Investigating the role of *Brettanomyces* and *Dekkera* during winemaking

by

Adriaan Oelofse

Dissertation presented for the the degree of
Doctor of Philosophy (Science)

at
Stellenbosch University
Institute for Wine Biotechnology, Faculty of AgriSciences

Promoter: Prof. M. du Toit
Co-promoters: Prof. I.S. Pretorius
                   Prof. A. Lonvaud-Funel

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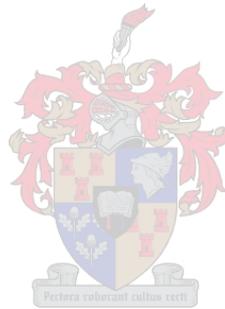
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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 21/10/2008

This dissertation is dedicated to my family, especially my parents,

Hierdie proefskrif is opgedra aan my familie, veral aan my ouers,

Rudie and Julie.

BIOGRAPHICAL SKETCH

Adriaan Oelofse *was born in Wynberg, Cape Town on the 10th of January 1977. He attended Vredenburg High School and matriculated in 1994. Adriaan enrolled at Stellenbosch University in 1995 and obtained a BSc degree in 1998, majoring in Biochemistry and Microbiology. In 2000, Adriaan enrolled at the Institute for Wine Biotechnology and obtained an Honours degree in Wine Biotechnology in December of that year. He enrolled for his Master's degree in Wine Biotechnology at the same Institute in 2001. After the graduation of his MSc degree in April 2003, Adriaan enrolled for his PhD in Wine Biotechnology.*

ACKNOWLEDGEMENTS

PREFACE

This dissertation is presented as a compilation of eight chapters, consisting of four research chapters with each of the chapters being introduced separately. All chapters are written according to the style of the *South African Journal of Enology and Viticulture*.

Chapter 1   **GENERAL INTRODUCTION AND PROJECT AIMS**

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SUMMARY

Wine quality is greatly influenced by the number of microorganisms, which occur throughout the winemaking process. These microorganisms are naturally present on the grapes and in the cellar from where they can be introduced to the winemaking process at any given time and consequently impart specific contributions to the wine quality. However, these microorganisms can be seen either as beneficial or as wine spoilage microorganisms, depending on the conditions under which they can proliferate during the winemaking process. Wine yeasts (*Saccharomyces* spp.) are typically responsible for the alcoholic fermentation; lactic acid bacteria (LAB) are responsible for malolactic fermentation (MLF), while acetic acid bacteria (AAB) and other wild yeasts (non-*Saccharomyces* spp.) are typically associated with the formation of off-flavours under poorly controlled winemaking conditions.

In recent years, evidence from the wine industry has highlighted a specific group of non-*Saccharomyces* yeast species as a serious cause for wine spoilage that required more research investigations. Yeast of the genus *Brettanomyces* or its teleomorph *Dekkera* has been identified as one of the most controversial spoilage microorganisms during winemaking as they can produce several compounds that are detrimental to the organoleptic quality of wine. This has triggered the research initiative behind this doctoral study on the significance of *Brettanomyces* and *Dekkera* yeasts during winemaking.

In this dissertation, various aspects of the detection, isolation and identification methods of *Brettanomyces* yeast from the winemaking environment were investigated. As a first objective, a culture collection of *Brettanomyces bruxellensis* wine isolates had to be established. This followed after the isolation of *Brettanomyces* yeasts from various red wine cultivars from South African wineries from different stages of the winemaking process. Different conventional microbiological methods such as plating on selective agar media and microscopy were investigated along with molecular identification techniques such as the polymerase chain reaction (PCR) in this regard.

Other focus areas of this study aimed at performing genetic characterisation and differentiation studies of *B. bruxellensis* wine isolates. For this purpose, different intraspecific identification methods were investigated on several strains, including strains of European origin. The application of molecular techniques allowing strain identification aided in the selection of specific strains that were evaluated for volatile phenol production in synthetic media and wine. The results obtained from this work indicated that a large degree of genetic diversity exists among *B. bruxellensis* strains and that the volatile phenol production differed between the strains after evaluation in synthetic media and wine.

In addition to the molecular intraspecific strain identification techniques that were investigated, a feasibility study was also performed that focused on evaluating

Fourier transform infrared (FTIR) spectroscopy combined with chemometrics as an alternative approach for differentiating between *B. bruxellensis* strains.

The two approaches of FTIR spectroscopy that were investigated involved the use of firstly, Fourier transform mid-infrared (FTMIR) spectroscopy to obtain spectral fingerprints of spoiled wines by different *B. bruxellensis* strains; and secondly, Attenuated total reflectance (FTIR-ATR) to obtain spectral fingerprints from whole cells of *B. bruxellensis* on microbiological agar media. The results of this study illustrated the potential of FTIR spectroscopy to become a reliable alternative to molecular based methods for differentiating between *B. bruxellensis* strains and for characterisation studies.

The formation of volatile phenols in wine by species of the genera *Brettanomyces* and *Dekkera* is one of the primary reasons for their classification as wine spoilage yeasts. The enzymatic activities of this reaction have been identified and involve a phenyl acrylic (phenolic) acid decarboxylase (PAD) and a vinyl phenol reductase (VPR). However, only a limited amount of information is available about these enzymes from *Brettanomyces/Dekkera* yeasts and no genetic data have been described. It was therefore imperative that this dissertation should include a genetic investigation into the phenylacrylic (hydroxycinnamic) acid decarboxylase from the species *B. bruxellensis* involved in the formation of volatile phenols. Strategies that were investigated included various molecular DNA techniques and protein purification procedures to obtain either genetic or protein sequence data. The decarboxylase activity of this yeast species towards *p*-coumaric acid was demonstrated and substantial genetic sequence data was obtained.

The results from this dissertation made a substantial contribution to the current available knowledge about *Brettanomyces/Dekkera* spp. and led to a better understanding of this wine spoilage yeast. This research developed a platform from which further investigations could follow and the knowledge gained will be invaluable for future *Brettanomyces* research projects at the Institute for Wine Biotechnology at Stellenbosch University.

OPSOMMING

Wynkwaliteit word beïnvloed deur 'n verskeidenheid van mikroörganismes wat regdeur die wynmaakproses teenwoordig is. Hierdie mikroörganismes kom natuurlik voor op druiwe en in die kelder vanwaar hulle op enige tydstip kan deel word van die wynmaakproses en gevolglik spesifieke bydraes tot die wynkwaliteit kan maak. Afhangende van die kondisies waaronder hulle kan groei gedurende die wynmaakproses, kan hierdie mikroörganismes óf as voordelig óf as bederfmikroörganismes gesien word. Wyngiste (*Saccharomyces* spp.) is tipies verantwoordelik vir die alkoholiese fermentasie en melksuurbakterieë (MSB) vir die appelmelksuurgisting (AMG). Asynsuurbakterieë (ASB) en ander wilde giste (nie-*Saccharomyces* spp.) word tipies geassosieer met die vorming van afgeure onder swak gekontroleerde wynmaakkondisies.

Die afgelope paar jaar het bewyse vanuit die wynindustrie klem gelê op 'n spesifieke groep nie-S*accharomyces* gisspesies as 'n ernstige oorsaak van wynbederf wat meer navorsing vereis. Gis van die genus *Brettanomyces* of sy teleomorf, *Dekkera,* is geïdentifiseer as een van die mees kontroversiële bederfmikroörganismes gedurende wynmaak omdat hulle verskeie komponente kan produseer wat nadelig is vir die organoleptiese kwaliteit van wyn. Dit was juis die navorsingsinisiatief agter hierdie doktorale studie oor die belang van *Brettanomyces* en *Dekkera* giste gedurende die wynmaakproses.

In hierdie skripsie is verskeie aspekte van die deteksie, isolering en identifikasie metodes van *Brettanomyces* gis in die wynmaakomgewing ondersoek. As eerste doelwit moes 'n kultuurversameling van *Brettanomyces bruxellensis* wynisolate opgestel word. Dit het gevolg na die isolasie van *Brettanomyces* giste vanuit verskeie rooi wynkultivars van Suid-Afrikaanse wynplase wat in verskillende fases van die wynmaakproses was. Verskillende konvensionele mikrobiologiese metodes soos uitplaat op selektiewe agar media en mikroskopie was ondersoek saam met molekulêre identifikasietegnieke soos die polimerase kettingreaksie (PKR).

Ander fokusareas van hierdie studie was gemik op genetiese karakterisering en differensiasie studies van *B. bruxellensis* wynisolate. Vir hierdie doel was verskillende intraspesifieke identifikasiemetodes op verskeie rasse ondersoek wat rasse van Europese oorsprong ingesluit het. Die toepassing van molekulêre tegnieke vir rasidentifikasie het gehelp in die seleksie van spesifieke rasse wat geëvalueer was vir die produksie van vlugtige fenole in sintetiese media en wyn. Die resultate van hierdie studie het gewys dat 'n groot mate van genetiese diversiteit bestaan tussen *B. bruxellensis* rasse en dat die vlugtige fenolproduksie verskil het tussen rasse na evaluasie in sintetiese media en wyn.

Bykomend tot die molekulêre intraspesifieke rasidentifikasietegnieke wat ondersoek is, is 'n moontlikheidstudie gedoen wat gefokus het op die evaluasie van Fourier Transformasie Infrarooi (FTIR) spektroskopie in kombinasie met

chemometrika as 'n alternatiewe benadering om te onderskei tussen *B. bruxellensis* rasse.

Die twee benaderings van FTIR spektroskopie wat ondersoek is het eerstens die gebruik van Fourier Transformasie mid-infrarooi (FTMIR) spektroskopie behels om spektrale vingerafdrukke van bederfde wyne deur verskillende *B. bruxellensis* rasse te bepaal; en tweedens Geattenueerde Totale Refleksie (FTIR-ATR) om spektrale vingerafdrukke van heel selle van *B. bruxellensis* op mikrobiologiese agar media te bepaal. Die resultate van hierdie studie het die potensiaal van FTIR spektroskopie geïllustreer as 'n betroubare alternatief tot molekulêr-gebasseerde metodes om te onderskei tussen *B. bruxellensis* rasse en vir karakteriseringstudies.

Die vorming van vlugtige fenole in wyn deur spesies van die genus *Brettanomyces* en *Dekkera* is een van die hoofredes vir hul klassifikasie as wynbederfgiste. Die ensimatiese aktiwiteite van hierdie reaksie is reeds geïdentifiseer en behels 'n fenielakriliese (fenoliese) suurdekarboksilase (PAD) en 'n vinielfenolreduktase (VPR).

Slegs 'n beperkte hoeveelheid informasie is egter beskikbaar oor hierdie ensieme van *Brettanomyces/Dekkera* giste en geen genetiese data is nog beskryf nie. Dit was daarom belangrik dat hierdie skripsie 'n genetiese ondersoek insluit van die fenielakriliese (hidroksiekaneel) suurdekarboksilase van *B. bruxellensis* wat betrokke is by die vorming van vlugtige fenole. Strategieë wat ondersoek was het verskeie molekulêre DNA tegnieke en proteïensuiweringsprosedures ingesluit om genetiese of proteïen volgordedata te bekom. Die dekarboksilase aktiwiteit van hierdie gisspesie teenoor *p*-koemariensuur was gedemonstreer en substansiële genetiese volgordedata was verkry.

Die resultate uit hierdie skripsie het 'n noemenswaardige bydrae gelewer tot die bestaande kennis en insig van die *Brettanomyces/Dekkera* bederfgis. Hierdie navorsing is 'n goeie basis waaruit verdere ondersoeke kan voortspruit en die kennis wat hiermee opgedoen is is baie waardevol vir toekomstige *Brettanomyces* navorsingsprojekte by die Instituut vir Wynbiotegnologie te Universiteit Stellenbosch.

CONTENTS

General Introduction
&
Project Aims

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

One of the oldest fermentation processes that have obtained considerable interest over the centuries is the process of winemaking. Winemaking is a great example of where the natural complexity of a product is a direct reflection of its quality. There are many factors throughout the winemaking process that can influence wine quality starting with vineyard practices, harvest conditions, cellar management, barrel ageing and bottling. However, the involvement of microorganisms during the winemaking process holds relation to all of these factors and plays a central role in wine production.

With the participation of microorganisms in the complex ecology of wine, recent years have seen a renewal of interest. Numerous investigations are intended to further understand the significance of these microorganisms during winemaking. Some of the different microorganisms involved in this process include the wine yeasts that are responsible for the alcoholic fermentation (*Saccharomyces cerevisiae*), the natural wild yeast population (non-*Saccharomyces*), lactic acid bacteria (LAB), which are responsible for the malolactic fermentation (MLF), and acetic acid bacteria (AAB) (Fugelsang, 1997; Ribéreau-Gayon *et al.*, 2000). Focusing on yeast, *S. cerevisiae* is the primary agent responsible for the transformation of grape juice into wine. However, a wide variety of indigenous non-*Saccharomyces* yeast species also exist that can consequently contribute to the taste and flavour of wine (Fleet and Heard, 1993; Romano *et al.*, 1997). The autochtonous microbial population of winemaking includes a diversity of yeast species that are introduced to the must and wine from the grapes and cellar environment (Rosini, 1984; Boulton *et al.*, 1996) and these can proliferate under favourable winemaking conditions. The metabolites derived from the non-*Saccharomyces* yeasts contribute to the flavour, aroma and taste of the final product (Lambrechts and Pretorius, 2000) along with the commercial starter cultures. These metabolic by-products can however impart negative elements to the wine character and the yeasts capable of causing unwanted modifications to the sensorial properties of wine are regarded as potential spoilage microorganisms (Loureiro and Malfeito-Ferreira, 2003). Depending on consumer preference, individual perception and wine style, it is sometimes difficult to distinguish between yeast activity that is detrimental and that which is beneficial and therefore microbial spoilage during winemaking is not easily defined.

It has been indicated that yeasts of the genera *Brettanomyces* and *Dekkera* (teleomorph form) can affect the chemical composition of the must and wine by producing various metabolites that are considered to be detrimental to the organoleptic properties of the final product. For this reason, *Brettanomyces/Dekkera* yeasts are regarded as spoilage microorganisms that occur throughout the winemaking process and are well recognised for their potential to produce undesirable flavours (Chatonnet *et al.*, 1995). Indications from the winemaking environment have revealed that these spoilage yeasts can cause turbidity and colour change in wine and produce substantial quantities of acetic acid, tetrahydropyridines and phenolic off-flavours (e.g. 4-ethylphenol, 4-ethylguaiacol), which all affect the

sensory quality of wine (Herezstyn, 1986; Chatonnet *et al.*, 1992; Ciani and Ferraro, 1997). For many years, barrel aging has been considered as a source of spoilage. However, better surveys of the yeast population and spoilage has clearly shown that the problem could occur even in stainless steel tanks and also during the alcoholic fermentation. An increasing number of winemakers have started to question the role of *Brettanomyces/Dekkera* during the winemaking process. *Brettanomyces*-associated problems have seemingly become more prominent in recent years as a consequence of lower sulfur dioxide (SO_2) usage due to pressing consumer demands, the increase of pH that lowers the SO_2 efficiency and the favourable conditions during aging in barrels (Du Toit *et al.*, 2005; Renouf *et al.*, 2006). This in conjunction with concerns about adequate cellar hygiene and less-severe processing regimes (Loureiro and Querol, 1999) has triggered a series of investigations.

With regards to wine spoilage, particularly from *Brettanomyces*/*Dekkera* yeasts, the tools currently available are limited in their ability to measure the presence of spoilage microbes (Loureiro and Malfeito-Ferreira, 2003). A great deal of uncertainty exists as many difficulties are encountered in the routine detection, isolation and identification of this yeast at genus level (Ibeas *et al.*, 1996). These issues make it difficult to employ strategies for the monitoring and control of these species. The current methods are time consuming, and thus faster, more accurate methods are needed. It is also of great importance to determine strain diversity among these strains to see if all strains are associated with spoilage. This will allow researchers to perform characterisation studies on these strains to establish the influence of the specific wine parameters (pH, SO_2, ethanol etc.) on their growth and survival during winemaking. Generation of such valuable data can be directly beneficial to the winemaker and can be considered alongside the implementation of control strategies for *Brettanomyces/Dekkera* in order to maintain good quality control.

1.2 PROJECT AIMS

This study forms an integral part of a larger research project on wine spoilage caused by microorganisms that is being conducted at the Institute for Wine Biotechnology. This study started with the investigation of the occurrence and distribution of the different species of *Brettanomyces/Dekkera* throughout the winemaking process in South Africa, specifically in red wines. *Brettanomyces*/*Dekkera* was chosen as spoilage yeast as very little knowledge was available at the time. The wine industry experienced more problems with *Brettanomyces* and therefore the project was initiated to firstly build a culture collection of South African strains and then to gain fundamental knowledge on their spoilage potential. The specific aims of this study were as follows:

 (i) to establish contact with the industry to obtain participants for the project;
 (ii) to evaluate and improve current methods of isolation, detection and identification;
(iii) to isolate *Brettanomyces* yeasts from various wines throughout the winemaking
        process;
(iv) to establish a protocol for routine detection and identification of *Brettanomyces*
        yeasts in the winemaking environment;

(v) to investigate different molecular techniques for inter- and intraspecies identification;

(vi) to select genetically diverse strains of *Brettanomyces* and evaluate their volatile phenol production in synthetic media and wine;

(vii) to investigate the use of Fourier transform infrared (FTIR) spectroscopy for the discrimination of wines contaminated with *Brettanomyces*;

(viii) to investigate the use of Fourier transform infrared – attenuated total reflectance (FTIR-ATR) spectroscopy for *Brettanomyces* strain discrimination (whole intact cells);

(ix) to purify the phenolic acid decarboxylase enzyme involved in the formation of volatile phenols by protein purification;

(x) to isolate the phenolic acid decarboxylase gene involved in the formation of volatile phenols by molecular methods.

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Literature Review

The significance of *Brettanomyces* and *Dekkera* during winemaking: a synopsis of scientific focus areas

2. SIGNIFICANCE OF *BRETTANOMYCES* AND *DEKKERA* DURING WINEMAKING: A SYNOPSIS OF SCIENTIFIC FOCUS AREAS

2.1 INTRODUCTION

Winemaking comprises a diverse set of factors that play a crucial role during the transformation of grapes to wine. The most important factors generally considered by winemakers include vineyard management, grape quality, winemaking practices and commercial wine yeast selection. However, the microbiology behind the wine should also be considered because this is one of the parameters often neglected as a quality control constraint. Wine microbiology entails a complex interaction of a variety of microorganisms that play an essential role on the outcome of the final product and, if the microbiology of wine is disregarded, there will simply be no wine.

The involvement of microorganisms in the fermentation of alcoholic beverages has been a subject of interest for centuries. As early as the mid-1800s, Louis Pasteur observed the conversion of grape juice into wine by the action of yeast and noticed the presence of bacteria that were capable of causing wine spoilage (Drysdale and Fleet, 1988). Since then the microbiology of wine has been the topic of many investigations and a large diversity of microorganisms that are present during the winemaking process have been identified (Fugelsang, 1997; Loureiro, 2000).

Apart from the principal wine yeast *Saccharomyces cerevisiae,* different genera and species of bacteria and non-*Saccharomyces* yeasts have been identified*.* These microorganisms form a natural part of the active biomass involved in the winemaking process as they are found on grapes, in the must and wine and can therefore contribute to the organoleptic properties of the final product (Heard and Fleet, 1988; Fleet and Heard, 1993; Lambrechts and Pretorius, 2000). The contributions are, however, not always positive with regard to the wine's flavour because microbial activity often results in wine spoilage.

Wine spoilage is a serious problem for the wine industry because it renders the products unacceptable and can lead to large economic losses. For this purpose, research is targeted towards the microorganisms that are responsible for spoilage during the winemaking process. The typical focus areas include: methods of detection, identification and characterisation of spoilage microorganisms; the monitoring and control of spoilage compounds; and fundamental investigations to gain more knowledge on the metabolism and activities of spoilage microorganisms.
One of the controversial yeasts that has gained increasing attention in recent years, specifically as it is associated with wine spoilage, belongs to the genera *Brettanomyces* and *Dekkera* well-known for the production of ethylphenols.

This review presents a summary of some the above-mentioned major scientific research focus areas about the yeasts *Brettanomyces* and *Dekkera* during winemaking.

2.2 *BRETTANOMYCES/DEKKERA* DURING WINEMAKING

2.2.1 HISTORY OF *BRETTANOMYCES*

The first reference to the genus *Brettanomyces* dates back to 1904 when N.H. Claussen isolated a yeast from a slow secondary fermentation of an old English stock beer (Gilliland, 1961). The flavours produced by this yeast became characteristic of the British beers of that time and so the name 'Brettanomyces' was derived from 'British brewing fungus'. It was not until the 1940s, when M.T.J. Custers performed the first systematic study on *Brettanomyces* yeast, that *Brettanomyces* was associated with wine (Custers, 1940). Although this study included 17 strains, of which most were isolated from beer, one strain originated from a French wine (Krumbholz and Tauschanoff, 1933).

2.2.2 DIFFERENT SPECIES IN WINE

The taxonomy of the genus *Brettanomyces* has seen numerous reclassifications over the years from the handful of species that were initially identified. Originally, these species included *B. bruxellensis*, *B. lambicus, B. clausenii, B. anomalus* and *B. intermedius,* which reproduced asexually by means of budding (Custers, 1940; Van der Walt and Van Kerken, 1958). The genus *Dekkera* was introduced to the taxonomy in 1964 after the production of ascospores (sporulating-form) was observed (Van der Walt, 1984). Currently, the five species jointly belonging to the genera *Brettanomyces* and *Dekkera* are: *Brettanomyces custersianus, Brettanomyces naardenensis, Brettanomyces nanus, Brettanomyces anomalus* and *Brettanomyces bruxellensis* (Kurtzman and Fell, 2000; Cocolin *et al.,* 2004). Teleomorphs (perfect state) are known for the last two species, *Dekkera anomala* and *Dekkera bruxellensis* (Kurtzman and Fell, 2000). The details regarding the morphological, biochemical and physiological characteristics of these species are well described in recent classification manuals (Barnett *et al.*, 2000; Boekhout *et al.*, 2002; Kurtzman and Fell, 2000). From the five species currently known, the species primarily associated with winemaking is *B. bruxellensis* (*D. bruxellensis*) (Egli and Henick-Kling, 2001; Stender *et al.,* 2001; Cocolin *et al.,* 2004), although *B. anomalus* (*D. anomala*) and *B. custersianus* isolations from must fermentations have been reported in two instances (Querol *et al.*, 1990; Esteve-Zarzoso *et al.*, 2001). With advances in DNA-based methods, recent wine-related investigations often include *D. anomala* along with the predominant species *D. bruxellensis* as conventional methods had showed difficulty in differentiating between these two species (Loureiro and Malfeito-Ferreira, 2006). Although current taxonomical classifications suggest that *Dekkera* should be used in reference with the species *bruxellensis* and *anomala* (Boekhout *et al.*, 1994)*,* many discrepancies exist and some authors frequently prefer using the technically incorrect naming of *Brettanomyces bruxellensis* and *B. anomalus* when referring to these yeasts in a winemaking context. This is largely

attributed to the fact that the sexual or sporulating form, *Dekkera,* is yet to be found in wine.

