0000	LR1-3(11)
obk0048.sp	1.0000	LR1-3(8)
obk0034.sp	1.0000	LR1-2(14)
obk0047.sp	1.0000	LR1-3(7)
obk0029.sp	1.0000	LR1-2(9)

1: isolate number;2: yeast strain sample no. 8 from Buitenverwachting on site 1, sampled for the 3rd time

4.4 CONCLUSION

From the results obtained in this study, it was concluded that FT-NIR spectroscopy can be used to differentiate between the commercial wine yeast strains used in the South African wine industry. Furthermore, strains from a particular farm and site

always recognised each other first, before recognising the rest of the similar spectra in the database using spectral comparisons and PCA. This might be related to differences in the existing ecosystems, i.e. the soil composition, climate, age of the vineyard, the geographical location of the vineyard, grape variety and harvest technique (Querol *et al.*, 1992). The wine estates are located in different climatological areas. Constantia, Botrivier and Stellenbosch are in the cooler coastal regions, while Robertson, Rawsonville and Riebeeck Kasteel are in the warmer inland regions.

Using FT-NIR, none of the unknown isolates could be identified as commercial wine yeasts, although seven commercial wine yeast strains were identified with CHEF, six of which were VIN13 and one of which was N96. These were found among a total of 1600 isolated yeast strains. With electrophoretic karyotyping, a specific strain, WK1-5(6), was found that gave the same karyotype as VIN13, but confirmation with PCR (using sequences in the δ region) showed that this yeast strain was not VIN13 (Muller *et al.*, 2003). It is possible that the yeast strains that were examined in nature might be undergoing mutations. In another experiment, three genetically modified (*S. cerevisiae* [VIN13] strains that were genetically restructured) were compared with the commercial wine yeast strains, but none were recognised (Data not shown). This clearly showed that the FT-NIR method is very sensitive and specific towards the composition and genetic structure of the strains examined.

FT-NIR spectroscopy is a rapid, economical and simple technique for analytical purposes. Samples were ready for FT-NIR analysis within three days and results could be obtained within a few minutes and with a single scan. FT-NIR spectroscopy may be used by yeast producers to implement and perform rapid quality control and assurance. Large numbers of yeast strains may be isolated from the vineyard, characterised and even commercialised to be used as starter cultures in wine fermentations. It would also be possible to determine if genetically modified yeasts would spread in nature and, furthermore, to what extent they influence the existing microflora. However, preliminary data indicate that fears of commercial yeasts (genetically non-modified and modified) ultimately dominating the natural microflora in grapegrowing environments seem to be exaggerated.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

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5.1 GENERAL DISCUSSION AND OTHER PERSPECTIVES

The population constitution and dynamics of yeasts in nature are complex and varied. The literature shows that it is mainly *Kloeckera* and its anamorph, *Hanseniaspora*, that are found on the surfaces of grapes, but prevailing *Saccharomyces cerevisiae* strains complete the fermentation process. In general, the yeast *S. cerevisiae* is an important factor contributing to the quality of wines and is therefore one of the main focus points of research. Since the development of biotechnology, researchers are continuously trying to improve the physiological and biochemical properties of yeasts through genetic manipulation, but the release of the recombinant yeast strains is subject to strict statutory approval. Since existing commercial wine yeast strains are used for these manipulations, it is necessary to know to what extent these strains survive and spread in nature and to what extent they influence the fermentations of the following year.

Chapter 2 reviewed the techniques being used for the identification and differentiation of yeasts. In this review, some of the more important techniques used in the current identification and classification systems in the wine industry and biotechnology were discussed. Originally, the identification of yeast species and strains were based on morphological and physiological abilities (Kreger-van Rij, 1984; Barnett *et al.,* 1990; Guillamón *et al.*, 1998), but these techniques are expensive, time consuming and require great expertise and skill. Because yeasts play a very important role in the wine and food industry, and are being produced commercially on a large scale, an accurate and fast identification system is required. Techniques that are rapid, need little or no sample preparation and are convenient to use with a high degree of accuracy are being optimised and used in the identification and classification of yeast strains. Therefore, some of the more important molecular techniques used in the current identification and classification systems in the wine industry and biotechnology were also discussed. Electrophoretic karoytyping was selected as the starting point for genetic identification because of its use in differentiating between strains of *S. cerevisiae* (Vezinhet *et al.*, 1990; Yamamoto *et al.*, 1991; Shütz & Gafner, 1994). Although karyotyping showed differentiated chromosome patterns for every strain, it did not provide any information on species level. Other techniques, employing computer technology, have also been used to identify yeast strains, with the application of FT-NIR spectroscopy being highlighted in the review. The most common application of FT-NIR spectroscopy in the wine industry has been for the determination of the alcohol content of wine (Baumgarten, 1987; Van den Berg *et al.*, 1997; Dambergs *et al.*, 2001; Manley *et al.*, 2001). It has also been applied to routine analyses of the methanol, glycerol, fructose, glucose and total residual sugars present during the production of alcoholic beverages (Van den Berg *et al.*, 1997). Other applications include the determination of the malolactic