Some authors have made the point that the separation of *Brettanomyces* and *Dekkera* in the context of wine is meaningless because current molecular DNA techniques reveal no distinction between the anamorph and teleomorph forms (Loureiro and Malfeito-Ferreira, 2006). This might explain why it is not uncommon to see the use of '*Brettanomyces*/*Dekkera* spp.' in wine research. In this review, the same context will be used in which the original authors used the naming in their publications. This can either be *B. bruxellensis* or *D. bruxellensis.*

2.2.3 OCCURRENCE AND DISTRIBUTION DURING WINEMAKING

Brettanomyces/*Dekkera* spp. are ubiquitously distributed in nature and their occurrence and spoilage activities have been well summarised by Loureiro and Malfeito-Ferreira (2006). The majority of reports associate *Brettanomyces*/*Dekkera* spp. with fermented food products ranging from cheeses and fermented milk to various alcoholic beverages including wine, beer, cider, kombucha (fungus-tea) and tequila (Davenport, 1976; Kumara and Verachtert, 1991; Lachance, 1995; Kosse *et al.*, 1997; Gadaga *et al.*, 2002; Teoh *et al.,* 2004; also see references cited in Licker *et al.*, 1998; Loureiro and Malfeito-Ferreira, 2006). Less frequent reports of their isolations from other sources (bees, fruit-flies, olives and carbonated drinks) are also available (Van der Walt and Van Kerken, 1958; Phaff *et al.*, 1978; Deák and Beuchat, 1995; Kotzekidou *et al.*, 1997). *Brettanomyces*/*Dekkera* spp. have been and still are isolated from wines and wineries all around the world, predominantly from red wines. Although these yeasts are also isolated from white wines (Licker *et al.*, 1998; Dias *et al.*, 2003b) this is less frequent which is their loss of viability and the consequent non-existence of ethylphenol levels in white wines is largely ascribed to the efficiency of sulfur dioxide (SO2) at lower pH conditions (Loureiro and Malfeito-Ferreira, 2006). Hence, the focus of the research on these yeasts has primarily fallen on their occurrence in red wine.

The winemaking process hosts multiple sources where *Brettanomyces*/*Dekkera* spp. can survive and numerous debates about the initial source and dispersion of these yeasts have occurred (Licker *et al.*, 1998). The vineyard provides many sources, including the soil, rootlets, bark, leaves and grapes. Davenport (1976) investigated all of these but could not isolate any *Brettanomyces* spp. In 1987 however, Guerzoni and Marchetti reported their isolation from grapes damaged by sour rot (Loureiro and Malfeito-Ferreira, 2006). This agrees with recent knowledge suggesting a connection between *Brettanomyces*/*Dekkera* and damaged grapes (*Botrytis*-affected) (Taillandier, 2007). Surprisingly, only one investigation has been successful in recovering *Brettanomyces/Dekkera* spp. from grapes (Renouf and Lonvaud-Funel, 2006) despite the fact that they have been isolated many times from fermenting musts during earlier research (Licker *et al.*, 1998; Pretorius, 2000; Jolly *et al.*, 2003; Prakitchaiwattana *et al.*, 2004; Van de Water, 2004). The poor detection

of *Brettanomyces/Dekkera* spp. on grapes has been speculated to be the result of their low cell numbers amid a diverse microbial ecosystem where other wild yeast and bacterial species dominate. Renouf and Lonvaud-Funel (2006) have, however, overcome this problem by developing an enrichment medium that enabled them to detect *B. bruxellensis* on grape berries. They were subsequently able to detect this yeast from several vineyards and at different stages of grape berry development.

Following the initial stages of winemaking, *Brettanomyces/Dekkera* spp. have been more consistently associated with wine and cellar equipment (Fugelsang, 1998). As their populations are usually minor in the presence of numerous other rapidly fermenting yeasts, their increase in numbers only during more nutritionally favourable conditions that suit their slow-growing characteristics (Fugelsang *et al.*, 1993). These conditions are created once alcoholic fermentation is completed and traces of residual sugars allow them to proliferate more easily. Malolactic fermentation (MLF) and ageing in used barrels have therefore been recognised as the most critical stages of wine production for *Brettanomyces/Dekkera* contamination (Chatonnet *et al.*, 1992; Fugelsang *et al.*, 1993; Chatonnet *et al.*, 1995; Licker *et al.*, 1998; Renouf *et al.*, 2006b; Suárez *et al.*, 2007). During MLF, *Brettanomyces/Dekkera* spp. is presented with conditions of low free SO_2, residual sugar concentrations and yeast autolysis with the release of nutrients occurring along with modest microbial competition. The main characteristics of oak barrels (new and old) that are beneficial to *Brettanomyces/Dekkera* growth are the porous microstructure, which allows the influx of small amounts of oxygen (Swaffield and Scott, 1995; Loureiro and Malfeito-Ferreira, 2006) and the presence of cellobiose that can serve as sugar resource (Boulton *et al.*, 1996). In addition, difficulty of sanitation (old barrels) is favourable to established *Brettanomyces/Dekkera* populations and promotes contamination of wine (Pollnitz *et al.*, 2000; Yap *et al.*, 2007). When MLF is performed in barrels these characteristics can aid the growth of *Brettanomyces/Dekkera* during this phase of winemaking. That these yeasts have also been recovered from wines in concrete or stainless steel tanks is more likely due to other reasons of survival than those pertaining in barrels (Chatonnet *et al.*, 1992; Rodrigues *et al.*, 2001). Furthermore, numerous finished and bottled wines have also been known to host *Brettanomyces/Dekkera* populations. These wines have been linked to prior conditions of long periods of barrel ageing, lower SO_2 concentrations and less filtration prior to bottling (Herezstyn, 1986a; Arvik *et al.,* 2002).

Wineries and equipment that have been investigated revealed the presence of *Brettanomyces/Dekkera* yeasts in winery air samples and on cellar walls, drains, pumps, transfer lines and other pieces of equipment that are difficult to sterilise (Van der Walt, 1984; Alguacil *et al.*,1998; Fugelsang, 1998; Connel *et al.*, 2002). It is therefore not surprising that wineries are often considered as the primary source of *Brettanomyces/Dekkera* contamination, as opposed to grapes. The yeast *D. bruxellensis* (*B. bruxellensis*) is regarded as a frequent contaminant of the winemaking process. However, as its occurrence is often inconsistent, each winery

can present a unique situation that requires the determination of the specific origin and route of contamination.

2.2.4 DETECTION AND IDENTIFICATION METHODS

2.2.4.1 Isolation media

The isolation of *Dekkera/Brettanomyces* spp. from winemaking environments is not easy as they are slow growing and have a relative low occurrence (Fugelsang, 1997).

Additionally, it has been described that *Dekkera/Brettanomyces* yeasts are difficult to recover from materials heavily contaminated with other microorganisms (Van der Walt and Van Kerken, 1960). For this purpose, several authors have investigated different possibilities of selective media by altering the main constituents and carbon sources (Heard and Fleet, 1986). The earlier media for *Brettanomyces* isolation included maltose and sucrose as carbon sources and it was reported that the use of sorbate, ethanol and cycloheximide as antimicrobials was not satisfactory (Van der Walt and Van Kerken, 1960; Wright and Parle, 1974). More recent studies included glycerol and trehalose with sucrose as carbon sources with a wider range of antimicrobial agents (gentamicin, oxytetracycline, cycloheximide and sorbic acid) to suppress the growth of unwanted yeasts and bacteria (Chatonnet *et al.*, 1992; Fugelsang *et al.*, 1997; Alguacil *et al.*, 1998). Furthermore, vitamins such as thiamine and biotin have also been suggested as these can be beneficial to the growth of *Dekkera/Brettanomyces*, although some authors do not regard this as necessary (Fugelsang *et al.*, 1997; Loureiro and Malfeito-Ferreira, 2006). The development of a selective or differential medium specifically for the isolation of *Dekkera/Brettanomyces* spp. was presented by Rodriguez *et al.* (2001). This medium named DBDM (*Dekkera/Brettanomyces* Differential Medium) was reported as being able to recover less than 1% of the target yeasts from a total microbial population in combination with the Most Probable Number (MPN) technique. Along with yeast nitrogen base (YNB) this medium contained two antimicrobial agents (ethanol and cycloheximide), a pH indicator (bromocresol green) to indicate media acidification and a substrate (*p*-coumaric acid). The latter compound was included as its degradation results in a distinct phenolic off-flavour that can be indicative of *Dekkera/Brettanomyces* activity. For a more comprehensive list of media that have been tested for the detection of *Dekkera/Brettanomyces* spp. refer to Rodriguez *et al.* (2001). The development of a selective liquid medium that enabled the detection of *Dekkera/Brettanomyces* spp. followed the works of Rodriguez *et al.* (2001) and Couto *et al.* (2005a). This WLN (Wallerstein Laboratory Nutrient)-based medium was aimed at the development of a simple detection system for *Brettanomyces/Dekkera* yeast that could be used on a routine basis in the wine industry (Couto *et al.*, 2005a). Liquid media have been described as having a resuscitation function that could be beneficial for the recovery of some microorganisms (e.g. yeast) while reducing mould growth (Loureiro and Malfeito-Ferreira, 2006).

As mentioned before, the prevalence of *Brettanomyces/Dekkera* spp. on grapes has been remarkably low and the lack of recoveries has been ascribed to the short-comings of optimal isolation media and poor detection limits. Renouf and Lonvaud-Funel (2006) proposed the use of an enrichment medium to overcome this problem and obtained good success with the detection of *D. bruxellensis* on the surface of grape berries. The use of enrichment steps has previously proven very useful for the detection of scarcely represented *S. cerevisiae* and *S. paradoxus* on grapes (Van der Westhuizen *et al.*, 2000; Redzepovic *et al.*, 2002) and should definitely be considered at times when the presence of *Brettanomyces/Dekkera* is uncertain. It is also necessary to emphasize the importance of incubation time while performing detection and isolation of *Brettanomyces/Dekkera* spp. from the winemaking environment. Their low growth rate and fastidious nutritional requirements demand incubation times of up to two weeks (Rodrigues *et al.*, 2001) making the general incubation periods (three to six days at 25-30°C) used for other yeasts inadequate for routine microbiological screenings.

Direct methods of enumeration by plating on selective growth media can be inaccurate resulting from the possible viable but non-culturable (VBNC) state of microorganisms. Cells in the VBNC state are metabolically active but unable to undergo cellular division for growth in liquid or on agar and are therefore non-culturable (Oliver, 1993). Moreover, evolution to a VBNC state is related to the intensity of the stress (Oliver *et al.*, 1995) and there are hypotheses currently about whether SO_2 and ethanol could induce this state amongst *Brettanomyces/Dekkera* spp. (Millet and Lonvaud-Funel, 2000; Arvik *et al.*, 2005; Du Toit *et al.*, 2005). This is especially important for *Brettanomyces/Dekkera* spp. as we have found instances where wines contained objectionable levels of ethyl phenols but yielded no culturable cells.

2.2.4.2 DNA-based identification techniques

As discussed in the previous section, the conventional identification methods for *Brettanomyces/Dekkera* spp. are insufficient, especially during the winemaking process where a period of a week is crucial. The low relative occurrence, prolonged incubation times and variable identification results often obtained due to their mixed morphological features (Smith, 2002) prompted development of more rapid and reliable identification techniques of these spoilage yeasts. Therefore recent years have seen the development of several molecular DNA-based techniques (Loureiro and Querol, 1999).

Stender *et al.* (2001) developed a technique that does not require DNA extraction and utilises microscopic visualisation of fluorescent *Brettanomyces/Dekkera* cells after *in situ* hybridisation of species-specific PNA (peptide nucleic acid) probes to the 26S ribosomal RNA (RNA-FISH hybridisation). The authors have assigned a high specificity to this method which uses pelleted *D. bruxellensis* cells from a centrifuged wine. Considering the difficulties encountered

with microscopic identification of cell morphologies this method can be very useful. A similar study described the use of FISH probes on sequence regions beyond the D1/D2 domains of the 26S rRNA gene that can successfully detect all of the five currently known *Brettanomyces/Dekkera* species *(D. bruxellensis, D. anomala, B. custersianus, B. nanus* and *B. naardenensis*) (Röder *et al.*, 2007).

Other direct molecular techniques that are fast, sensitive and accurate involve the polymerase chain reaction (PCR). Specific sequences spanning the 5.8S ribosomal RNA genes and their flanking internal transcribed spacer (ITS1 and 2) regions can be targeted for species identification of *Brettanomyces/Dekkera* (Esteve-Zarzoso, 1999; Egli and Henick-Kling, 2001). The 5.8S rRNA and ITS regions have been documented in many studies for yeast identification (White *et al.*, 1990; Guillamon *et al.*, 1998; Esteve-Zarzoso, 1999; Granchi *et al.*, 1999) and can include restriction fragment length polymorphisms (PCR-RFLP) for interspecies discrimination of *Brettanomyces/Dekkera* yeasts (Esteve-Zarzoso, 1999; Nisiotou and Gibson, 2005). A nested PCR method comprising two primer sets has been developed for the direct detection of *Brettanomyces/Dekkera* strains in sherry (Ibeas *et al.*, 1996). This approach is very efficient for identification of *D. bruxellensis* strains from intact yeast cells. Another highly specific PCR (targeting the D1-D2 loop of the 26S rRNA) was developed by Cocolin *et al.* (2004) that form amplification products only with the species *B. bruxellensis* and *B. anomalus*. Differentiation between these two species could be achieved after restriction enzyme analysis (*Dde*I) of the amplified products. The use of denaturing gradient gel electrophoresis (PCR-DGGE) for the characterisation of yeast diversity within wine fermentations has also been indicated to detect *Brettanomyces* yeasts (Cocolin *et al.*, 2004; Renouf *et al.*, 2006a).

One of the concerns about direct PCR methods is that the sensitivity can depend on the level of contamination (Loureiro and Malfeito-Ferreira, 2006) and that only a high detection limit ($\geq 1 \times 10^4$ cfu/mL) may provide a positive result. Several authors have reported that wines could be tainted with a phenolic off-flavour character by *Brettanomyces* counts below this value (Ibeas *et al.*, 1996; Phister and Mills, 2003; Cocolin *et al.*, 2004) and therefore PCR detection limits of less than 10^4 cfu/mL are required. Phister and Mills (2003) employed real-time PCR and showed detection of D. bruxellensis in wine at levels as low as one to 10 cells/mL, depending on the dilution factor of the sample. In contrast however, Delaherche *et al.* (2004) obtained a detection limit of 10^4 cfu/mL with real-time PCR and this currently questions the routine use of this technique. A change of the DNA extraction has greatly improved the detection limit to 10 cfu/mL by the same authors (personal communication, 2007). Another recent suggestion for achieving detection levels of about 10 cfu/mL for *Brettanomyces/Dekkera* yeasts has included the use of a loop-mediated isothermal amplification (LAMP) method (Hayashi *et al.*, 2007).

2.2.4.3 Genetic diversity and techniques for strain discrimination

The identification of *D. bruxellensis* as the primary spoilage species during winemaking was soon followed by investigations that focused on determining the genetic diversity amongst this species. Intraspecies identification of *Brettanomyces/Dekkera* yeasts has not been frequently reported and some of the first techniques that have been described used random amplified polymorphic DNA (RAPD-PCR) and amplified fragment length polymorphisms (AFLPs) (de Barros Lopes *et al.*, 1999; Mitrakul *et al.*, 1999). Genetically different strains of *D. bruxellensis* wine isolates were revealed from different vintages and exhibited different chromosomes (three or four) and consequently different chromosomal fingerprints (Mitrakul *et al.*, 1999). It was also shown that the wine strains of *D. bruxellensis* were genetically different from reference strains. Several studies have since been performed that allow for strain identification and they included techniques such as, AFLPs (Bellon *et al.*, 2003; Curtin *et al.*, 2007); PCR fingerprinting with microsatellite primers; intron splice site-PCR (de Barros Lopes *et al.*, 1998); sequencing a portion of the 26S rRNA gene (Conterno *et al.*, 2006); restriction enzyme analysis of mitochondrial DNA and RAPD-PCR with OPA-primers (Martorell *et al.*, 2006); restriction enzyme analysis - pulsed field gel electrophoresis (REA-PFGE) (Miot-Sertier and Lonvaud-Funel, 2007); and PCR-DGGE (Renouf *et al.*, 2006c). Genetic characterisation studies have relevance in the wine industry because they connect different *D. bruxellensis* strains with geographic origin, vintage year and wine variety (Conterno *et al.*, 2006). Renouf *et al.* (2006c) found three different chromosomal patterns (after digestion with restriction enzymes) for *D. bruxellensis* isolates from different French wineries, but concluded that the same strains were predominant throughout the winemaking process at the specific wineries. Conterno *et al.* (2006) found that a total of 47 wine isolates of *B. bruxellensis* could be grouped into six clusters. The same authors also found that physiological traits were highly variable and did not correlate with the groupings from the DNA analysis. Therefore, the genetic diversity that exists among species with related genomes should be further explored to obtain correlations between phenotype (visible and biochemical properties) and genetic composition (Bellon *et al.*, 2003).

In a large study using AFLP analysis for the characterisation of *D. bruxellensis* isolates from Australian wineries, eight genotypes have been found (Curtin *et al.*, 2007). These eight strain groupings originated from a total of 244 *D. bruxellensis* isolates from 31 red winemaking regions, in which some strains regularly prevailed. It was also found that the wine strains were highly divergent from the *D. bruxellensis* type strain (Bellon *et al.*, 2003; Curtin *et al.*, 2007).

Future studies on strain identification might give clearer information on the origin of these species during the vinification process. By tracing the routes of *Brettanomyces/Dekkera* contamination genetically, it might be possible to gather beneficial information for the winemakers which could be considered along with preventative measures (Miot-Sertier and Lonvaud-Funel, 2007).

2.3 WINE SPOILAGE BY *BRETTANOMYCES/DEKKERA* SPP.

Brettanomyces/Dekkera yeasts obtained their significance in wine due to the formation of various spoilage compounds that are detrimental to wine quality. For this reason, most of the investigations performed on these yeast species focussed on their wine spoilage capabilities in order to establish the importance of their occurrence during winemaking.

This section will summarise the current knowledge of the main compounds and describe their impact on the organoleptic properties of wine.

2.3.1 PRODUCTION OF VOLATILE PHENOLS

The production of phenolic off-flavours (POF), specifically volatile phenols, defines the importance of *Brettanomyces/Dekkera* yeasts during winemaking and has been well documented (Heresztyn, 1986a; Chatonnet *et al.*, 1992, 1995, 1997; Edlin *et al.*, 1995; Licker *et al.*, 1998; Suárez *et al.*, 2007). Volatile phenols represent a large family of aromatic compounds of which the vinyl- and ethylphenols are implicated with *Brettanomyces* spoilage (Chatonnet *et al.*, 1992). These volatile phenols, especially the ethylphenols, are responsible for off-odours that have been described as 'animal', 'medicinal', 'Elastoplast', 'sweaty leather', 'barnyard', 'spicy' and 'clove-like' and are detrimental to the aroma profile of wines at high concentrations (Chatonnet *et al.*, 1992; 1995; Suárez *et al.*, 2007).