fermentation status in rebate wine and the determination of the free amino nitrogen (FAN) and °Brix values of must samples (Manley *et al.*, 2001). NIR has also been used for the identification of yeast strains (Halász *et al.*, 1997). Mid-infrared spectroscopy has been used for the differentiation of brewing yeast strains (Timmins *et al.*, 1998), the identification of bacteria, including *Lactobacillus* (Curk *et al.*, 1994), *Staphylococcus*, *Streptococcus*, *Clostridium*, *Legionella* and *Escherichia coli* (Helm *et al.*, 1991), and the identification of food-borne yeasts (Kümmerle *et al.*, 1998).

Due to the complexity of the vinification process and the widespread practice of seeding must with dry commercial wine yeast cultures with favourable characteristics, it has become increasingly important to use an easily identifiable strain to ensure consistency of wine type, style and quality (Pretorius & Van der Westhuizen, 1991). In an effort to achieve this, much attention has been paid to the genetic improvement of wine yeast strains to, amongst others, produce colour- and aroma-liberating enzymes (Laing & Pretorius, 1993; Pérez-González *et al.*, 1993; Lambrechts & Pretorius, 2000; Lilly *et al.*, 2000; Van Rensburg & Pretorius, 2000), convert malic acid into lactic acid (Ansanay *et al.*, 1996; Bony *et al.*, 1997; Volschenk *et al.*, 1997), and increase glycerol production (Remize *et al.*, 1999; Eglinton *et al.*, 2002). Existing commercial wine yeast strains are generally used for these manipulations, but the release of recombinant yeast strains (genetically modified organisms, GMOs) is, however, subject to statutory approval, because of concerns that these GMOs may out compete the natural microflora due to their newly acquired genetic traits. In order to establish a foundation from which knowledgeable decisions regarding the release of recombinant yeast can be made, research needs to be done on their behaviour in nature. The aim of this study was therefore to use electrophoretic karyotyping (CHEF) and FT-NIR spectrocopy to investigate the ability of current commercial wine yeast strains to survive and spread in nature. This study can be used as a stepping stone from which future studies on the monitoring of recombinant wine yeasts in nature can be undertaken.

Chapter 3 highlighted the research results obtained with the CHEF identification method. A few other methods are also mentioned in this chapter, although the focus is primarily on the results obtained with CHEF electrophoretic karyotyping. These results indicate that commercial wine yeast strains are not easily dispersed from the cellar to the vineyard. 1600 samples were isolated from various locations in the vineyard, and only seven seems to be commercial wine yeast strains, six of them which are Vin13, while the other one is N96. Not surprisingly, these strains are widely used in the South African wine industry. The commercial wine yeast strains are also more likely to be found near the cellar and the places where the grape skins are dumped. Therefore, should a recombinant yeast strain be used in wine making, it will not be dispersed from the cellar to the vineyard.

In conjunction with CHEF, a cost-effective, fast and less time-consuming identification technique, namely FT-NIR, was used. Chapter 4 describes the research results obtained with this technique. This method can be used as a rapid,

non-destructive, simple technique for analytical purposes and the inexpensive identification of yeasts. From the results obtained in this study, it is concluded that FT-NIR spectroscopy can be used to differentiate between some of the commercial wine yeast strains used in the South African wine industry. When FT-NIR was used, none of the isolated strains could be identified as commercial wine yeast strains. Several reasons might be offered as a possible explanation i.e the natural yeast flora can undergo mutations or sporulation. As confirmation, to a certain extent to this, the database was spiked with certain genetically modified VIN13 strains. These strains had not been recognised as being commercial wine yeast strains. However, spectral comparisons and principal component analysis (PCA) showed that yeast strains from the same farm and even from the same site seemed to recognise each other with high recognition rates (an average of over 70%).

In conclusion, the commercial wine yeast strains used in the South African wine industry and cellars do not disperse from the cellar to the vineyard. The very few commercial wine yeast strains founded were near the cellar and the places where the grape skins are dumped. Concerns that genetically modified yeast strains would suppress the natural micropopulation seem to be overrated.

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