The formation of volatile phenols by *Brettanomyces/Dekkera* yeast has been shown to be the result of enzymatic transformation of phenolic (hydroxycinnamic) acids present during winemaking (Heresztyn, 1986a; Chatonnet *et al.*, 1992). Hydroxycinnamic acids are naturally present in grape juice and wine and originate from the grapes, where they are generally esterified with tartaric acid or anthocyanin esters (Dugelay *et al.*, 1993). The action of enzymes with cinnamoyl-esterase activity releases these weak acids to their free forms (Gerbaux *et al.*, 2002), in which they can be inhibitory towards the growth of many microorganisms (Stead, 1995; Zaldivar and Ingram, 1999; Barthelmebs *et al.*, 2001). However, *Brettanomyces/Dekkera* spp. overcome the toxicity problem by converting these acids into volatile phenols. The formation of volatile phenols by *Brettanomyces/Dekkera* spp. is shown in the graphical representation in Figure 2.1. The free hydroxycinnamic acid precursors (*p*-coumaric, ferulic and caffeic acid) are decarboxylated into hydroxystyrenes (4-vinylphenol, 4-vinylguaiacol and 4-vinylcatechol, respectively), and then reduced into their corresponding ethyl-derivative forms (4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol, respectively) (Heresztyn, 1986a; Chatonnet *et al.*, 1992; Hesford *et al.*, 2004). It is believed that the free available hydroxycinnamic acids can be caused by the action of fungal enzymes or by grape juice heating (Gerbaux *et al.*, 2002), although other mechanisms may exist. It is speculated that *Brettanomyces/Dekkera* spp. might be able to hydrolyse bound phenolic acids, but there is no scientific proof for this. The bound or the free hydroxycinnamic forms are however not the sole requirement for these yeasts to produce the ethyl-derivatives as

B. bruxellensis has been shown to produce 4-ethylphenol directly from 4-vinylphenol as substrate (Dias *et al.*, 2003b).

The two enzymes that facilitate the biotransformation of phenolic acids involve a phenolic (cinnamic) acid decarboxylase (PAD) for the formation of the vinyl derivatives and a vinyl phenol reductase (VPR) for the formation of the ethyl derivatives thereafter. The decarboxylation step has been linked to the *POF1* (phenolic off-flavour) or *PAD1* (phenylacrylic acid decarboxylase) gene of *S. cerevisiae* (Clausen *et al.*, 1994). Similar decarboxylase activities exist in numerous bacteria, fungi and yeast species, of which some are present during the winemaking process (Heresztyn, 1986a; Chatonnet *et al.*, 1992; Cavin *et al.*, 1993; Degrassi *et al.*, 1995; Edlin *et al.*, 1995; Cavin *et al.*, 1997; Edlin *et al.*, 1998; Shinohara *et al.*, 2000; Van Beek and Priest, 2000; Barata *et al.*, 2006; Couto *et al.*, 2006). The reduction step and ethylphenol formation occurs less frequently in microorganisms (Chatonnet *et al.*, 1995; Barthelmebs *et al.*, 2001), but is particularly effective in wine by the species *D. bruxellensis* and *D. anomala* (Edlin *et al.*, 1995; Chatonnet *et al.*, 1997; Dias *et al.*, 2003a). Furthermore, *S. cerevisiae* are not able to produce ethylphenols (Chatonnet *et al.*, 1993), and LAB, predominantly *Lactobacillus* spp., are only capable of producing low amounts under oenological conditions (Chatonnet *et al.*, 1995; Couto *et al.*, 2006). Recently, strains of *Pichia guilliermondii* have also been reported as producing considerable quantities of ethylphenols in grape must, to an extent similar to *D. bruxellensis* strains (Dias *et al.*, 2003a). As *P. guilliermondii* have been recovered from grapes, grape juice and grape juice-related environments such as winery equipment, they have great significance for wine spoilage through the production of volatile phenols. However, these species are not capable of producing high levels of 4-ethylphenol in wine (Barata *et al.*, 2006).
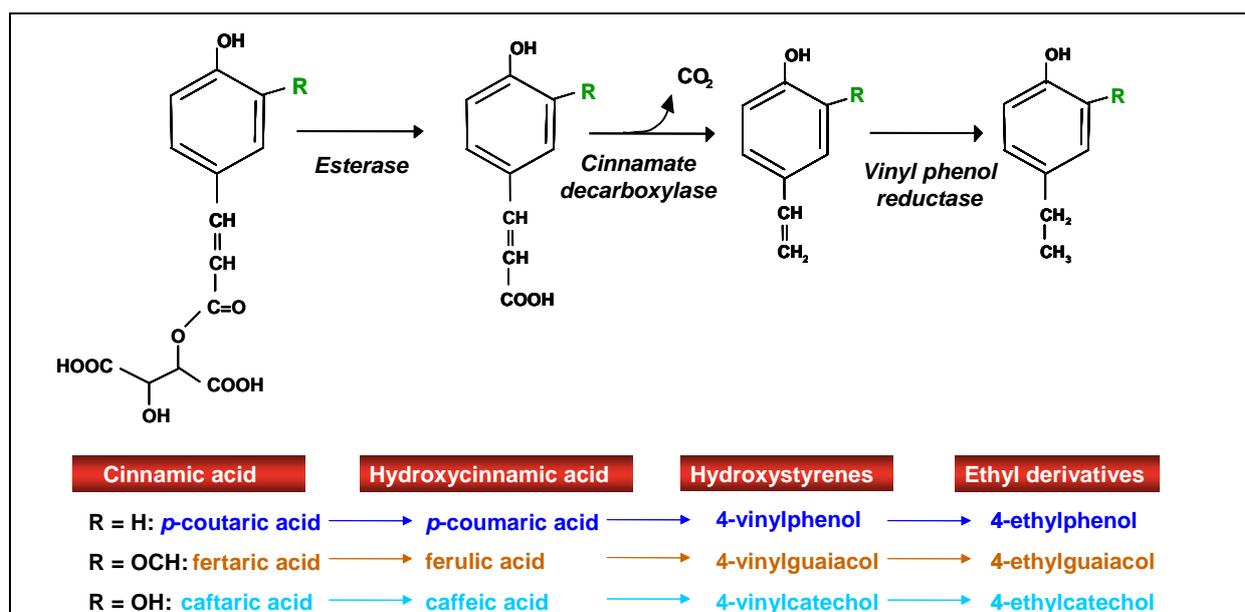
FIGURE 2.1.
Formation of volatile phenols via the decarboxylation of hydroxycinnamic acids.

Numerous studies have been performed to elucidate the role of volatile phenolic compounds and the formation of '*Brettanomyces* character' in wine (Heresztyn, 1986a Chatonnet *et al.*, 1992; 1995; 1997; Edlin *et al.*, 1995; Licker *et al.*, 1998; Dias *et al.*, 2003b; Coulter *et al.*, 2004; Hesford and Schneider, 2004; Francis and Newton, 2005). It has been found that the threshold concentrations of these compounds (Table 2.1), especially the ethyl derivatives, vary substantially and the perception of the individual aromas is greatly influenced by the wine style, cultivar and the consumer's perceptive abilities.

For more detailed overviews of wine spoilage by volatile phenols and the relevance of *Brettanomyces/Dekkera* yeasts also refer to Loureiro and Malfeito-Ferreira (2006) and Suárez *et al.* (2007).

TABLE 2.1.
Aroma threshold values of volatile phenols in wine (taken from Curtin *et al.*, 2005).

Compound	Concentration in red wine (µg/L)	Aroma threshold (µg/L)	Aroma descriptor
4-Vinylphenol	8.8-43	440*/600**	Phenol Medicinal
4-Vinylguaiacol	0.2-15	33*/110**	Clove-like
4-Ethylphenol	118-3696	30-60**	Horsey
4-Ethylguaiacol	1-432	20***	Spicey, clove
4-Ethylcatechol	27-427	10*	Phenol Band-Aid® Medicinal Banyard

*model wine, **red wine, ***water.

2.3.2 OTHER SPOILAGE FAULTS

Brettanomyces/Dekkera yeasts have also been responsible for turbidity or haziness in wine (Van der Walt and Van Kerken, 1958; Van Zyl, 1962 in Licker *et al.*, 1998) along with the production of several other metabolites that can contribute to wine spoilage. However, the conditions under which some of these are produced in wine and the exact mechanisms involved are not fully understood.

2.3.2.1 Volatile acidity (VA) and other volatile fatty acids

Brettanomyces/Dekkera spp. have long been known for their ability to affect wine quality negatively through the formation of acetic acid, which constitutes more than 90% of wine's volatile acidity (VA) (Van der Walt and Van Kerken, 1958). Elevated levels of acetic acid can be detrimental to wine quality as it imparts a vinegary/acetone-like aroma (Eglinton and Henschke, 1999) and has also been associated with sluggish/stuck fermentations (Bisson, 1999). *Brettanomyces/Dekkera* yeasts consist of a particular metabolism that enables them to produce acetic acid

(Licker *et al.* 1998; Loureiro and Malfeito-Ferreira, 2006). M.T.J. Custers was the first to describe that the presence of oxygen stimulated glucose fermentation and that this led to the introduction of a biochemical characteristic known as the "negative Pasteur effect" (or 'Custers' effect') (Scheffers and Wiken, 1969; Wijsman *et al.*, 1984; Licker *et al.*, 1998). Custers also determined that several strains of *Brettanomyces* were capable of producing considerable quantities of acetic acid under conditions of aerobiosis and found that anaerobic conditions inhibited glucose fermentation (Licker *et al.* 1998).

Recently, studies have shown that the availability of oxygen presents a favourable scenario for the development of *Brettanomyces*/*Dekkera* yeasts during winemaking as it supports their growth and survival and also acetic acid production (Ciani *et al.*, 1997; Freer *et al.*, 2003; Aguilar-Uscanga *et al.*, 2003). In contrast, anaerobosis during alcoholic fermentation may well impede *Brettanomyces*/*Dekkera* growth, but would not necessarily prevent their development (Ciani *et al.*, 1997). Therefore, the risk involved with VA formation by *Brettanomyces*/*Dekkera* spp. can be reduced by minimising the wine's exposure to oxygen (during racking and transfers) in combination with effective SO_2 usage (Du Toit *et al.* 2005). Furthermore, aerobic conditions during winemaking, in particular barrel ageing and storage of red wine, should best be avoided because other microorganisms, such as acetic acid bacteria (AAB), are more likely to produce large quantities of acetic acid (Du Toit, 2000).

Other important volatile fatty acids produced by these yeasts that can have an impact on wine quality include: isovaleric acid (3-methylbutanoic acid), 2-methylbutyric and isobutyric acid (Olsen, 1994; Fugelsang, 1997; Licker *et al.*, 1998). However, the focus of this review will fall on isovaleric acid as it can have a major sensory impact on wine aroma. Isovaleric acid has previously been found to be the dominant odorant in wines that were classified as containing a high degree of '*Brettanomyces* character' (Licker *et al.*, 1998). The aroma character of isovaleric acid has been described as 'rancid' following Gas Chromatography-Olfactometry (GC-O) analysis, although sensory panels often refer to 'sweaty' and 'cheesy' aromas when describing this compound (Coulter *et al.*, 2004). Furthermore, although high concentrations of isovaleric acid do not correlate with high levels of ethylphenols, it is believed that its presence may enhance the overall perception or intensity of other *Brettanomyces*-derived characters (Coulter *et al.*, 2004). The exact cause and the conditions under which isovaleric acid is produced in wine are yet to be determined, but it is known that the amino acid degradation of L-leucine, L-isoleucine and L-valine are involved in the formation of isovaleric acid, 2-methylbutyric and isobutyric acid, respectively.

The metabolic pathway of each of these volatile fatty acids can be seen in Figure 2.2.
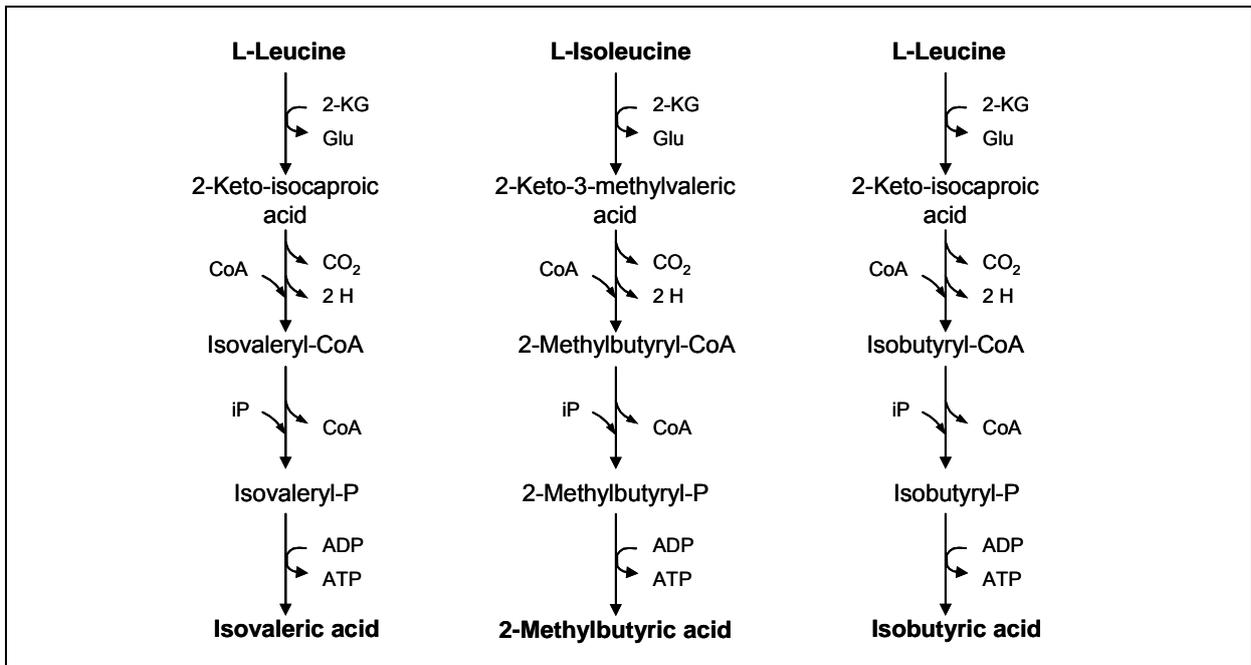
FIGURE 2.2.
Formation of isovaleric acid (3-methylbutanoic), 2-methylbutyric and isobutyric acid (Harwood and Canale-Parola, 1981).

2.3.2.2 Mousiness

Another microbiologically produced off-flavour that can render the aroma and taste of wines unacceptable is 'mousiness'. Mousy off-flavour in wine was first reported by Heresztyn (1986b) who isolated and characterised the compounds responsible for this unpleasant aroma from wines contaminated with species of *Lactobacillus* and *Brettanomyces*. In light of the recent in-depth review on mousy off-flavour by Snowdon (2006) only the main aspects entailing *Brettanomyces*/*Dekkera* spp. will be mentioned.

Three chemical compounds have been identified as being responsible for mousy off-flavour in wine: 2-acetyltetrahydropyridine (ATHP), 2-ethyltetrahydropyridine (ETHP) and 2-acetylpyrroline (APY) (Heresztyn, 1986b; Grbin *et al.*, 1995). Of these, *Brettanomyces*/*Dekkera* yeasts are capable of producing only the first two (Fig. 2.3).
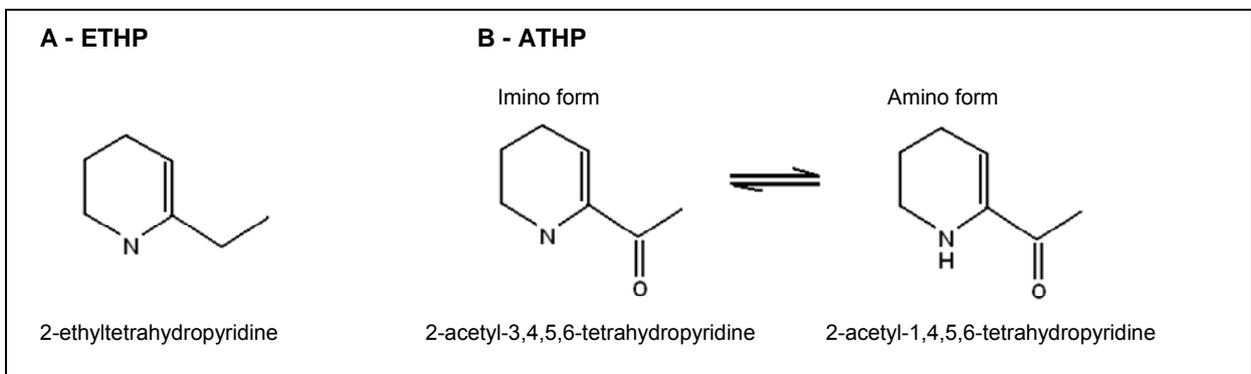
FIGURE 2.3.
Chemical compounds produced by *Brettanomyces*/*Dekkera* yeasts responsible for mousy off-flavour (Snowdon *et al.*, 2006).

ATHP appears to be the more prevalent in wine because this compound is normally detected in concentrations (4.8-106 μg/L) (Grbin _et al._, 1995) above its threshold value (1.6 μg/L) (Teranashi _et al._, 1975). ETHP has a much higher threshold value (150 μg/L) (Craig and Heresztyn, 1984) but has only recently been detected in wines in much lower concentrations (Grbin _et al._, 1995).

The amino acid L-Lysine is essential for the formation in these two chemical compounds (Heresztyn, 1986b; Grbin _et al._, 1995; Grbin and Henschke, 2000) and ethanol is a necessary precursor for the flavour to occur in wine (Snowdon _et al._, 2006). Additionally, oxygen has been indicated to have a stimulatory effect on the production of ATHP and ETHP (Grbin, 1998), but this is probably due to higher biomass formation of _Brettanomyces/Dekkera_ strains under aerobic conditions. The aroma characters associated with ATHP are reminiscent of 'cracker biscuit' or 'popcorn', however due to the pH of wine, it is more apparent as a bitter, metallic palatable aftertaste. Mousy off-flavours occur infrequently in wine for reasons not fully understood. Chatonnet said: "These compounds are not of major significance when performing sensory screenings of '_Brettanomyces_ character' in wine".

2.3.2.3 Loss of colour

Wines contaminated with _Brettanomyces/Dekkera_ strains generally have an undesirable colour. A few reports are available that show glycosidic activity (β-glucosidase) amongst _Brettanomyces/Dekkera_ strains (Fugelsang _et al._, 1993; McMahon _et al._, 1999; Mansfield _et al._, 2002; Potgieter, 2004; Fia _et al._, 2005). In these cases, these yeasts may produce wines with enhanced aroma and complexity. On the other hand, a large part of the total glycoside concentration of grapes comprises mono-glucosylated anthocyanins, which are considered as the primary red pigments in _Vitis vinifera_ (Somers _et al._, 1988). The hydrolysis of glucose usually results in the formation of a corresponding anthocyanin that can be converted to a colourless pseudobase, consequently affecting the colour negatively (Mansfield _et al._, 2002). This may be a reason why some wines contaminated with _Brettanomyces/Dekkera_ spp. have an undesirable colour (Suárez _et al._, 2007).

Another possible theory for loss of colour caused by _Brettanomyces/Dekkera_ spp. is presented here. Wine colour is largely determined by various grape-derived anthocyanin pigments. Three types of pyranoanthocyanin pigments, namely vitisins, pyranoanthocyanin-flavanols and vinylphenolic adducts, have recently been described to increase and stabilise the colour of the wine, particularly during ageing (Morata _et al._, 2007). The vinylphenolic pyranoanthocyanins result from the condensation of vinylphenols with grape anthocyanins, mainly malvidin-3-O-glucoside. Among the group of vinylderivatives, vinylcatechol, 4-vinylguaiacol and vinylphenol have been associated with the formation of vinylphenol malvidin adducts (Fulcrand _et al._, 1996; Francia-Aricha _et al._, 1997; Schwarz _et al._, 2003). The role of yeasts was explained by Morata _et al._ (2007) in that the fermentation by _S. cerevisiae_ strains containing hydroxycinnamate decarboxylase activity is favourable for the

formation of vinylphenolic adducts. In Figure 2.4, it can be seen how vinylphenols, formed from the decarboxylation of hydroxycinnamic acids, combine with grape anthocyanins (malvidin-3-O-glucoside) to generate vinylphenolic pyranoanthocyanins.

Along with the wine yeast *S. cerevisiae* and numerous other wine microorganisms, *Brettanomyces/Dekkera* yeasts also contain hydroxycinnamate decarboxylase activity (Heresztyn, 1986a; Chatonnet *et al.*, 1992; Edlin *et al.*, 1995) and are capable of producing vinylphenols (see section 2.4.1) (Clausen *et al.*, 1994; Chatonnet *et al.*, 1997; Couto *et al.*, 2006). However, *Brettanomyces/Dekkera* yeasts are more likely to reduce the available vinylphenols to ethyl derivatives, consequently influencing the formation of vinylphenolic pyranoanthocyanins. Although this is highly speculative at this stage, it might explain the loss of colour as seen in severe cases of *Brettanomyces/Dekkera* contamination.
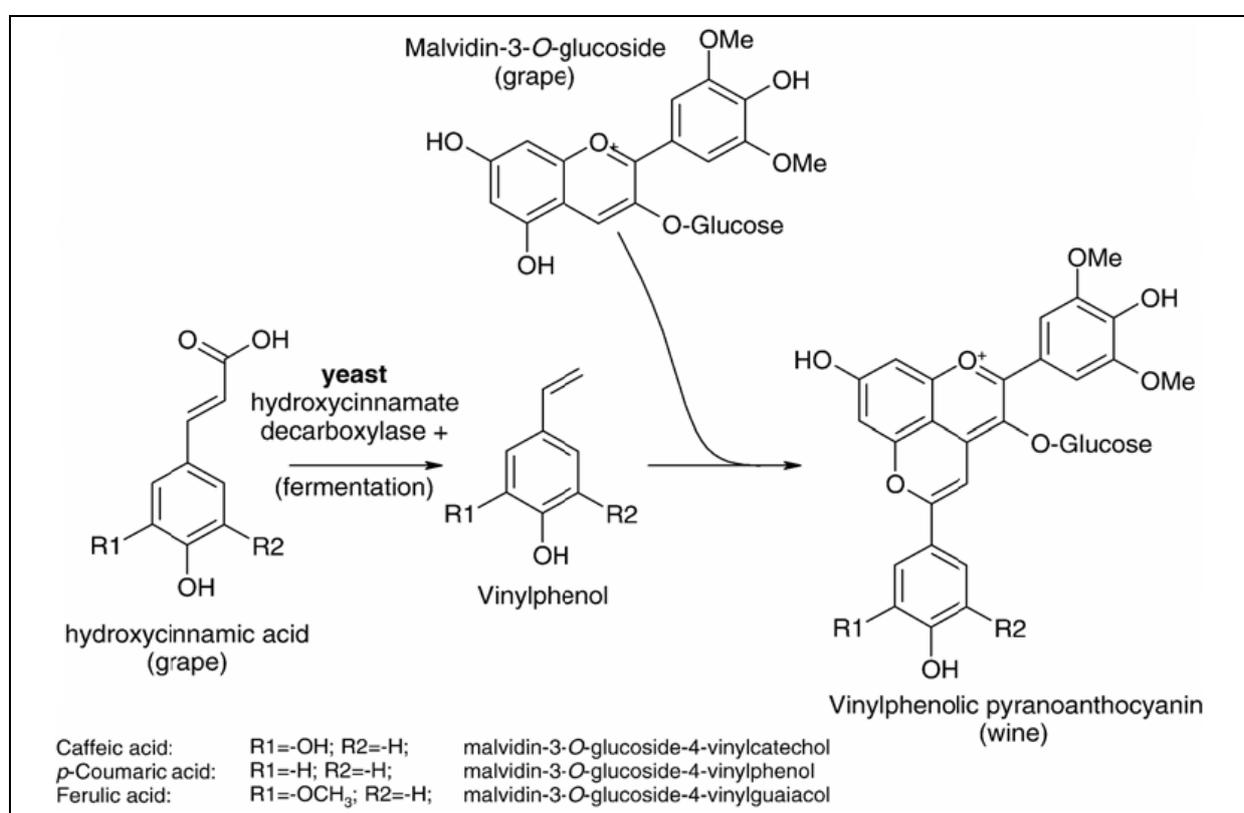
FIGURE 2.4.
Formation of vinylphenolic pyranoanthocyanins during fermentation with yeasts showing hydroxycinnamate decarboxylase activity (Morata *et al.*, 2007).

2.3.2.4 Biogenic amines

The metabolic activities of microorganisms may also give rise to the formation of biogenic amines (BA). These amines are produced via the decarboxylation of amino acids; e.g. histidine leads to the formation of histamine (Caruso *et al.*, 2002). Most BA research has been undertaken into lactic acid bacteria, especially in fermented foods (Stratton *et al.* 1991; Simon-Sarkadi and Holzapfel, 1994) but also in wine (Lonvaud-Funel, 2001). However, very few studies report on BA production by yeasts during winemaking. Caruso *et al.* (2002) evaluated the production of BA by various wine-

related yeasts, including *B. bruxellensis*. They found that *B. bruxellensis* produced the highest concentration of total BA (average of 15 mg/L) in comparison with other yeasts, despite exhibiting the weakest fermentative ability. The biogenic amines that were produced included: ethanolamine, methylamine, tryptamine, putrescine, cadaverine, histamine, agmatine and 2-phenylethylamine. Except for the last two amines, the majority of these were barely detectable. Also, considerable strain variability was seen with agmatine produced by *B. bruxellensis* (Caruso *et al.*, 2002)*.* In another study the formation of 2-phenylethylamine accounted for approximately 50% of the total BA produced by *B. bruxellensis* in a grape must fermentation (Granchi *et al.*, 2005). Similar to the findings of Caruso *et al.* (2002), *B. bruxellensis* was the yeast species that produced the highest concentration of BA with an average value of 20 mg/L.

The interest of BA in wine has been triggered as these compounds have been linked with unwanted physiological effects in people who show 'amine intolerance' (Gafner, 2003). The resulting symptoms include: headaches, nausea, diarrhoea and red skin colouration, to name a few. The ability of the human body to degrade BA is drastically affected by alcohol consumption as ethanol inhibits the enzyme diamine oxidase activity (DAO) responsible for converting BA to harmless products (Gafner, 2003). The toxicological importance of BA in wine still needs to be established along with the individual toxic thresholds. Nevertheless, the ability of *Brettanomyces*/*Dekkera* spp. to produce biogenic amines contributes to their general aptitude for spoiling wine.

2.4 INVESTIGATIONS ON *BRETTANOMYCES* MANAGEMENT

Due to the importance of *Brettanomyces*/*Dekkera* yeasts and their association with wine spoilage, it became evident that strategies for control, monitoring and risk management were needed. The incidences of *Brettanomyces*/*Dekkera* spoilage in wine have become more prominent during the past decade. Yap *et al.* (2007) attributed this largely to:

   (i)   recent trends in some winemaking styles – such as wines with higher pH values and residual sugar;

   (ii)   trends in winemaking practices - decreased use of filtration and SO_2;

   (iii) general poor cellar hygiene along with improper cleaning and sanitisation of barrels - a critical source of *Brettanomyces/Dekkera* contamination of wine;

   (iv) the spread of *Brettanomyces/Dekkera* between wineries and regions due to the use of contaminated barrels which are traded in the second hand barrel market; and

   (v)   importation of *Brettanomyces/Dekkera*-contaminated wine from other affected wineries.

The past decade has yielded reports that present valuable recommendations and strategies to the wine industry regarding the control and monitoring of

Brettanomyces/*Dekkera* spp. This section will focus on and summarise the specific research investigations cited in peer-reviewed journals that focused on these aspects as well as highlight other more general considerations.

2.4.1 PREVENTATIVE MEASURES TO CONSIDER

As *Brettanomyces*/*Dekkera* yeasts form a natural part of the winemaking process, they cannot entirely be eliminated. The spoilage caused by this species is inevitable in conditions favouring their proliferation. However, there are a few preventative measures that can be considered, although the issues relating to the complexity of microbiological spoilage in wine are not simply resolved with individual factors, but rather require a holistic approach.

Although there are still conflicts in literature about the origin of *Brettanomyces*/*Dekkera* spp*.,* the vineyard can definitely play a role and grapes of sound quality can decrease some of the risks leading to the production of phenolic off-flavours (Loureiro and Malfeito-Ferreira, 2006; Taillandier, 2007). In addition, the concentrations of the hydroxycinnamic acid precursors directly responsible for *Brettanomyces*/*Dekkera* phenolic off-flavours can depend on grape variety, quality (Phister and Mills, 2003) and maceration practices. Gerbaux *et al.* (2002) studied the effect of maceration temperature (heating) and intensity on the release of hydroxycinnamic acids from grape skins as factors influencing the formation of volatile phenols by *Brettanomyces*/*Dekkera* spp. These factors, along with enzymes used for colour extraction and clarification, such as pectolytic and other enzymes containing cinnamoyl esterase activity, can lead to increased levels of volatile phenol production by *Brettanomyces*/*Dekkera* spp. (Dugelay *et al.*, 1993; Gerbaux *et al.*, 2002).

Yeast selection can also play a crucial role. There are several criteria to consider when it comes to selection of commercial *S. cerevisiae* starter cultures (Degré, 1993). These are especially important for the prevention of stuck or sluggish fermentations (Malherbe *et al.*, 2007), which create an environment for any form of microbial spoilage. The specific aspect of wine yeasts relating to *Brettanomyces*/*Dekkera* produced off-flavours evolves around their ability to produce vinylphenols via the decarboxylation of cinnamates (Clausen *et al.*, 1994). *S. cerevisiae* strains differ in their ability to produce vinylphenols (Van Wyk and Rogers, 2000; Nelson, 2007) and higher levels of vinyl derivatives can consequently lead to objectionable concentrations of ethylphenols by *D. bruxellensis*. Conversely, vinylphenols are involved in the formation of pyranoanthocyanins and at higher levels these can also be beneficial to the stability and intensity of colour in wine (Morata *et al.*, 2007). It remains to be ascertained which aspect of hydroxycinnamic acid decarboxylation by wine yeast is more important.

2.4.2 FACTORS FOR CONTROLLING *BRETTANOMYCES/DEKKERA*

2.4.2.1 Sulfur dioxide (SO$_2$)

The antimicrobial potential of SO$_2$ makes it ideal for wine preservation, in particular for reducing microbiological instabilities (Romano and Suzzi, 1993). Regarding, SO$_2$ and its effect on the yeast *D. bruxellensis,* studies have yielded incoherent results as this species is regarded as either sensitive or resistant (Loureiro and Malfeito-Ferreira, 2006). Some authors found this yeast to be sensitive to free SO$_2$ concentrations exceeding 30 mg/L (Chatonnet *et al.*, 1992; Gerbaux *et al.*, 2002), explaining why it is frequently isolated from wines with low SO$_2$ protection (Heresztyn, 1986a). Others observed yeast growth with concentrations of free SO$_2$ of above 30 mg/L, reflecting the resistance of certain *D. bruxellensis* strains (Van der Walt and Van Kerken, 1961; Froudière and Larue, 1988). This controversy, however, does not lie in the free form of SO$_2$, but rather in the actual effectiveness of its molecular form (Boulton *et al.*, 1996; Margalit, 1997; Ribéreau-Gayon, 2000), which is dependent on many variations in wine composition (pH, ethanol, temperature, anthocyanin levels and nutrient content) (Smith, 1996 in Licker *et al*. 1998). For example, the concentration of molecular SO$_2$ is pH-dependent and 30 mg/L of free SO$_2$ can release 0.4 mg/L of molecular SO$_2$ at pH 3.7, and 0.8 mg/L at pH 3.4 (Margalit, 1997). The use of 0.5 to 0.8 mg/L molecular SO$_2$ has been recommended to control *Brettanomyces/Dekkera* spp. (Henick-Kling *et al.*, 2000). The effectiveness of molecular SO$_2$ on a strain of *B. bruxellensis* has also been linked with oxygen availability (Du Toit *et al.*, 2005). The authors reported that 0.25 mg/L of molecular SO$_2$ drastically affected the cultureability of the strain, however, the strain remained viable and increased in numbers after exposure to oxygen. This is especially of importance during racking and transfers throughout barrel ageing. Barrels can also reduce SO$_2$ levels over a period of four to six months of ageing (Chatonnet *et al.*, 1993) and therefore SO$_2$ management is crucial during this time. This agrees with anecdotal evidence that new barrels can absorb up to 15 mg/L of free SO$_2$ over the same time period (Coulter *et al.*, 2004).

A survey done by the Australian Wine Research Institute (AWRI) revealed a tendency among Australian winemakers to use many smaller SO$_2$ additions, as opposed to larger, less frequent additions during winemaking. This is not advised as it can lead to the unintentional selection or build-up of more resistant yeast species, including *Brettanomyces/Dekkera* spp. (Coulter *et al.* 2004).

2.4.2.2 Other additives

In addition to SO$_2$ usage, alternative additives have also been investigated as growth inhibitors for *Brettanomyces/Dekkera* yeasts (Loureiro and Malfeito-Ferreira, 2006; Suárez *et al.*, 2007). Dimethyl dicarbonate (DMDC), also commercially known as Velcorin[®], has been evaluated for *Brettanomyces/Dekkera* yeast inhibition. It was found that DMDC could not completely inhibit the growth of *B. anomalus* with a

dosage of 400 mg/L, but 250 mg/L inhibited the fermentation of *B. bruxellensis* (Delfini *et al.*, 2002). A very recent study described the effectiveness of DMDC for the prevention of *B. bruxellensis* in wine and evaluated its use during different winemaking stages (Renouf *et al.*, 2007). The authors obtained variable results with strains of *B. bruxellensis* showing moderate resistance with 150 mg/L DMDC in grape must and 250 mg/L causing only a transitory inhibition during MLF. The effectiveness of DMDC was, however, dependent on ethanol content (Malfeito-Ferreira *et al.*, 2004). It was also found that DMDC did not completely eliminate *B. bruxellensis* populations in the presence of lees at 200 mg/L (Renouf *et al.*, 2007). On the other hand, in finished wines the *B. bruxellensis* population declined to less than 100 cfu/mL. Renouf *et al.* (2007) advised against the use of DMDC before the end of MLF as it can act on fermenting species such as *S. cerevisiae* and *Oenococcus oeni* and instead recommended the use of DMDC prior to bottling. Regular additions up to 200 mg/L (maximum permitted level in wine) can help to control *Brettanomyces* growth during barrel maturation (Loureiro and Malfeito-Ferreira, 2006) in countries where its use is allowed.

The effect of DMDC is not directly pH dependent (Threlfall and Morris, 2002) and it yields no residual odours or flavours (Ough, 1983). The use of DMDC requires an approved dosing machine and must be carefully handled.

Weak acids, such as sorbic, benzoic and fumaric acids have also been investigated for use against *Brettanomyces*/*Dekkera* spp. as they contain antifungal activity. *D. bruxellensis* are regarded as one of the most tolerant species to sorbic acid and dissolved carbon dioxide (Ison and Gutteridge, 1987; Loureiro, 1997). Although these weak acids are included in selective media (Chatonnet *et al.*, 1992, Rodriquez *et al.*, 2001), they are not favourable during winemaking and face consumer resistance (Pretorius, 2000; Suárez *et al.*, 2007). Weak-acid preservatives have been shown to be more effective at low pH values, and that inhibition depended on the actual intracellular concentration of the individual preservatives (Quintas *et al.*, 2005). An indirect measure to prevent ethylphenol formation during ageing involves the use of antioxidants such as, ascorbic and erythorbic acids (Suárez *et al.*, 2007). Antioxidants can be used to reduce the presence of oxygen, thereby being indirectly detrimental to the proliferation of *Brettanomyces*/*Dekkera* yeasts.

2.4.2.3 Fining agents

Protein or microbial instabilities are well known for causing turbidity or haziness in wines. This has also been addressed as an issue relating to wine spoilage by *Brettanomyces*/*Dekkera* after wineries, which apparently practiced effective SO_2 and pH management, barely showed a reduction of 4-ethylphenol concentrations (Coulter *et al.* 2004). In such instances, the effectiveness of SO_2 was compromised as it is rapidly bound. Suárez *et al.* (2007) summarised numerous investigations of fining agents and their impact on *Brettanomyces*/*Dekkera* yeasts during winemaking. *Brettanomyces* populations can be reduced by 40 to 2000-fold by treatments with

fining proteins (Murat and Dumeau, 2003). *Brettanomyces*/*Dekkera* yeasts in a red wine have also been decreased from an initial population of 10^4 cfu/mL to 170 cfu/mL after fining with liquid gelatine at a dosage of 0.6 ml/L (Suárez *et al.*, 2007).

2.4.2.4 Filtration

Studies that pertain to wine filtration for the removal of *Brettanomyces*/*Dekkera* cells have also been performed (Calderón *et al.*, 2004). The authors reported effective removal of *Brettanomyces* cells using membranes with a pore size smaller than 0.45 µm. Millet and Lonvaud-Funel (2000) studied the VBNC (viable but non-culturable) state of wine microorganisms during storage. It was found that non-culturable cells could pass through the 0.45 µm filtration and it is believed that *Brettanomyces* cells can possibly reduce their cellular size when entering the VBNC state. This is a very interesting phenomenon considering that the average size of *Brettanomyces* spp. is (5-8) x (3-4) µm (Millet and Lonvaud-Funel (2000). This might explain why some wines are still subjected to *Brettanomyces*/*Dekkera* spoilage after they have been declared sterile by agar plate enumerations. The VBNC state of microorganisms, particularly of *Brettanomyces*/*Dekkera* spp., is an area that needs to be further explored. Moreover, the importance of molecular DNA-based identification techniques is amplified by the shortcomings of microbiological plating techniques. The use of smaller pore sizes during filtration (e.g. cross-flow with 0.22 µm) is recommended for wines destined to be used for topping-up barrels during ageing (Oelofse and Du Toit, 2006). However, filtration poses similar problems to fining as it can be detrimental to the colloidal structure of wine and can lead to a loss of colour (Suárez *et al.*, 2007). It should therefore be carefully considered.

2.4.2.5 Alternative methods

The use of a polysaccharide derived from chitin, called chitosan, has been reported to exert a selective pressure on the growth of *B. bruxellensis* in a mixed bioethanol fermentation with *S. cerevisiae* (Gómez-Rivas *et al.*, 2004). The presence of 3-6 g/L of chitosan drastically decreased the growth of *B. bruxellensis* and *B. intermedius* in this study.

The application of high pressure on wine has also been investigated. Total microbial populations comprising AAB, LAB and yeasts (including *Brettanomyces* spp.) could be reduced by 99% by pressures of 400 or 500 MPa for 5 or 15 min at 4 or 20°C, respectively (Puig *et al.*, 2003). Wine pasteurisation using high hydrostatic pressures have been shown not to cause major modifications to the physiochemical and sensorial properties of wine (Mok *et al.*, 2006). However, the application of this treatment in oenology still requires a lot of study for the development of appropriate equipment.

The use of temperature for the inactivation of *Brettanomyces*/*Dekkera* yeasts in wine has also been investigated. Couto *et al.* (2005b) found that a population of 10^6 cfu/mL could be thermally inactivated with 37.5°C for 6 min and 41°C for 0.6 min

treatment. The concern about this approach is its impact on the aroma and flavour characteristics of wine, if the treatment is not carefully controlled.

An alternative strategy to chemical preservation involves the use of antimicrobial agents as part of biopreservation (Pretorius, 2000). Biological control with various antimicrobial agents, such as zymocins is currently being considered, but their efficiency in wine is yet to be determined (Du Toit and Pretorius, 2000). Recently, a potential application of antimicrobial agents active on *Brettanomyces/Dekkera* yeasts during wine ageing and storage has also been hypothesised. Comitini *et al.* (2004) described the use of two killer toxins produced by *Pichia anomala* (DBVPG 3003) and *Kluyveromyces wickerhamii* (DBVPG 6077) that have fungicidal activity against *D. bruxellensis*. The two toxins named Pikt and Kwkt are stable in wine for at least 10 days and show potential use for the future. However, the purification and use of antimicrobial agents might be expensive.

2.4.3 CURATIVE MEASURES FOR VOLATILE PHENOLS

In addition to the methodologies that have been investigated for controlling the microbiological aspects of *Brettanomyces/Dekkera* spp. contamination, there are also some control strategies pertaining to the specific chemical aromas or phenolic off-flavours produced. The volatile phenols, specifically the ethylphenols, which result from a *Brettanomyces/Dekkera* contamination, can also be reduced.

By using reverse osmosis and adsorption, Ugarte *et al.* (2005) obtained a 77% reduction in the total ethylphenols (4-EG and 4-EP). The three hour process comprised a hydrophobic absorbent resin and a membrane with tangential-flow filtration. However, a reduction in some aromatic compounds, namely ethyl- and methyl vanillate and other esters, was also obtained.

Other absorbents commonly used during winemaking have also been reported to reduce off-flavours and odours. Polyvinylpolypyrrolidone (PVPP) and charcoal are used by some winemakers to lower ethylphenol levels (Suárez *et al.*, 2007). The prescribed amounts vary and range from 60-480 mg/L for PVPP and 15-240 mg/L for charcoal, depending on the intensity of the off-flavours. Fining agents such as casein and potassium caseinate also present an absorptive function and have been used to reduce low levels of ethylphenols (Ruiz-Hernández, 2003). The adsorption of volatile phenols by using active dried wine yeast and yeast lees as a biosorbent was also investigated (Chassagne *et al.*, 2005). This came after decreases in the contents of 4-EP and 4-EG were found in red wine containing yeast lees compared to the same wine aged without lees (Guilloux-Benatier *et al.*, 2001). The authors found that active dried yeast of *S. cerevisiae* removed 33% and 26% of the 4-EP and 4-EG concentrations, respectively, in a model wine solution (Chassagne *et al.*, 2005). It was speculated that the rapid adsorption process occurs predominantly by yeast surface binding. The affinity of yeast lees for volatile phenol adsorption was sensitive to the level of yeast autolysis and physicochemical parameters, such as ethanol content, temperature and pH.

2.4.4 CONSEQUENCES OF OAK BARRELS AND CONTROL

Another aspect of winemaking that is increasingly gaining substantial attention, involves the role of oak barrels and their effect on microbial wine spoilage. Wooden barrels are particularly known as an ecological niche where microbial spoilage can occur, especially by yeasts such as *D. bruxellensis* (Swaffield and Scott, 1995; Laureano *et al.*, 2005). Due to the difficulty of sanitising barrels several concerns have also been raised regarding the control of *Brettanomyces*/*Dekkera* spp. during barrel ageing and storage of wine.

Brettanomyces/*Dekkera* yeasts are known to survive in barrels in areas where they are protected against treatments such as SO_2. These include the yeast lees, around bung holes and in the oak structure (Laureano *et al.*, 2005). The penetrative capacity of the wine serves as a vector for carrying these yeasts deep into the cracks and crevices of staves (up to 8 mm) (Fugelsang, 1997; Laureano *et al.*, 2005). Here, the cells have a large degree of protection against SO_2 gas allowing them to establish themselves (Fugelsang, 1997; Swaffield *et al.*, 1997). The survival of established microbial populations becomes a greater sanitary issue with used barrels (Chatonnet *et al.*, 1999) as the pores become impregnated or blocked by microbial cells, colour pigments and other colloidal materials. This is further complicated by the presence of fungal growth and the formation of microbial biofilms (Yap *et al.*, 2007). The influence of biofilms on wood structure and wine spoilage is not well characterised. However, biofilms are reportedly up to 1000 times more resistant to chemical cleaning agents and sanitisers (Kumar and Anand, 1998; Lewis, 2001). In this regard, Joseph and Bisson (2004) found that 50% from a total of 35 *Brettanomyces/Dekkera* isolates could form biofilms which adds another dimension to their control.

Barrel cleaning and sanitation is very difficult and there are many inconsistencies in literature as to which methodologies are most effective. Chatonnet *et al.* (1992) advised that the sanitation of barrel wood requires at least 7 g of SO_2 gas per barrel. Henick-Kling *et al.* (2000) recommends that filled barrels should receive approximately 30-35 mg/L free SO_2 during summer months. A recent study by Laureano *et al.* (2005) evaluated different treatments on used French barrels (third fill) that contained red wine with high ethylphenol levels and culturable *D. bruxellensis* cells. These included: (i) cold water rinse followed by three hot water rinses at 70°C and air-drying; (ii) same as above plus filling with an aqueous solution of SO_2 (200 mg/L, pH 3) and storing for one month; (iii) cold water rinse, followed by filling the barrel three-quarters full with hot water at 90°C for 10 min; and (iv) cold water rinse, followed by a 70°C hot water rinse and steaming under low pressure (0.5 kg/cm) for 10 min. It was found that the treatment with steam was the most effective, although none of the approaches was able to significantly reduce the microbial populations as *D. bruxellensis* cells were still recovered from the external surfaces of grooves, side surfaces of staves (2-4 mm) and at the bunghole (4-6 mm).

Studies concerning the use of ozone have also been reported. Cantacuzene *et al.* (2003) evaluated the effect of aqueous ozone and ozone gas on *B. bruxellensis*

contaminated oak cubes. The authors found a reduction of the *Brettanomyces* population with the ozone gas and with a hot water treatment (82°C for 20 min), but not with the aqueous ozone. In contrast, *Brettanomyces* populations were reduced by up to 99% with ozonated water in another investigation (Coggan, 2003). Despite many anecdotal reports, ozone sanitation has been used with good results and is strongly recommended for stainless steel tanks.

Barrel shaving and re-firing have also been investigated by Pollnitz (2000) who found that the wine contained up to 85% less 4-EP and 4-EG after being stored in shaved and re-fired barrels in comparison to control barrels (untreated barrels). This was attributed to a reduced microbial population on the inner surface of barrels, which confirmed the prevalence of viable *Brettanomyces/Dekkera* cells in the wood structure (Pollnitz, 2000).

One of the latest additions to barrel cleaning and disinfection techniques use high-power ultrasonics (Yap *et al.*, 2007). Laboratory tests have proven that ultrasound or sonification could effectively kill viable cells of *D. bruxellensis* in synthetic media. More than 97% of the population of 4.4 x 10^6 cfu/mL were destroyed with ultrasound power at 50 watts for 90 to 120 s. Further trials directly in wine are anticipated.

Regarding barrel cleaning, there currently appears to be no substantial scientific evidence on which approach will guarantee complete sterilisation. Barrels are often, if not in most cases, impossible to sterilise and the effectiveness of all the above-mentioned procedures is highly doubted (Boulton *et al.*, 1996; Pollnitz *et al.*, 2000; Arvik and Henick-Kling, 2002; Malfeito-Ferreira *et al.*, 2004). In addition to the uncertainties, there are currently no reliable techniques that allow for the direct detection for *Brettanomyces/Dekkera* spp. in wood. This should be considered for future investigations in order to reveal the true value of the various barrel sanitation techniques.

The possibility that new oak barrels can be beneficial for the growth and survival of the *Brettanomyces/Dekkera* population that is carried by the wine has also been hypothesised (Lonvaud-Funel and Renouf, 2005). This speculation has value because new barrels provide greater sugar resources (higher cellubiose levels) and oxygen contributions than older barrels (Swaffield and Scott, 1995; Boulton *et al.*, 1996; Loureiro and Malfeito-Ferreira, 2006; Yap *et al.*, 2007). However, new oak is not a source of contamination, since wood is not the natural habitat for the yeast. Moreover, it is expected that the toasting process eliminates new barrels as a source of *Brettanomyces/Dekkera* contamination. Problems related to new barrels are most likely to be caused by poor barrel management at wineries, e.g. pre-rinsing with non-sterile water.

Several recommendations for the control, monitoring and curative procedures of *Brettanomyces/Dekkera* spoilage in wine have been formulated over the years (Godden *et al.*, 2004). Combined results from scientific investigations and empirical findings currently indicate that the success relating to the control of

Brettanomyces/Dekkera-associated spoilage in wine evolves around a holistic approach. More detailed recommendations for the control of *Brettanomyces/Dekkera* yeasts during winemaking can also be found in Coulter *et al.* (2004), Loureiro and Malfeito-Ferreira (2006), Oelofse and Du Toit (2006) and Suárez *et al.* (2007). A summary of all the scientific investigations on the control of *Brettanomyces/Dekkera* yeasts discussed in this overview can be seen in Table 2.2.

TABLE 2.2.
Summary of investigations on *Brettanomyces/Dekkera* control.

Treatment	Reference
Protein clarification Gelatine Egg white Potassium caseinate Caseins	Murat and Dumeau, 2003 Ruiz-Hernández, 2003
Filtration Membranes (0.45 µm) Ultrafiltration	Millet and Lonvaud-Funel, 2000 Calderón *et al*., 2004
Physicochemical variables Low aging temperature Low pH Reduction of oxygen content Avoidance of micro-oxygenation High alcohol levels	Gerbeaux *et al*., 2000
Reduction of precursor concentration Low maceration temperature Avoidance of pectolytic enzymes and enzymes with cinnamoyl esterase activity	Gerbeaux *et al*., 2002
Additives SO2 DMDC Chitosan Sorbic acid Benzoic acid Fumaric acid Ascorbic acid Erythorbic acid	Ison and Gutteridge, 1987 Delfini *et al*., 2002 Renouf *et al*., 2007 Gómez-Rivas *et al*., 2004
High pressure processing 400-500 MPa	Puig *et al*., 2003
Biological techniques Zymocins/killertoxins Bacteriological enzymes	Du Toit and Pretorius, 2000 Comitini *et al*., 2004
Genetic egineering Transgenic yeasts	Du Toit and Pretorius, 2000
Other alternatives Ozone Reverse osmosis Absorbents PVPP Charcoal Ultrasonics	Guilloux-Benatier, 2001 Coggan, 2003 Cantacuzene *et al*., 2003 Chassagne et al., 2005 Yap *et al*., 2007

2.5 CONCLUSIONS

The microbiology of wine is very complex and this often makes it difficult to pinpoint the exact problem and its origin in a habitat such as wine when spoilage does occur. Nonetheless, large scale investigations on controlling wine spoilage have been conducted for many years in an attempt to improve wine quality and great progress has been made in the past decade.

With regards to *Brettanomyces/Dekkera* yeasts, only small steps have been taken and there are still many factors to be researched. These yeast species are only a few of the many organisms that exist in the winemaking environment and, because they are living entities, there will be a lot of diversity among their species. Despite their economic importance and the increasing amount of interest by the wine industry, *Brettanomyces/Dekkera* spp. have been largely understudied at genetic level. It is for this reason that a genome sequence project on this wine spoilage yeast has recently been initiated (Woolfit *et al.*, 2007). Preliminary results indicate that the proteome of *D. bruxellensis* is rich in transporters and genes involved in lipid and nitrogen metabolism. This may well elucidate their ability to survive in an environment with high ethanol and nutrient limitation. Future studies on the genetic characterisation of this species will resolve their true significance during winemaking.

2.6 LITERATURE CITED

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Research Results

Detection, isolation and identification of *Brettanomyces* yeasts from red wine: a South African case study

3. RESEARCH RESULTS

Detection, isolation and identification of *Brettanomyces* yeasts from red wine: a South African case study

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ABSTRACT

Yeasts of the genus *Brettanomyces,* or its teleomorph *Dekkera,* can persist throughout the harsh winemaking process and have the potential to cause wine spoilage by producing various products, including volatile phenols such as 4-ethylphenol and 4-ethylguaiacol. These compounds often result in phenolic off-flavours described by terms such as 'medicinal', 'barnyard-like', 'leathery', 'wet dog' and 'Band-aid'. It is these phenolic off-flavours that are detrimental to the sensorial quality of wine. The main objective of this study was to isolate *Brettanomyces* yeasts from different stages of the winemaking process from various South African red wines. Sampling stages included the vineyard, grape juice, alcoholic fermentation, malolactic fermentation, barrels and finished wines. During this study, conventional microbiological methods such as plating on selective agar media (DBDM and WLN) and microscopy were investigated in combination with molecular identification techniques such as the polymerase chain reaction (PCR) method. Colonies obtained on the selective media were analysed for morphological traits that could aid in the detection and identification process of wine species of *Brettanomyces*. Five different PCR primer sets specific for *Brettanomyces* spp. identification were tested. From a total of 307 samples obtained, 41 isolates positively identified as *Brettanomyces bruxellensis* were found.

Keywords: *Brettanomyces, Dekkera, red wine, spoilage, detection, isolation, identification, selective media, PCR.*

3.1 INTRODUCTION

The microbiology of wine is complex and, of the range of microorganisms found during winemaking, many are implicated in wine spoilage. Yeast of the genus *Brettanomyces* has been identified as one of the most serious and controversial spoilage microbes of the winemaking process (Ibeas *et al.* 1996; Cocolin *et al.,* 2004; Chatonnet *et al.,* 1995). Unlike the other wild yeasts, *Brettanomyces* is highly adapted to proliferate in red wine due to their relative resistance to the normal concentrations of sulfur dioxide found in wine and better ethanol tolerance (Licker *et al.,* 1998).

Wines that have been spoiled by *Brettanomyces* are recognised by descriptive aroma expressions such as 'medicinal', 'elastoplast', 'Band-aid', 'smoky', 'farmyard', 'animal' and 'sweaty leather' and these off-flavours are characteristic of volatile phenols (Heresztyn 1986; Chatonnet *et al.,* 1992; Edlin *et al.,* 1995). The formation of volatile phenols involves a two-step conversion of hydroxycinnamic acid precursors (*p*-coumaric, ferulic and caffeic acid) firstly into vinylphenols (4-vinylphenol, 4-vinylguaiacol and 4-vinylcatechol) and then to ethylphenols (4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol) (Chatonnet *et al.,* 1992, 1995, 1997; Joseph and Bisson 2004; Loureiro and Malfeito-Ferreira 2003; Hesford *et al.,* 2004). The ethylphenols are mainly associated with *Brettanomyces* spoilage and the production of these compounds by *Brettanomyces* has been exploited in recent years in the development of selective media that enables the detection of this yeast species (Rodrigues *et al.,* 2001; Couto *et al.,* 2005).

In addition to the phenolic off-flavours, *Brettanomyces* produces other secondary metabolites that can be detrimental to the organoleptic quality of wines. These include the production of acetic acid (volatile acidity) and tetrahydropyridines ('mousy' off-flavours) (Fugelsang *et al.,* 1993; Aguilar-Uscanga *et al.,* 2003; Snowdon *et al.,* 2006). Severe cases of *Brettanomyces* spoilage that affected the appearance of wine have also been reported. These included turbidity, loss of colour due to anthocyanin degradation and film formation (Grbin and Hensckhe 2000; Mansfield *et al.,* 2002; Joseph and Bisson, 2004; Snowdon *et al.,* 2006; Curtin *et al.*, 2007; Hayashi *et al.*, 2007).

The risk of wine contamination by *Brettanomyces* spp. can cause wineries significant economic losses. For this reason numerous international efforts towards monitoring and control have been initiated. The classic microbiological methods used for identification of *Brettanomyces* spp. are not adequate because of their inability to detect viable populations along with the slow-growing nature and mixed morphological features of this species (Millet and Lonvaud-Funel, 2000). Therefore, molecular techniques have been developed in recent years to improve detection and identification of these species from the winemaking environment (White *et al.*, 1990; Ibeas *et al.,* 1996; Esteve-Zarzoso *et al.,* 1999; Egli and Henick-Kling, 2001 and Cocolin *et al.,* 2004). Molecular techniques, such as the polymerase chain reaction (PCR) method, have become a powerful tool for elucidating classic issues concerning

taxonomy. The taxonomical nomenclature of *Brettanomyces* has seen many reclassifications (Boekhout *et al.,* 1994; Cocolin *et al.,* 2004). Today, there are five species belonging to the genera *Brettanomyces* and *Dekkera*, namely *Brettanomyces custersianus, Brettanomyces naardenensis, Brettanomyces nanus, Brettanomyces anomalus* and *Brettanomyces bruxellensis*. The teleomorphs, *Dekkera anomala* and *Dekkera bruxellensis*, are known to exist for their *Brettanomyces* counterparts for the last two species which are also the only species that have been associated with wine (Barnett *et al.*, 2000; Egli and Henick-Kling, 2001; Stender *et al.,* 2001; Kurtzman and Fell, 2002; Cocolin *et al.,* 2004). Oenologists commonly refer to these yeast species as *Brettanomyces* or simply 'Brett'.

The principal aim of this study was to detect, isolate and identify yeasts of the genera *Brettanomyces* and *Dekkera* from South African red wines and consequently establishing a culture collection. Grape, juice and wine samples from various stages of the winemaking process from different wineries were used along with different available methods of detection and identification.

3.2 MATERIALS AND METHODS

3.2.1 CONTACT WITH INDUSTRY

An industry resource that specialises in cellar hygiene (Thalès Wine Cellar Services, South Africa) aided in the selection of wineries in the Western Cape region of the South African winelands that could participate in this project. The wineries approached were informed about the project's details via email, fax and personal appointments. There were two main purposes in the participation of the wineries: firstly, to obtain samples throughout the winemaking process from which *Brettanomyces* yeasts could be isolated. Secondly, to establish the critical stages during the winemaking process at which *Brettanomyces* yeast are regarded as problematic. The 11 wineries willing to participate did not necessarily have a history of *Brettanomyces*-associated problems and had control over the timing of sampling, sample volumes and numbers, cultivar selection and stage of winemaking.

3.2.2 SAMPLE COLLECTION

Grape, juice and wine samples were collected from the participating wineries over three consecutive harvest seasons (2004, 2005 and 2006). The cultivar distribution was targeted towards the main red wine cultivars in South Africa, namely Cabernet Sauvignon, Merlot, Shiraz and Pinotage. Isolations were performed from various stages of the winemaking process, namely the vineyard, grape must, alcoholic fermentation, malolactic fermentation, barrel ageing and bottled or finished wine. Samples were also obtained from random wines that were received for analysis over the three year period.

3.2.2.1 Grape samples

Grape samples were aseptically taken from specific vineyard blocks (representing a specific cultivar) one week before harvesting. Representative sampling from the vineyard blocks was performed by sampling berries from various places throughout the block and pooling them. Where this was not possible, grape bunches were collected from the crushing bins on the day of harvest or collected from the winemaker. The grape berries or bunches were crushed directly in 'Ziploc' bags to obtain grape juice. The mixture of grape berries, stems and grape juice was homogenised and kept at room temperature for 30 min before samples were plated out for the identification of *Brettanomyces* yeast.

3.2.2.2 Grape juice and wine samples

Grape juice and wine samples (volumes of 250 – 750 mL) were collected directly from the sampling taps of large scale fermentation tanks in the cellars. Wine samples from barrels were typically drawn by the winemaker by means of a "wine thief". Alternatively, wine samples were collected by inserting a plastic hose to the bottom of the barrel and collecting a mixture of wine and yeast lees. Sampling containers were typically pre-rinsed with the juice or wine sample before filling. Samples were taken to the laboratory as soon as possible thereafter and plated out within 1 – 6 h after sampling. Prior to plating on selective agar media, 1 mL of the sample was serially diluted with sterile water. One hundred µL of each dilution was directly plated out on the selected agar media.

3.2.3 YEAST STRAINS

The yeast strains used in this study are listed in Table 3.1. These included the strains used for the evaluation of the media and the evaluation of the different primer sets for the identification of *Brettanomyces* yeast by PCR.

3.2.4 CULTURE CONDITIONS

The evaluation of media for the isolation of non-*Saccharomyces* yeasts were performed with various agar nutrient mediums. These included: YPD agar medium (yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L and agar 20 g/L) (Biolab, Merck, Wadeville, Gauteng); GYP agar medium (glucose 20 g/L, yeast extract 5 g/L, peptone 10 g/L, calcium carbonate 5 g/L and agar 20 g/L) (Biolab); EYP agar medium [ethanol 5% (v/v), yeast extract 5 g/L, peptone 10 g/L, calcium carbonate 2 g/L and agar 20 g/L] (Biolab); DBDM (*Dekkera/Brettanomyces* Differential Medium) agar medium [yeast nitrogen base (without amino aids) 6.7 g/L, bromocresol green 22 mg/L, *p*-coumaric acid 100 mg/L, ethanol 6% (v/v) and agar 20 g/L] and WLN agar medium [Wallerstein Laboratory Nutrient media 60 g/L (Sigma), sorbic acid 0.25 g/L, trehalose 5 g/L, *p*-coumaric acid 100 mg/L, agar 15 g/L, and pH 5.5].

The following antibiotics were included in the WLN and DBDM agar media for the inhibition of unwanted microorganisms: kanamycin sulphate 25 mg/L (Roche, Germany) for the inhibition of acetic acid bacteria; chloramphenicol 50 mg/L (Roche) for the inhibition of lactic acid bacteria; cycloheximide 50 mg/L (Sigma) for the inhibition of *Saccharomyces* and certain non-*Saccharomyces* yeasts; biphenyl 0.1% (w/v) (Sigma) was included for the inhibition of fungi when grape juice was plated out. Incubation occurred over a period of 5 to 11 days at 30ºC where after yeast growth was observed.

TABLE 3.1
List of yeast strains used in this study.

Yeast species	Sample ID	Isolated from	Source
Saccharomyces cerevisiae	VIN13		Anchor yeast
Saccharomycodes ludwigii			IWBT
Kloeckera apiculata			IWBT
Zygosaccharomyces bailii			IWBT
Schizosaccharomyces pombe			IWBT
Kluyveromyces sp.			IWBT
Pichia sp.			IWBT
Rhodotorula sp.			IWBT
Torulaspora sp.			IWBT
Debaryomyces sp.			IWBT
Brettanomyces TF		french red wine	TF
Brettanomyces W1		french red wine	TF
Brettanomyces W2		french red wine	TF
Brettanomyces bruxellensis	ISA 1649	lambic beer	CBS72^T

IWBT - Institute for Wine Biotechnology, Stellenbosch University, South Africa.
TF - Thalès (member of Chène and Cie), France.
ISA - Instituto Superior de Agronomia, Lisbon, Portugal.
CBS - Central Bureau voor Schimmelcultures, Delft, The Netherlands.

3.2.5 COLONY INVESTIGATIONS AND MICROSCOPY

Colony types obtained on DBDM and WLN agar media were investigated and photographed. The cell morphologies from these colonies were investigated by means of standard light microscopy (Microscope - Nikon Optiphot-2) under 100x magnification and photographed (Nikon Coolpix 4500 with microscope adapter). Microscopic sample preparation comprised taking a minor portion of an individual colony from the agar plate and re-suspending it in 5 µL distilled water directly on a microscopic slide prior to viewing.

3.2.6 *BRETTANOMYCES* IDENTIFICATION

3.2.6.1 Evaluation of PCR for *Brettanomyces* identification

A selection of five different PCR primer sets was evaluated for the identification of *Brettanomyces* isolates. The details regarding the primers can be seen in Table 3.2. The PCR with the DB1/DB2 primers was performed according to Ibeas *et al.* (1996).

The PCR methods using the universal ITS1/ITS4 primers and the pA1, pB2, pN1, pC1/ITS4 primer combinations were performed as described by Egli and Henick-Kling (2001). The PCR method with the DB90/DB394 primers was according to Cocolin *et al.* (2004). The PCR with the Br-F/Br-R primers was performed as per specifications received from E. and J. Gallo Winery (personal communication) and specifically targeted *B. bruxellensis*. PCR reactions of 50 µL final volumes were performed with genomic DNA (0.5 µg) that were isolated according to the procedure described by Sambrook *et al.* (1989). The primers were tested on reference strains and wine isolates of *Brettanomyces* spp.

TABLE 3.2
List of primers evaluated for PCR identification of *Brettanomyces/Dekkera* species.

Primer	Sequence (5'- 3')	Species	Target	Size
	Universal (Esteve-Zarzoso *et al*. 1999)			
ITS1	TCCGTAGGTGAACCTGCGG	*Brettanomyces /*	partial ITS	
ITS4	TCCTCCGCTTATTGATATGC	*D. bruxellensis*	elements	485 bp
	***Brettanomyces* specific (Cocolin *et al*. 2004)**			
DB90F	GAYACTAGAGAGAGRRGGARGGC	*D. anomala*		
DB394R	ACGAGGAACGGGCCGCT	*D. bruxellensis*	26S rRNA	305 bp
	Nested PCR (Ibeas *et al*. 1996)			
DB1	AGAAGTTGAACGGCCGCATTTGCAC	*D. bruxellensis*	RAD4	470 bp
DB2	AGGATTGTTGACACTCTCGCGGAGG			
	Species-specific (Egli & Henick-Kling, 2001)			
pA1	TATAGGGAGAAATCCATATAAAAC	*D. anomala*		490 bp
pB2	AGAGTGAGGGGATAATGATTTAAGG	*D. bruxellensis*		130 bp
pC1	CATTAGCATACAAACACAACAAACC	*B. custersianus*	5.8S rRNA	400 bp
pN1	CGTTTCATTTTTGTGACGTCCC	*B. naardenensis*		450 bp
ITS4	TATAGGGAGAAATCCATATAAAAC			
	Gallo (personal communication)			
Br-F	n/a	*D. bruxellensis*		350 bp
Br-R				

3.2.6.2 Identification of wine isolates

Colonies obtained on the WLN selective agar media were pre-screened to identify potential *Brettanomyces/Dekkera* isolates destined for PCR identification. The criteria used were: (i) acid production indicated by yellow zone formation on the blue WLN agar plates; (ii) the formation of cream to lime-coloured colonies on WLN media; (iii) presence of a phenolic taint indicative of 4-ethylphenol formation; and (iv) typical 'boat-shaped' and 'mixed' cell morphologies visible with microscopy. The isolates that matched these criteria were selected for identification by PCR.

Potential *Brettanomyces/Dekkera* isolates were cultivated in 10 mL YPD liquid media for 5 days, after which standard genomic DNA isolations followed, as described above. Isolates were subjected to PCR identification using the primer sets ITS1/ITS4 and DB90F/DB394R.

3.3 RESULTS AND DISCUSSION

3.3.1 INDUSTRY PARTICIPATION

The participation of the initial 11 wineries changed over the research period because of a combination of factors including logistical problems, miscommunications regarding sampling times and lack of commitment from wineries. These, along with the fact that the *Brettanomyces* detection procedures were far from optimised at the time, made it difficult to monitor *Brettanomyces* development accurately over the years. However, the purpose of participation from wineries was mainly to obtain samples from which isolations could be performed and this was achieved.

3.3.2 SAMPLES

A total of 307 samples from various stages of the winemaking process were obtained for the isolation of *Brettanomyces* yeast. These samples included the four red wine cultivars, Cabernet Sauvignon, Merlot, Pinotage and Shiraz, originally targeted as well as wine blends of these four, and a satisfactory representation of the different stages of the winemaking process was obtained.

The cultivar distribution (Fig. 3.1) revealed that the largest amount of samples received belonged to Shiraz and Cabernet Sauvignon. The amount of Merlot and Pinotage samples obtained was substantially lower. The reason for this can be explained in the fact that Shiraz and Cabernet Sauvignon are the main red wine cultivars in South Africa and are typically present at most wineries. Therefore, the participant wineries provided samples of either or both of these cultivars. Some of the wineries did not produce Merlot or Pinotage wines. In addition, there is an interesting tendency among winemakers to be more concerned about *Brettanomyces*-produced off-flavours in Shiraz and Cabernet Sauvignon wines, and thus participant wineries normally included these cultivars for analysis. Similarly, it was found that the random wine samples received from other sources (non-participating wineries) for *Brettanomyces* analysis also predominantly belonged to these two cultivars.

The distribution of the samples from the different stages of the winemaking process is shown in Figure 3.2. Most of the samples obtained included wines undergoing malolactic fermentation (MLF) and barrel ageing. This resulted as there were instances where more than one sample was collected from the same MLF batch or barrel at a later stage. For example; MLF samples were drawn from the same tank (batch) after one week of MLF initiation (MLF beginning) and again two months later (MLF completion). In the case of barrel ageing there were instances where samples were collected from the same barrel after 3-month and 6-month periods. This resulted in more samples during these specific winemaking stages. It was originally planned to collect samples at the different times of the specific stages, e.g. MLF beginning, middle and end. However, due to logistical reasons and miscommunication, this was not always possible.
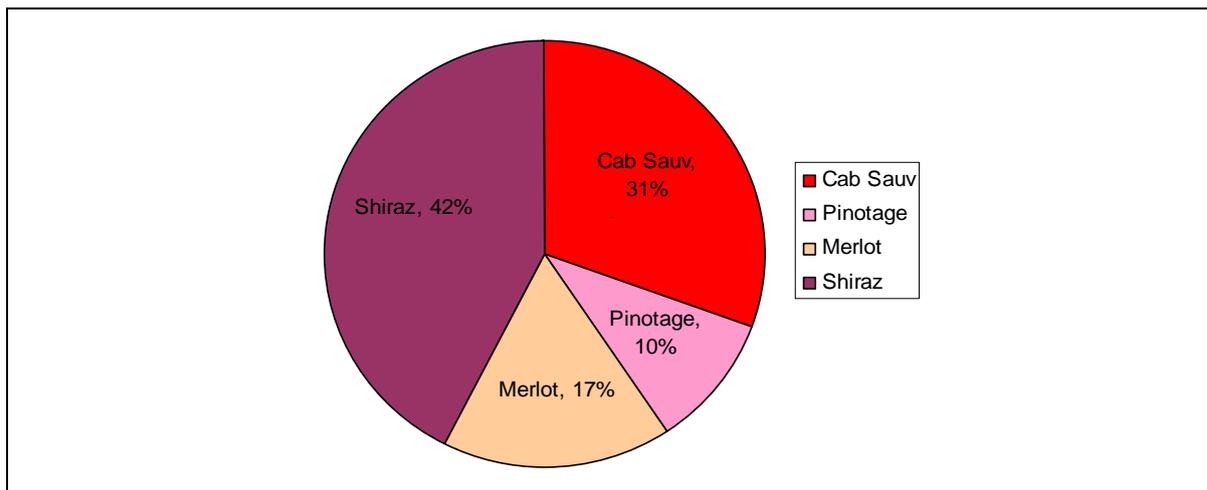
FIGURE 3.1
Cultivar distribution from all the samples collected from 2003 to 2005.
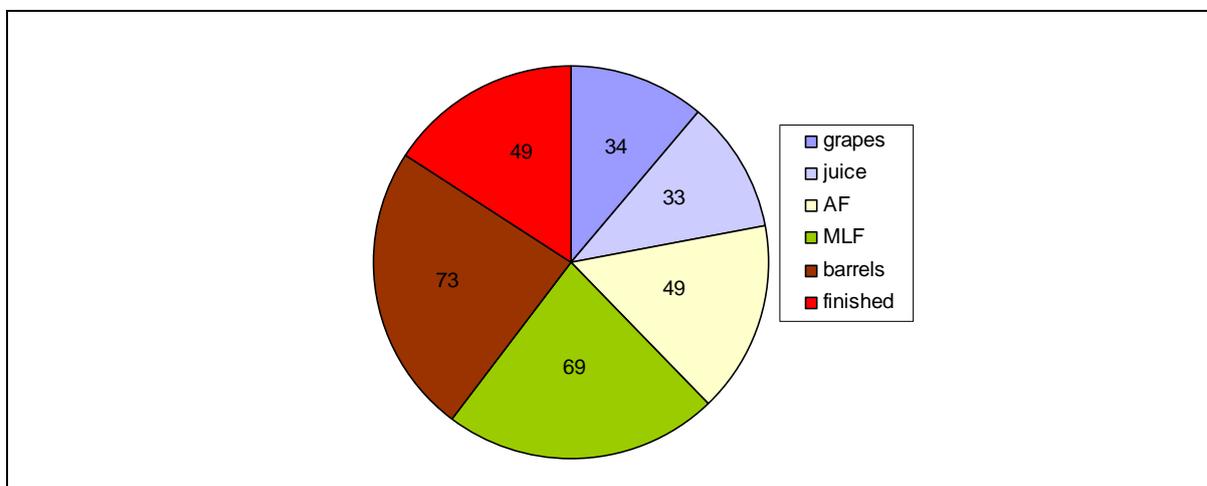
FIGURE 3.2
Total sample distribution and amount of samples collected from the various winemaking
stages over the three winemaking seasons (2003 to 2005).

From all the samples received, it was possible to isolate a large variety of wild yeasts,
including yeasts of the genus *Brettanomyces.* The latter group of isolates, which were
positively identified by means of PCR, were used to establish a *Brettanomyces*
culture collection at the Institute for Wine Biotechnology (IWBT) for all the other aims
of this study.

3.3.3 EVALUATION OF AGAR MEDIA FOR *BRETTANOMYCES* GROWTH AND DETECTION

Excluding DBDM and WLN differential media selected from literature (Rodrigues
et al., 2001), the YPD, GYP and EYP agar media evaluated for *Brettanomyces*
growth were selected from the standard range of media used at the IWBT for yeast
growth. The purpose of the evaluation of these standard media was mainly to see if
they could support *Brettanomyces* growth and recovery from a winemaking
environment and if it was possible to differentiate between *Brettanomyces* colonies

and other yeasts.

The results of the different agar media tested for *Brettanomyces* and other yeast growth are shown in Table 3.3. All the *Saccharomyces*, non-*Saccharomyces* and *Brettanomyces* strains tested were capable of growing on the general purpose YPD, GYP and EYP agar plates. In most cases it was not visually possible to discriminate between *Brettanomyces* colonies and other colonies on these agar plates and these standard media were not favoured for the purpose of *Brettanomyces* detection. YPD agar (without antibiotics) was nevertheless always included for plating out purposes as this gave an indication of the microbial load of the samples analysed.

TABLE 3.3
Evaluation of different agar plate media for *Brettanomyces* growth.

Media	YPD	GYP	EYP	DBDM (50)*	DBDM (100)	DBDM (150)	DBDM (200)	WLN (50)	WLN (100)
Yeasts									
Brettanomyces TF	+	+	+	+	+	+	+	+	+
Brettanomyces W1	+	+	+	+	+	+	+	+	+
Brettanomyces W2	+	+	+	+	+	+	+	+	+
Saccharomyces cerevisiae	+	+	+	-	-	-	-	-	-
Saccharomycodes ludwigii	+	+	+		+	+	+	+	+
Kloeckera apiculata	+	+	+	+	+	±	±	+	+
Zygosaccharomyces bailii	+	+	+	±	-	-	-	-	-
Schizosaccharomyces pombe	+	+	+	±	-	-	-	+	+
Kluyveromyces sp.	+	+	+	+	+	+	+		
Pichia sp.	+	+	+	+	+	±	±	+	+
Rhodotorula sp.	+	+	+	-	-	-	-	-	-
Torulaspora sp.	+	+	+	-	-	-	-	-	-
Debaryomyces sp.	+	+	+	+	+	±	±	+	+

* Number in brackets indicate the concentration (mg/L) of cycloheximide used
(±) poor growth
(+) clear growth
(-) no growth

Following this, DBDM and WLN differential agar media were evaluated for the detection and isolation of *Brettanomyces* yeasts. These two agar media have been described by previous authors (Rodrigues *et al.,* 2001) specifically for the detection of *Brettanomyces/Dekkera* yeasts.

In this study it was found that both these agar media were suitable for the detection of *Brettanomyces* spp. but that variable growth was also obtained with some non-*Saccharomyces* spp. *Saccharomyces cerevisiae* could not grow on either of the DBDM or WLN media. During the development of the DBDM media, Rodrigues *et al.* (2001) manipulated the media with different concentrations and combinations of antimicrobial agents such as cycloheximide, ethanol and sorbic acid (in the case of WLN media), to improve the selectivity for *Brettanomyces* spp. Despite the fact that results showed improved growth inhibition for some of the important wine contaminant yeasts, e.g. *Kloeckera apiculata, Candida vini*, *Pichia* spp., *Zygosaccharomyces bailii* and *S. cerevisiae*, other species could also grow on these media (Rodrigues *et al.,* 2001; Couto *et al.,* 2005).

It was specifically during the initial winemaking stages, such as grape crushing, juice preparation and the early stages of alcoholic fermentation (<5% alcohol), that the growth of non-*Brettanomyces* yeasts was evident on both DBDM and WLN agar in this study. In addition, the growth of bacteria and fungi was often observed despite the presence of the antimicrobial agents used in the media. It is important to mention that these observations varied and were dependent on the grape quality and sulfur dioxide levels used at crushing because these parameters determined the microbial populations of the samples. For this reason, it was decided to include the antibiotics kanamycin, chloramphenicol and biphenyl for the inhibition of acetic acid bacteria, lactic acid bacteria and fungi respectively. This was followed by increasing the cycloheximide concentration from 10 mg/L to 50 mg/L in a further attempt to improve selectivity. The inclusion of these antibiotics in the agar media proved to be more selective, especially during the initial winemaking stages and consequently simplified the *Brettanomyces* screenings that followed.

Stages of sampling post-alcoholic fermentation yielded lower amounts of non-*Brettanomyces* yeasts on the selective media due to the antimicrobial effect of the ethanol levels (>6%) in the wine that consequently diminish the natural wild yeast populations in the wine samples (Fleet, 1990; Fleet and Heard, 1993).

As the resistance of yeast species to cycloheximide can vary (Barnett *et al*., 2000), it was decided to evaluate different concentrations of cycloheximide in DBDM and WLN as indicated in Table 3.3. This was aimed at improving the selectivity of the media for isolation purposes. It was found that cycloheximide concentrations above 50 mg/L appeared not to be inhibitory towards the growth of the yeast species of *Kloeckera, Pichia* and *Debaryomyces* tested. The use of ethanol (~6% v/v) as sole carbon source and antimicrobial agent in DBDM has however been shown to inhibit *Kloeckera apiculata* (Rodrigues *et al.,* 2001). The *Brettanomyces* strains were not affected even when a maximum concentration of 200 mg/L cycloheximide (DBDM media) was used. It was however observed that the colonies appeared smaller in comparison to those plates containing lower concentrations and this indicated that the growth rates were slightly slower. In support of this finding Rodrigues *et al.* (2001) mentioned that sorbic acid and cycloheximide in WLN media at concentrations higher than 250 mg/L and 50 mg/L respectively, did not improve the selectivity for *Brettanomyces* spp. but rather increased the incubation time for *Brettanomyces* colony formation. The same result occurred with the inclusion of ethanol (6%) and cycloheximide (10 mg/L) in the DBDM media. Increased cycloheximide concentrations have been shown to extend the growth period of a single *D. bruxellensis* strain in liquid media (Couto *et al.,* 2005). However, the risk when increasing the cycloheximide concentration is that *Brettanomyces* strains that are more sensitive to cycloheximide may not be detected. For this reason, it was finally decided to use 30 mg/L cycloheximide as this provided a good compromise between 10 mg/L (described in literature) and 50 mg/L, specifically for a media that allows for the detection and isolation of *Brettanomyces* spp. from wine. In addition, it was

believed that the inclusion of ethanol in the agar media in combination with antibiotics could affect the recovery of *Brettanomyces* cells. For this reason ethanol was not included in the WLN agar medium.

In this study, the WLN agar medium proved to be more successful for the detection and isolation of *Brettanomyces* yeasts in practice. After numerous routine screenings, there were occasions where *Brettanomyces* colonies were observed on WLN and no or significantly fewer colonies were found on DBDM agar after plating out from the same wine. For this reason, WLN agar medium was preferred and was further investigated.

3.3.4 COLONY INVESTIGATIONS AND MICROSCOPY OF *BRETTANOMYCES* ISOLATES

The majority of the colonies obtained from different yeast genera on the standard media YPD, GYC and EYP were similar in colour (cream) and morphology. *Brettanomyces* colonies could not be visually distinguished and therefore these media were not suitable for screening procedures. For example, *S. cerevisiae* colonies and *Brettanomyces* colonies showed a similar cream colour on YPD agar (without cycloheximide) and could not be distinguished from each other (Fig. 3.3). However, it was noticed that single *S. cerevisiae* colonies may appear larger in size than the slower growing *Brettanomyces* colonies.

B. bruxellensis
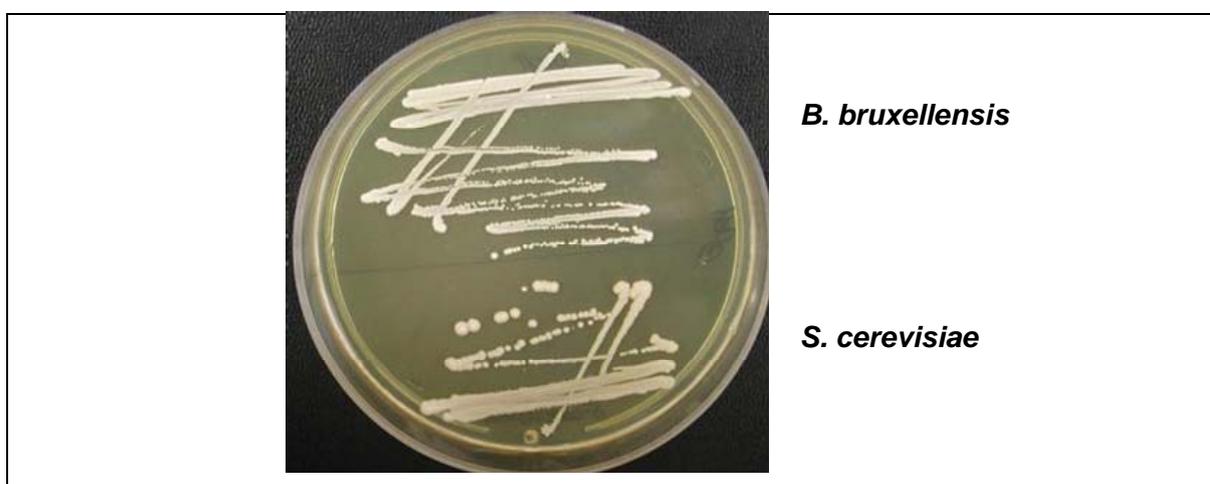
S. cerevisiae

FIGURE 3.3
YPD agar showing the growth of *S. cerevisiae* VIN13 and *B. bruxellensis* ISA 1649.

Different colony morphotypes were found on both the DBDM and WLN agar plates. The colony types, however, varied between cultivars and depended on the stage of the winemaking process from which they were isolated. Examples of the different colonies observed on DBDM and WLN agar media can be seen in Figure 3.4. Although different coloured colonies were obtained on both these agar media, numerous yeast species had colonies of similar colour. Randomly selected colonies revealing similar morphologies and colour (light-creamy green) were investigated with microscopy. It was evident that colonies with a similar appearance could represent

different yeast species. This observation complicated the identification of *Brettanomyces* yeasts based purely on colony identification on selective agar plates. In an attempt to simplify the plate screening method, characteristic *Brettanomyces* colony and cellular morphologies were determined. This was achieved by investigating each of the colonies found on the selective agar media by microscopy and comparing these to the specific cellular morphologies of known *Brettanomyces* species.

It was found that *Brettanomyces* colonies were typically yellow-cream to green colonies (often also pin-pointed) when they appeared on DBDM and WLN agar plates. With prolonged incubation, a colour change towards a darker green typically occurred (see Figure 3.5A). This corresponded to the finding of previous authors (Rodrigues *et al.,* 2001; Dias *et al.*, 2003). It was also observed that certain non-*Brettanomyces* colonies could similarly be visualised as different shades of green (data not shown). The green colouration of the cells is a result of the gradual diffusion of the bromocresol green indicator present in the nutrient media into the cells causing a colouration of the cytoplasm. The cells within a colony therefore determine the colony colour, which can intensify with prolonged incubation. In Figure 3.5B it can be seen how cells of *D. bruxellensis* changed colour over a period of time. In addition, the agar media changed colour from blue (WLN) or blue-green (DBDM) to yellow due to acid production (Ciani and Ferraro, 1997; Dias *et al.*, 2003; Rodrigues *et al.,* 2001) (see Figures 3.5A and B).
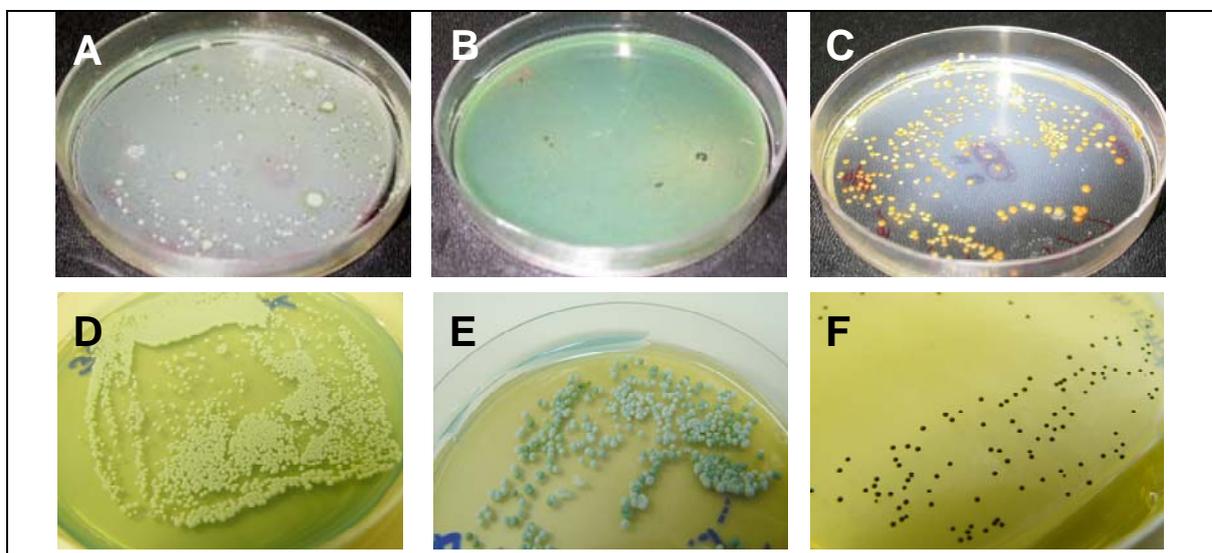

FIGURE 3.4
Different colony types found on WLN and DBDM agar plates after isolations from a winemaking environment. (A) grape juice on DBDM; (B) wine from alcoholic fermentation on DBDM; (C) wine after alcoholic fermentation on WLN; (D) from barrel shavings on WLN, (E) barrel aged red wine on WLN; and (F) finished wine on WLN.
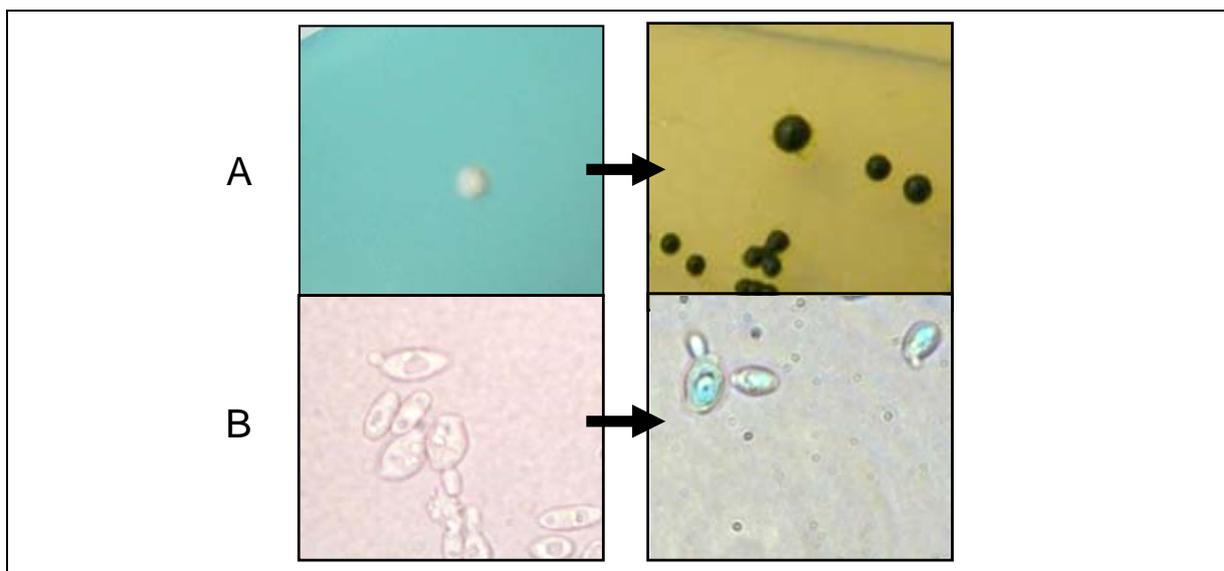
FIGURE 3.5
Change of *Brettanomyces* colony on WLN agar media over time. (A) colony colour change;
and (B) cellular change.

The incubation time needed for *Brettanomyces* growth may differ. The latter was found to be dependent on the recovery and/or re-culturability of the cells which in turn depended on the media used. When *Brettanomyces* cells were cultivated from a previous culture (re-streaked or plated-out), incubation times would be shorter. Colonies would be observed on standard YPD agar (containing 50 mg/L cycloheximide) after 3 to 5 days and on WLN or DBDM media after 5 to 7 days. The recovery of *Brettanomyces* cells from materials with complex nutritional compositions and mixed microbial populations, such as wine, would result in increased incubation times. Colony formation on WLN and DBDM media was found to vary between 5 to 14 days and, in some cases, it was found that colonies could only be observed after 11 days of incubation at 30°C. This is why up to two weeks of incubation time is recommended for the detection of yeasts of the genera *Brettanomyces* or *Dekkera*. Colonies that appear after 2 to 3 days of incubation are unlikely to be *Brettanomyces* and this can additionally be used as discriminatory criteria (Loureiro and Malfeito-Ferreira, 2003).

In this study it was evident that the DBDM and WLN agar media were only semi-selective. Despite the inclusion of antibiotics in these media other unidentified yeast species were seen that complicated the screening and detection of *Brettanomyces*. It was observed that the variety of colonies detected decreased with the progression of the winemaking process and that the initial stages hosted the most complexity.

Some of the non-*Brettanomyces* colonies that were capable of growing on the WLN and DBDM agar media can be seen in Figure 3.6.
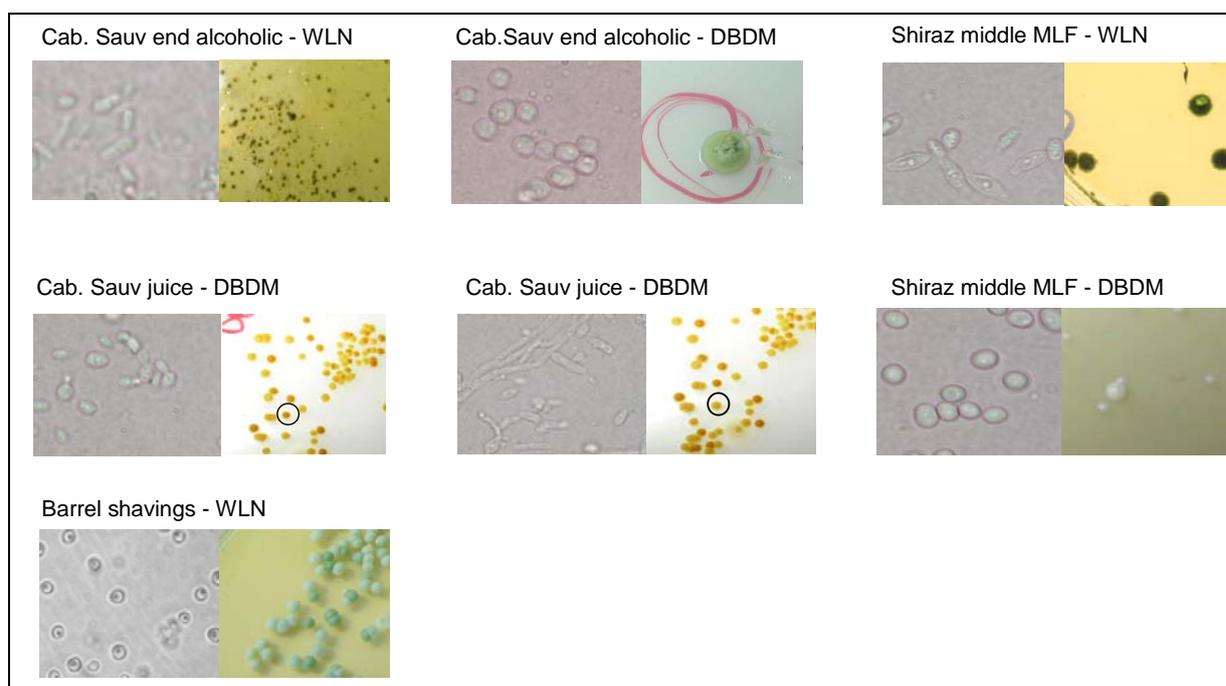
FIGURE 3.6
Non-*Brettanomyces* colonies obtained with WLN and DBDM agar media from isolations of different stages of the winemaking process.

In order to establish 'typical' *Brettanomyces* cellular morphologies pure cultures of different *Brettanomyces* spp. were investigated. The pure cultures of three strains, *B. bruxellensis* TF, W1 and W2, consisted of mixed cellar morphologies and included oval, ogival, carrot-shaped, elongated and boat-shaped cells. These morphologies (Fig. 3.7) were in agreement with other authors who describe the analysis of the genera *Brettanomyces* and *Dekkera* (Smith *et al.,* 1998). Budding cells of the genus *Dekkera* were described as spherical, subglobose to ellipsoidal, frequently ogival and cylindroidal to elongated. The existence of mixed cellar morphologies, however, pose great difficulty in the identification of microorganisms by simple microscopy, especially as the cell shapes that were found can represent numerous yeast species.

Despite the large variety of cell shapes that were found, a specific morphological character was identified that aided the identification process. It was observed that certain individual cells within a pure culture contained flat (uneven) ends on opposite sides. These flat jagged ends are the result of multiple budding scars and it was often observed that a bud cell was attached at this point. Moreover, in the case where cells displayed one flat end, the opposite side ended with a pointed arch. These cells are typically regarded as boat-shaped figures and this became the 'trademark' cellular morphology of *B. bruxellensis* in this study. Colonies that were obtained on selective media were investigated and, if boat-shaped cells were observed among a mixed cellular morphology, they would be selected as potential *Brettanomyces* yeasts.

FIGURE 3.7
The different cellular morphologies of a pure culture of a wine isolate identified as
B. bruxellensis (1000x magnification, phase-contrast microscope). The block marked with 'X'
indicates the typical boat-shaped morphology.

Despite the value of the microbiological techniques (selective plating followed by microscopy) many uncertainties remained. It was therefore inevitable that positive *Brettanomyces* identification could only be guaranteed with the application of molecular techniques (PCR). Therefore, a screening procedure for the identification of potential wine isolates of *B. bruxellensis* was established that included the selective plating, microscopic observation and molecular identification with PCR. This can be seen in Figure 3.8.

Selective plating (WLN)   Microscopic observation   PCR identification
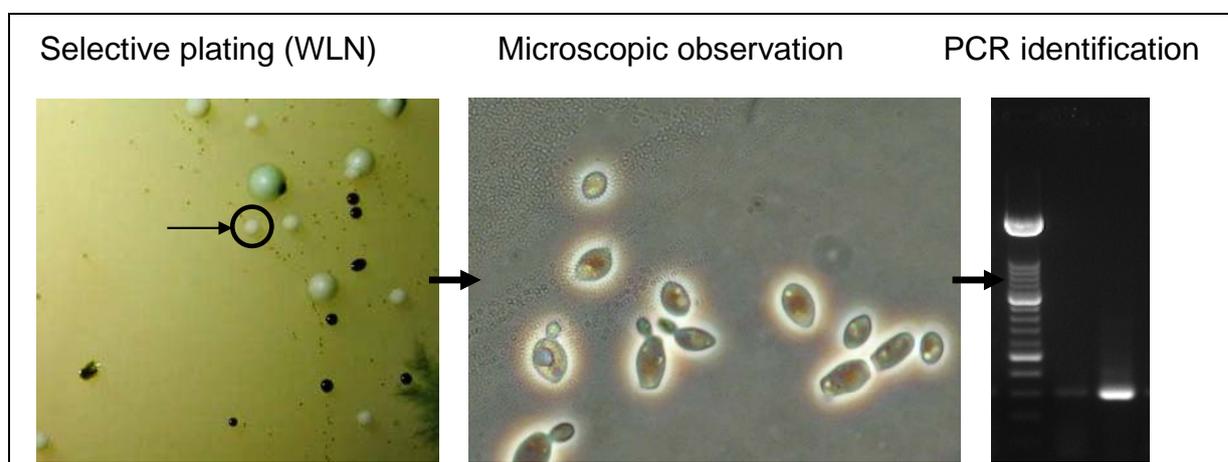
FIGURE 3.8
Identification procedure used for *Brettanomyces* yeasts isolated from wine. Potential *Brettanomyces* colonies obtained on the selective agar medium were investigated with microscopy. Cellular morphologies that resembled typical *Brettanomyces* morphologies were subjected to PCR identification.

3.3.5 *BRETTANOMYCES* DETECTION WITH PCR

The results obtained from the specific amplifications of the different primer sets are shown in Figures 3.9A-E. The different PCR primer sets tested were all capable of identifying yeasts belonging to the species *B. bruxellensis*.

The universal ITS1/ITS4 primers amplified the region spanning the 5.8S rDNA gene and the partial areas of its flanking intergenic spacer regions, ITS1 and ITS2, from all the isolates. In Figure 3.9A, it can be seen that isolates belonging to the species *B. bruxellensis* yielded a PCR product of approximately 485 bp and isolates belonging to the species *B. anomalus* yielded a product of approximately 550 bp (data not shown). These data corresponded to the findings of Egli and Henick-Kling (2001) and Esteve-Zarzoso *et al.* (1999) for species of *B. bruxellensis*, but not for *B. anomalus*. Esteve-Zarzoso *et al.* (1999) obtained an amplicon of approximately

800 bp for the latter species. The universal primers were found to be very useful especially because they could also identify the presence of other common wine yeasts including *S. cerevisiae, Cryptococcus curvatus, Rhodotorula glutinis, Hanseniaspora uvarum, Candida guillermondii* and *Pichia anomala* (Egli and Henick-Kling, 2001). It should however be mentioned that *Pichia kluyveri* will generate a similar sized PCR product (~490 bp) as *B. bruxellensis,* but discrimination can be obtained by investigating the cellular morphologies.

The DB1/DB2 primers could amplify a fragment of approximately 470 bp (Fig. 3.9B) for *B. bruxellensis* but no amplification could be obtained for *B. anomalus.* This primer set is complemented with another primer pair DB3/DB4 in a nested PCR reaction (Ibeas *et al.,* 1996) that allows for the detection of *B. bruxellensis* strains after the second primer set was used.

The primer set DB90/DB394 targeting the D1-D2 loop of the 26S rDNA gene successfully amplified a fragment of approximately 305 bp for the species *B. bruxellensis* (Fig. 3.9C). The same primer set also allows for the identification of *B. anomalus* and will result in a similar sized amplicon of 305 bp (Cocolin *et al.,* 2004). The discrimination of *B. bruxellensis* and *B. anomalus* can be achieved by restriction enzyme analysis with *Dde*I (Cocolin *et al.,* 2004). It was found that this primer set was reliable and accurate because it formed PCR products only with species of *Brettanomyces.*

The mixture of species-specific primers (pA1, pB2, pN1, pC1/ITS4) tested often resulted in inconsistent and unspecific amplification (data not shown). As *B. bruxellensis* is the predominant *Brettanomyces* species that exists in wine (Egli and Henick-Kling, 2001; Loureiro and Malfeito-Ferreira, 2003), the primer set pB2/ITS4 could be used for the sole detection of *B. bruxellensis*. This minimised the unspecific amplification and ambiguities originating from the mixture of primers that also allow for detection of the other *Brettanomyces/Dekkera* species. Although positive amplifications of approximately 130 bp were obtained for the species *B. bruxellensis* (Fig. 3.9D), this primer set was not preferred. It was found that the small size of the PCR product could not be consistently amplified and additionally required the use of a higher percentage of agarose and/or longer electrophoresis time than the other protocols.

The Br-F/Br-R primers successfully amplified a DNA fragment of approximately 350 bp (Fig. 3.9E) from *B. bruxellensis* as specified by the supplier (E. and J. Gallo Winery). However, as neither the targeted genetic sequence nor the primer sequences were available, this primer set was not used for further identification of isolates.

From the different approaches of identification used on the wine isolates, the use of ITS1/ITS4 and DB90F/DB394R primer sets was preferred for identification of *Brettanomyces* yeast. The high specificity of the DB90F/DB394R primer set was selected in instances where the cellular morphologies typically resembled that of *B. bruxellensis* as was established in this study. In cases where the cellular

morphologies appeared uncharacteristic the ITS1/ITS4 primer set was selected because it could be indicative of both *Brettanomyces* and non-*Brettanomyces* yeasts.
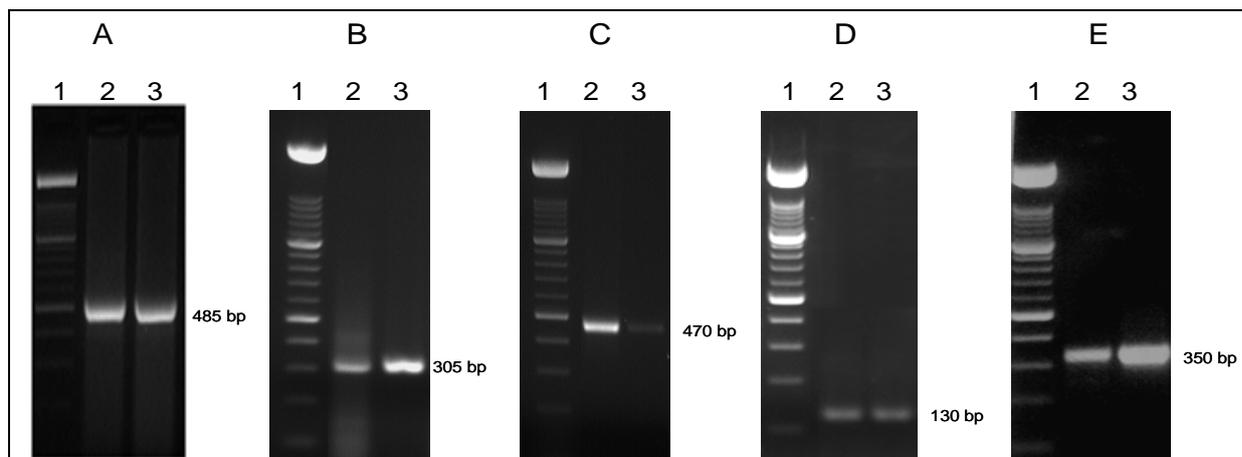
FIGURE 3.9
Evaluation of the different PCR primer sets for the identification of *B. bruxellensis*. The different primer sets are: (A) ITS1/ITS4; (B) DB90F/DB394R; (C) DB1/DB2; (D) BrF/BrR; and (E) pB2/ITS4. Lane 1, 100 bp molecular ladder (Roche Marker XIV); lane 2, *B. bruxellensis* W2 (wine isolate); and Lane 3, *B. bruxellensis* ISA 1649.

3.3.6 WINEMAKING STAGES OF *BRETTANOMYCES* DETECTION

In this study *Brettanomyces* yeasts were isolated from various stages of the winemaking process. From the 307 samples investigated for the presence of this yeast species, 41 yeast isolates positively identified as *B. bruxellensis* by PCR were obtained. A list of the isolates is given in Table 3.4. The PCR identification of the isolates was verified by two different primer sets as can be seen in Figure 3.10.

The majority of the *B. bruxellensis* strains were isolated from wines undergoing MLF and finished wines (Table 3.5). These included samples from tanks, barrels and bottled wine and represented the variety of practices winemakers follow. For example, many winemakers prefer performing MLF in barrels where others first complete MLF in tanks, after which they introduce the wines to barrel contact for ageing. During the first stages of the winemaking process significantly fewer *Brettanomyces* isolates could be detected. Only three and six isolates were obtained from grape juice and alcoholic fermentation samples respectively. No *Brettanomyces* yeasts could be isolated from the grapes.
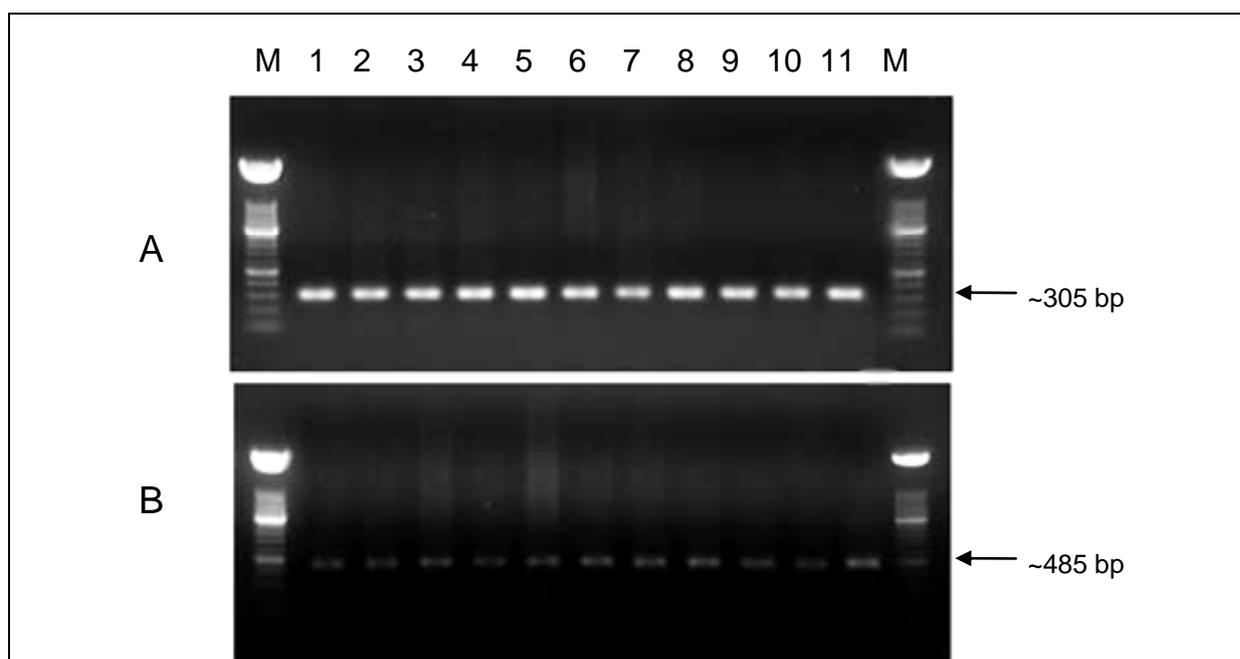
FIGURE 3.10
The identification of wine isolates as *B. bruxellensis* by means of two different PCR primer sets. (A) DB90F/DB394R; and (B) ITS1/ITS4. Lane M contained 100 bp molecular DNA ladder (Roche Marker XIV). Lanes 1-10 contained wine isolates of *Brettanomyces* and lane 11 contained *B. bruxellensis* ISA 1649 that served as positive control.

These findings agreed with the findings of many authors who also stated that the detection of very low amounts of *Brettanomyces* spp. during the initial stages of the winemaking process could be observed (Fugelsang, 1993; Ciani and Ferraro, 1997; Silva *et al.*, 2004). The poor detection or low numbers of *Brettanomyces* found during the initial stages have been explained by the fact that these slow-growing yeasts, if at all present, are out-competed by the large variety of yeast species that dominate on the grapes and in the juice (Egli and Henick-Kling, 2001). More recently, other authors have stated that *Brettanomyces* species might require the use of enrichment media for their detection during the initial stages (Renouf and Lonvaud-Funel, 2006). In addition, a few randomly selected samples from the bark of vines and from the soil directly beneath the vines were also investigated for *Brettanomyces* in this study. None of these revealed the presence of *Brettanomyces* yeast.

The isolation of *Brettanomyces* species during alcoholic fermentation yielded six isolates out of the 49 samples that were investigated. Four of the six samples were obtained from alcoholic fermentation stages that had been declared as sluggish or stuck ferments. Sluggish or stuck fermentations, especially during the late stages of alcoholic fermentation, provide an ideal environment for *Brettanomyces* cells to proliferate. In such cases the performance of the rigorous fermenting yeast *S. cerevisiae* is minimal, the sulfur dioxide levels have decreased and substantial amounts of sugar (glucose and fructose) are still available, which all favour the growth of *Brettanomyces* (Froudiere and Larue, 1988; Bisson, 1999).

During the malolactic fermentation stages, 14 *Brettanomyces* isolates were obtained from 69 samples that were analysed. As mentioned above, these samples included

MLF samples from tanks and barrels. MLF has been indicated as one of the most critical areas of the winemaking process where *Brettanomyces* can develop (Chatonnet *et al.*, 1992; Fugelsang *et al.*, 1993; Chatonnet *et al.*, 1995; Licker *et al.*, 1998; Renouf *et al.*, 2006). The poor antimicrobial potential of the wine as a consequence of low sulfur dioxide levels in combination with the presence of yeast lees (increased source of nutrition) present ideal conditions for *Brettanomyces* spp. to grow.

From the 49 finished wine samples (referring to wines after MLF) that were obtained 15 *Brettanomyces* isolates were found. These samples included wines undergoing barrel ageing, finished wine in tanks (before bottling) and wines that were already bottled. In the latter two cases the wines were in contact with barrels at an earlier stage. *Brettanomyces* contamination has long been associated with barrels and numerous authors regard particularly used barrels as the most notorious habitat of this yeast species (Ibeas *et al.,* 1996; Fugelsang *et al.*, 1993; Chatonnet *et al.,* 1995).

With regards to cultivars, *Brettanomyces* yeast was isolated from all the main red wine cultivars targeted in this study, however, no correlation could be drawn about which cultivar was more prone to act as host to this yeast species. One of the finished wines received for analysis included the white wine cultivar Chardonnay. White wines are not commonly associated with *Brettanomyces* spoilage due to the lower pH levels of white wine (directly resulting in a higher antimicrobial potential caused by the sulfur dioxide) and lower levels of phenolic acid precursors responsible for volatile phenolic off-flavours (Loureiro and Malfeito-Ferreira, 2006). However, the fact that white cultivars such as Chardonnay undergo barrel ageing may well explain the detection.

3.4 CONCLUSIONS

This study has covered various aspects of the detection, isolation and identification methods of *Brettanomyces* yeast from a winemaking environment. As a limited understanding and information about these aspects were available at the time, this research was necessary to develop a platform from which further investigations could follow. It was possible to segregate numerous *Brettanomyces* isolates from a small number of samples from various red wines, comprising all red cultivars that were tested, at different stages of the winemaking process. These isolates were used to establish a culture collection at the Institute for Wine Biotechnology that will be used for further research. This study contributed to a better understanding of *Brettanomyces* yeasts during winemaking and the knowledge gained will be invaluable for further projects. The results show how this species is common during winemaking and that more sensitive methods are needed to demonstrate the constant existence of these yeasts in the wine environment. Further investigations should focus on the direct detection of *B. bruxellensis* in wines.

TABLE 3.4.
List of wine isolates of *B. bruxellensis* collected in this study.

No.	Sample ID	Isolate name	Year	Cultivar	Stage	Description	Species ID
1	SA13	Isolate B1G R10	2003	Cabernet Sauvignon	Juice		*B. bruxellensis*
2		Isolate A36	2003	Merlot	MLF		*B. bruxellensis*
3	SA14	Isolate A24	2003	Shiraz	MLF		*B. bruxellensis*
4	SA15	Isolate V1-4	2003	red wine	MLF		*B. bruxellensis*
5	SA16	Isolate A24	2003	Shiraz	Juice		*B. bruxellensis*
6	SA17	Isolate 6	2003	Barrel wash water	Water	before steaming	*B. bruxellensis*
7	SA18	Isolate 7	2003	Barrel wash water	Water	after SO_2	*B. bruxellensis*
8	SA19	Isolate 8	2003	Barrel wash water	Water	after SO_2	*B. bruxellensis*
9	SA20	Isolate 4	2004	Shiraz	MLF		*B. bruxellensis*
10	SA21	Isolate 14	2004	Cabernet Sauvignon	Juice		*B. bruxellensis*
11	SA22	Isolate 27	2004	Merlot	AF		*B. bruxellensis*
12	SA23	Isolate 44	2004	Merlot	AF		*B. bruxellensis*
13	SA24	Isolate 55	2004	Blend (Cab Sauv/ Merl)	MLF		*B. bruxellensis*
14	SA25	Isolate 59	2004	Shiraz	MLF		*B. bruxellensis*
15	SA26	Isolate 63	2004	Chardonnay	Finished	barrel	*B. bruxellensis*
16	SA27	Isolate 70	2004	Pinotage	MLF	old barrel	*B. bruxellensis*
17	SA28	Isolate 82	2004	Cabernet Sauvignon	MLF	new barrel	*B. bruxellensis*
18	SA29	Isolate 83	2004	Cabernet Sauvignon	MLF	old barrel	*B. bruxellensis*
19	SA30	Isolate 84	2004	Cabernet Sauvignon	MLF	new barrel	*B. bruxellensis*
20	SA31	Isolate 85	2004	Cabernet Sauvignon	MLF	tank	*B. bruxellensis*
21	SA32	Isolate 86	2004	Cabernet Sauvignon	MLF	tank	*B. bruxellensis*
22	SA33	Isolate 87	2004	Cabernet Sauvignon	MLF	tank	*B. bruxellensis*
23	SA34	Isolate 88	2004	Cabernet Sauvignon	MLF	old barrel	*B. bruxellensis*
24	SA35	Isolate S4	2005	Chenin blanc	Finished	barrel	*B. bruxellensis*
25	SA36	Isolate S5	2005	Merlot	AF	tank	*B. bruxellensis*
26	SA37	Isolate S6	2005	Merlot	AF	tank	*B. bruxellensis*
27	SA38	Isolate S7	2005	Merlot	AF	tank	*B. bruxellensis*
28	SA39	Isolate S11	2005	Shiraz	AF	barrel	*B. bruxellensis*
29	SA40	Isolate 35a	2005	Merlot	Finished	tank	*B. bruxellensis*
30	SA41	Isolate 35b	2005	Merlot	Finished	tank	*B. bruxellensis*
31	SA42	Isolate 37a	2005	Merlot	Finished	tank	*B. bruxellensis*
32	SA43	Isolate 37b	2005	Merlot	Finished	tank	*B. bruxellensis*
33	SA44	Isolate 38	2005	Cabernet Sauvignon	Finished	barrel	*B. bruxellensis*
34	SA45	Isolate 39a	2005	Merlot	Finished	barrel	*B. bruxellensis*
35	SA46	Isolate 39b	2005	Merlot	Finished	barrel	*B. bruxellensis*
36	SA47	Isolate 40a	2005	Cabernet Sauvignon	Finished	barrel	*B. bruxellensis*
37	SA48	Isolate 40b	2005	Cabernet Sauvignon	Finished	barrel	*B. bruxellensis*
38	SA49	Isolate 41	2005	Shiraz	Finished	barrel	*B. bruxellensis*
39		Isolate K54	2005	red wine	Finished	bottle	*B. bruxellensis*
40		Isolate K101	2005	red wine	Finished	bottle	*B. bruxellensis*
41		Isolate K102	2005	red wine	Finished	bottle	*B. bruxellensis*

TABLE 3.5.
Number of *B. bruxellensis* isolates obtained from the various stages of winemaking.

WINEMAKING STAGES				
Vineyard (grapes)	Pre-fermentation (juice)	Alcoholic fermentation	Malolactic fermentation	Finished wine
nd[#]	3	6 (1B*)	14 (5B)	15 (7B)

[#]nd – not detected
*B - barrel

3.5 ACKNOWLEDGEMENTS

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Research Results

Molecular identification of *Brettanomyces bruxellensis* strains isolated from red wines and volatile phenol production

4. RESEARCH RESULTS

Molecular identification of *Brettanomyces bruxellensis* strains isolated from red wines and volatile phenol production

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ABSTRACT

The spoilage yeast *Brettanomyces/Dekkera* can persist throughout the winemaking process and has the potential to produce off-flavours that affect the sensory quality of wine. The main objective of this study was to select different strains of *Brettanomyces bruxellensis* isolated from red wines and to compare their volatile phenol production. From a collection of 63 strains, eight strains of *B. bruxellensis* were selected for volatile phenol production after the application of molecular techniques such as ISS-PCR, PCR-DGGE and REA-PFGE. All strains showed three large chromosomes of similar size with PFGE. However, unique restriction profiles of the chromosomes were visible after *Not*I digestion that clearly distinguished the strains. All strains were capable of producing large quantities of 4-ethylphenol and 4-ethylguaiacol from *p*-coumaric acid and ferulic acid, respectively in synthetic media. However, the diversity among strains for volatile phenol production differed between synthetic media and wine with regards to the maximum production levels of 4-ethylphenol and 4-ethylguaiacol. This study illustrated the diversity of *B. bruxellensis* strains that occur during winemaking.

Keywords: *Brettanomyces bruxellensis, Dekkera, red wine, spoilage, volatile phenols, identification, DGGE, PFGE.*

Abbreviations

p-**CA** *p*-coumaric acid; **FA** ferulic acid; **4-VP** 4-vinylphenol; **4-VG** 4-vinylguaiacol; **4-VC** 4-vinylcatechol; **4-EP** 4-ethylphenol; **4-EG** 4-ethylguaiacol; **4-EG** 4-ethylcatechol; **ISS** intron splice site; **DGGE** denaturing gradient gel electrophoresis; **REA-PFGE** restriction enzyme analysis - pulsed field gel electrophoresis

4.1 INTRODUCTION

The involvement of yeasts in the winemaking process has gained increasing attention over the years as they play a central role in determining wine quality. Yeast of the genus *Brettanomyces* or its teleomorph *Dekkera* are among the many naturally occurring types of yeast in the winemaking process. However, *Brettanomyces* yeasts, in particular *Brettanomyces bruxellensis,* are regarded as spoilage microorganisms with the potential to drastically alter the final outcome of red wine under uncontrolled conditions. These yeasts are highly adapted to grow and survive in red wine and can have detrimental effects on the visual and organoleptic quality of wines by causing film formation (Fugelsang *et al.*, 1993; Fugelsang, 1997), cloudiness (Fugelsang *et al.*, 1993; Fugelsang, 1997), loss of colour (Mansfield *et al.*, 2002), production of volatile acidity (Fugelsang *et al.*, 1993; Aguilar-Uscanga *et al.*, 2003), production of 'mousy' off-flavours (tetrahydropyridines) (Heresztyn, 1986; Snowdon *et al.*, 2006) and the formation of phenolic off-flavours (Chatonnet *et al.*, 1992; 1995; 1997; Loureiro and Malfeito-Ferreira, 2003; Dias *et al.*, 2003a; Joseph and Bisson, 2004). The latter is considered to be the most important indicator of *B. bruxellensis* activity in red wine. The origin of volatile phenols involves the sequential conversion of the hydroxycinnamic acid substrates (*p*-coumaric, ferulic and caffeic acid) into hydroxystyrenes (4-VP, 4-VG and 4-VC), which are further reduced into ethyl derivatives (4-EP, 4-EG and 4-EC) (Hesford *et al.*, 2004; Suárez *et al.*, 2007). The levels of ethyl derivatives produced can be indirectly influenced by certain winemaking practices (Oelofse and Du Toit, 2006), cultivar selection (different precursor concentrations) (Clifford, 1999) but mainly by the number of metabolically active *B. bruxellensis*. Depending on the wine style and concentrations of volatile phenols, the aromas perceived are described by terms such as 'medicinal', 'barnyard-like', 'inky', 'sweaty leather' and 'Band-aid' (Chatonnet *et al.*, 1992; Rodrigues *et al.*, 2001). Some consider the slightest hint of *Brettanomyces* character as an indication of spoilage. Others see the contribution of these contentious yeasts as an integral part of a red wine's complexity.

Brettanomyces yeasts are well adapted to survive during the winemaking process, largely due to their ethanol tolerance and relative resistance to the normal concentrations of sulfur dioxide found in wine (Licker *et al.*, 1998). Very little is known about the diversity of the strains of *Brettanomyces* yeasts that are present during winemaking. Therefore improved methods for the identification and genetic characterisation of strains are of utmost importance for the future. Molecular-based techniques that have been developed and investigated for both identification and interspecies differentiation include: PCR (Egli and Henick-Kling, 2001; Cocolin *et al.*, 2004), restriction fragment length polymorphism (PCR-RFLP) (Mitrakul *et al.* 1999), nested PCR (Ibeas *et al.*, 1996), PCR-DGGE (Renouf *et al.*, 2006) and RNA fluorescence *in situ* hybridisation (Stender *et al.*, 2001). Techniques that have been investigated for intraspecific discrimination included karyotyping, random amplified

polymorphic DNA (RAPD)-PCR, RFLP (Mitrakul *et al.*, 1999), PCR fingerprinting with microsatellite primers, intron splice site PCR (de Barros Lopes *et al.*, 1998), amplified fragment length polymorphism (AFLP) (de Barros Lopes *et al.*, 1999) and REA-PFGE (Miot-Sertier and Lonvaud-Funel, 2007).

Although the above-mentioned research has focussed on the genetic discrimination of *Brettanomyces* strains, very few studies included the evaluation of the strains for volatile phenol production in synthetic media and wine following their molecular discriminations. The objective of this study was to select different strains of *B. bruxellensis* that have been isolated from European red wines. For this purpose the molecular techniques of PCR-DGGE, ISS-PCR and REA-PFGE were used. Selected strains were evaluated for volatile phenol production in synthetic media and red wine by GC-MS.

4.2 MATERIALS AND METHODS

4.2.1 YEAST STRAINS AND GROWTH CONDITIONS

The strains of *Brettanomyces* used in this study are listed in Table 4.1. These were all from the FOEB laboratory culture collection (Faculté d'Oenologie de Bordeaux, France) and were originally isolated from various wines from France, South Africa and Spain, except for the reference strain from Belgian beer. Yeasts were isolated from wines with a modified WLN agar medium (Wallerstein Laboratory Nutrient media 60 g/L, sorbic acid 0.25 g/L, trehalose 5 g/L, *p*-coumaric acid 100 mg/L, agar 15 g/L, cycloheximide 30 mg/L and pH 5.5) and maintained on YPG agar medium (glucose 20 g/L, yeast extract 10 g/L, peptone 10 g/L, agar 25 g/L, pH adjusted to 4.8 with *o*-phosphoric acid). Incubation occurred over a period of 5 to 11 days at 30°C. All the isolates were identified as *Brettanomyces* with PCR and included in the FOEB laboratory culture collection.

4.2.2 IDENTIFICATION AND STRAIN DIFFERENTIATION

Strains were cultivated in 10 mL YPG broth for 5 days after which standard genomic DNA isolations followed as described in Sambrook *et al.* (1989). Strains were identified by means of polymerase chain reaction (PCR) using three different primer sets (ITS1/ITS4, DB1/DB2, DB90/DB394) in three separate reactions. The PCR method with the DB1/DB2 primers was performed according to Ibeas *et al.* (1996). The PCR method with the universal ITS1/ITS4 primers was performed according to Egli and Henick-Kling (2001). The PCR method with the DB90/DB394 was performed as described by Cocolin *et al.* (2004).

For strain differentiation, three molecular techniques were applied: PCR-DGGE, ISS-PCR and REA-PFGE. The details of all the primers used in this study are listed in Table 4.2. PCR-DGGE was performed on all the isolates (not all listed in Table 4.1) and the other techniques were performed on the selected isolates in Table 4.1.

TABLE 4.1.
List of *Brettanomyces* strains used in this study.

Species	FOEB no.	Sample ID	Isolated from	Source[a]
Brettanomyces bruxellensis	Clib300	Clib300	Belgian beer	CLIB
Brettanomyces bruxellensis	CB12	3983	Red wine	FOEB
Brettanomyces bruxellensis	CB3	26	Red wine	FOEB
Brettanomyces bruxellensis	CB9	32	Red wine	FOEB
Brettanomyces bruxellensis	CB11	35	Red wine	FOEB
Brettanomyces bruxellensis	CB19	L01A1	Red wine	ITV
Brettanomyces bruxellensis	CB23	L02C1	Red wine	ITV
Brettanomyces bruxellensis	CB28	L02H2	Red wine	ITV
Brettanomyces bruxellensis	CB38	L04G1	Red wine	ITV
Brettanomyces bruxellensis	CB20	L154	Red wine	ITV
Brettanomyces bruxellensis	CB21	L02A1	Red wine	ITV
Brettanomyces bruxellensis	CB29	L02I1	Red wine	ITV
Brettanomyces bruxellensis	CB40	L04J1	Red wine	ITV
Brettanomyces bruxellensis	CB27	L02G1	Red wine	ITV
Brettanomyces bruxellensis	CB63	D018	Red wine	CSIC
Brettanomyces bruxellensis	CB68	D2400	Red wine	CSIC
Brettanomyces bruxellensis	CB53	D008	Red wine	CSIC
Brettanomyces bruxellensis	SA82	SA82	Red wine	IWBT
Brettanomyces bruxellensis	SAA	SAA	Red wine	IWBT
Brettanomyces anomalus	SAF	SAF	Red wine	IWBT
Brettanomyces anomalus	CB52	D007	Red wine	CSIC
Brettanomyces anomalus	CB60	D015	Red wine	CSIC
Brettanomyces anomalus	CB61	D016	Red wine	CSIC

[a]CLIB, Collection de Levures d'Intérêt Biotechnologique, Paris-Grignon, France;

FOEB, Faculté d'Oenologie de Bordeaux, Bordeaux, France;

ITV, Institut Technique de la Vigne et du Vin, Beaune, France;

CSIC, Consejo Superior de Investigaciones Cientificas, Madrid, Spain;

IWBT, Institute for Wine Biotechnology, Stellenbosch University, South Africa.

TABLE 4.2.
List of primers used for the identification of *Brettanomyces/Dekkera* yeasts.

Primer	Sequence (5'- 3')	Species	Target
	Universal (White *et al.,* 1990)		
ITS1	TCCGTAGGTGAACCTGCGG	*Brettanomyces /*	partial ITS
ITS4	TCCTCCGCTTATTGATATGC	*Dekkera*	elements
	Brettanomyces specific (Cocolin *et al.,* 2004)		
DB90F	GAYACTAGAGAGAGRRGGARGGC	*B. anomalus*	26S rRNA
DB394R	ACGAGGAACGGGCCGCT	*B. bruxellensis*	
	Nested PCR (Ibeas *et al.,* 1996)		
DB1	AGAAGTTGAACGGCCGCATTTGCAC	*B. bruxellensis*	*RAD*4
DB2	AGGATTGTTGACACTCTCGCGGAGG		
	Intron splice site (de Barros Lopes *et al.*, 1996)		
EI1	CTGGCTTGGTGTATGT	*S. cerevisiae*	5' intron-exon splice site
	26S DGGE (Cocolin *et al.,* 2000)		
NL1gc	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA GGA AAA G	*S. cerevisiae*	26S rRNA
LS2	ATT CCC AAA CAA CTC GAC TC		

4.2.2.1 PCR-DGGE

The PCR-DGGE targeting a portion of the D1-D2 region of the 26S rRNA gene region was carried out on genomic DNA according to Cocolin *et al.* (2000). The primers used for the amplification were the NL1gc forward primer containing a GC-clamp (underlined) and the LS2 reverse primer (Table 4.2). The PCR reaction was performed in a final volume of 50 μL containing 0.5 μm of each primer; 4 μL of a custom made PCR-mix [comprising 10 mM Tris–HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 0.2 mg/mL BSA, 3.12% v/v glycerol, 1.5 mM MgCl$_2$, 200 μM each dNTP, and 0.1 U/μL Taq DNA-polymerase] was used (QBiogene, Illkirch, France) and 1 μL of gDNA (0.5 μg). The reactions were performed as follows: Initial denaturation at 95°C for 5 min, then 40 cycles of 95°C for 1 min, 52°C for 45 s and 72°C for 1 min. A final 7 min extension step at 72°C was used. The DGGE separation followed the PCR and was performed on a DCode™ Universal Mutation Detection System (Biorad, Hercules, CA, USA). Electrophoresis was performed in polyacrylamide gels using a 30 to 55% denaturing gradient. The gels were subjected to a constant voltage of 120 V for 4 h at a constant temperature of 60°C. After electrophoresis, gels were stained for 15 min in 300 mL TAE (1X) with 30 μL SYBR Green I (Molecular Probes, Eugene, OR, USA). The gels were photographed under UV transillumination.

4.2.2.2 ISS-PCR

In this study the EI1 primer (Table 4.2) was used singularly in a PCR experiment as previously described (de Barros Lopes *et al.* 1998). PCRs were performed in 50 μL of reaction mixture containing 50 pmol primer; 4 μL of a custom made PCR-mix (QBiogene) and 1 μL of gDNA (0.5 μg). The reactions were run for 35 cycles: denaturation at 94°C for 1 min, annealing at 45°C for 2 min and extension at 72°C for 1.5 min. An initial 3 min denaturation at 94°C and a final 5 min extension at 72°C were used. Products of each amplification reaction were resolved on a 1.5% agarose gel (Pharmacia Agarose NA) containing ethidium bromide and photographed under UV transillumination.

4.2.2.3 REA-PFGE

This method was performed as described by Miot-Sertier and Lonvaud-Funel (2007). Strains were cultivated in 2 mL YPG media for 5 days at 30ºC while shaking. The pelleted cells were washed twice with 50 mM EDTA (pH 8.0) and finally resuspended in 150 μL of the same solution. This cell suspension was mixed with 150 μL of 1% agarose (Chromosomal Grade Agarose, Bio-Rad) that was pre-melted and kept at 60ºC. Aliquots were made into moulds to prepare plugs and were kept for 30 min at room temperature. The agarose plugs were removed and placed in 2 mL lysis buffer (0.5 M NaCl, 0.25 M EDTA, 0.125 M Tris-HCl, β-mercaptoethanol and pH 7.5) for 18 h at 37ºC. Lysis buffer was replaced with a pronase buffer (1 mg/mL of Pronase E from *Streptomyces griseus*), 1% Sarcosyl, 0.45 M EDTA and 10 mM Tris-HCl) and

incubated for 72 h at 42ºC. After this, the plugs were washed twice in TE buffer (10 mM Tris-HCl, 1 mM EDTA and pH 8.0) for 30 min at 50ºC and twice again for 30 min at room temperature. At this step, the plugs could be stored in TE for some months at 4ºC. The plugs were digested with *Not*I restriction endonuclease (New England Biolabs) in a final volume of 120 µL for 18 h at 25ºC according to manufacturer specifications. The plugs were rinsed with TE buffer at room temperature before electrophoresis. Chromosomes and digested chromosomal DNA were separated by PFGE using the CHEF-DR® III System (Bio-Rad). Electrophoresis was performed at 10ºC in a 1% agarose gel (Pulse Field Certified Agarose, Bio-Rad) in 0.5X TBE buffer (0.1 M Tris, 0.09 M boric acid, 0.01 M EDTA and pH 8.0) at 6 V/cm: interpolation pulse time of 70 s for 15 h and 120 s for 11 h. Chromosomes from *S. cerevisiae* YNN295 (Bio-Rad) were used as size standard. The gel was stained with ethidium bromide for 15 min while agitating and destained in distilled H_2O for 4h at 4ºC prior to photography under UV transillumination.

4.2.3 VOLATILE PHENOL PRODUCTION

4.2.3.1 In synthetic media

Eight strains selected for volatile phenol production were cultivated in the media described by Dias *et al.* (2003b), with some modifications. The base medium was composed of YNB (Yeast Nitrogen Base) without amino acids 6.7 g/L (Difco), cas-amino acids 3.0 g/L, biotin 0.6 g/L, thiamine 1.6 g/L and hydroxycinnamic acid precursors 100 mg/L [*p*-coumaric acid (*p*-CA) or ferulic acid (FA)], or both together at 200 mg/L (Sigma)]. The base medium was evaluated with different carbon source compositions and precursor combinations, listed in Table 4.3. The capital letters (A, B or C) symbolise the carbon source type and the roman numbering (I, II or III) indicates the specific precursor added. The pH was adjusted to 3.6 using *o*-phosphoric acid. Cultures destined for cultivation in media with ethanol as sole carbon source were pre-conditioned first. This was performed by adding ethanol to the media in increments of 2% per day until the necessary ethanol concentrations were achieved. All media (100 mL of medium in 250 mL Erlenmeyer flasks) were inoculated from conditioned cultures. The flasks were inoculated with 100 µL of each culture at an optical density (OD_{600nm}) of ~1.0. These were allowed to grow at 25ºC for 7 days until late exponential/ beginning stationary phase before samples were taken for volatile phenol analysis.

TABLE 4.3.
Code usage explaining the carbon sources and precursors used.

Carbon source	Precursors (hydroxycinnamic acids)		
	I	II	III
	p-coumaric acid	ferulic acid	*p*-coumaric & ferulic acid
A 20 g/L glucose	AI	AII	AIII
B 2 g/L glucose & 8% ethanol	BI	BII	BIII
C 12% ethanol	CI	CII	CIII

4.2.3.2 In wine

Selected strains were evaluated for volatile phenol production in a Cabernet Sauvignon red wine with the following parameters: alcohol 12.5%; pH 3.72; residual sugar 1.1 g/L; free measurable precursors: *p*-CA 1656 µg/L, FA 114 µg/L; volatile phenols: 4-EP <10 µg/L (no 4-VP, 4-VG or 4-EG could be detected); stage of wine – after alcoholic fermentation (AF), before malolactic fermentation (MLF). The wine was sterilised by filtration (0.45 µm, Millipore). Strains were pre-conditioned for growth in the wine by starting with the YNB media A without substrates, followed by adjustments to the media:wine ratio from 9:1, 7:3, 1:1 and finally 3:7 over two day increments until the final media predominantly consisted of wine. Prior to inoculation into the wine, cells were centrifuged at 4000 rpm and re-suspended in YNB base media (without glucose) to an OD_{600nm} of ~0.6, after which 1 mL of each culture was inoculated into 80 mL of wine (200 mL Erlenmeyer flasks were used). To ensure the presence of the substrates, the wine was spiked with *p*-CA and FA at 100 mg/L each. Incubation occurred at $25^{o}C$ without agitation and samples were taken for volatile phenol analysis after 7 and 30 days, respectively.

4.2.4 CHEMICAL ANALYSIS BY GC-MS

Volatile phenol analysis was determined according to an adaptation of the method described by de Revel *et al.* (2005). Quantitative determination was done in SIM mode selecting ions of M/Z = 107, 122, 137, 152 for 4-VP, 4-VG, 4-EP (107, 122), 4-EG (122, 137, 152) and M/Z= 83 for the internal standard (dodecan-1-ol). Two millilitres of the internal standard of 40 mg/L dodecan-1-ol (Aldrich, St Quentin Fallavier, France) in hydro-alcoholic solution (50 % vol) were added to 50 mL of the reference solution (medium or wine) and extracted successively with 4 mL, 2 mL and again 2 mL of dichloromethane with 5 min stirring. The organic phases from the extractions were collected, mixed, and 2 µL of the extract was injected into an Agilent HP 5890 GC (Les Ulis, France) coupled with a mass spectrometer (HP 5972, electronic impact 70 eV).
The column employed was a Carbowax 20 M (50 m x 0.22 mm x 0.25 µm). Helium was the carrier gas (pressure: 70 kPa). The injector (split/splitless) was heated to $250^{o}C$ with a splitless time of 30 s and a split flow of 30 mL/min. Transfer line temperature was kept at $250^{o}C$. The oven temperature was increased after the injection from $40^{o}C$ up to $220^{o}C$ at a rate of $3^{o}C/min$ with a final step of 40 min. Standard compounds for 4-VG, 4-EG and 4-EP were purchased from Lancaster (Morecambe, UK) and 4-VP was purchased from Aldrich Chemicals.

4.3 RESULTS AND DISCUSSION

4.3.1 YEAST IDENTIFICATION TO THE SPECIES LEVEL

From a collection of 80 isolates, all were positively identified as yeasts belonging to the genus *Brettanomyces* with 77 isolates belonging to the species *B. bruxellensis* and three belonging to *B. anomalus*. The universal ITS1/ITS4 primers amplified the region spanning the 5.8S rRNA gene and partial areas of its flanking intergenic spacer regions ITS1 and ITS2 from all the isolates. In Figure 4.1A it can be seen that isolates belonging to the species *B. bruxellensis* yielded a PCR product of approximately 490 bp and isolates belonging to the species *B. anomalus* gave a product of approximately 550 bp. The DB1/DB2 primers successfully amplified a fragment of approximately 470 bp corresponding to *B. bruxellensis* (Fig. 4.1B). This primer set is complemented with another primer pair DB3/DB4 in a nested PCR reaction (Ibeas *et al.*, 1996) that allows for the detection of *B. bruxellensis* and *B. anomalus*. However, the first primer set DB1/DB2 could amplify DNA and detect *B. bruxellensis* yeasts, but not *B. anomalus*. The identification of *B. anomalus* CB52 was verified with the primer sets ITS1/4 and DB90/DB394. Finally, the primer set DB90/DB394 targeting the D1-D2 loop of the 26S rRNA gene successfully amplified a fragment of approximately 305 bp for both species of *B. bruxellensis* and *B. anomalus* (Fig. 4.1C).
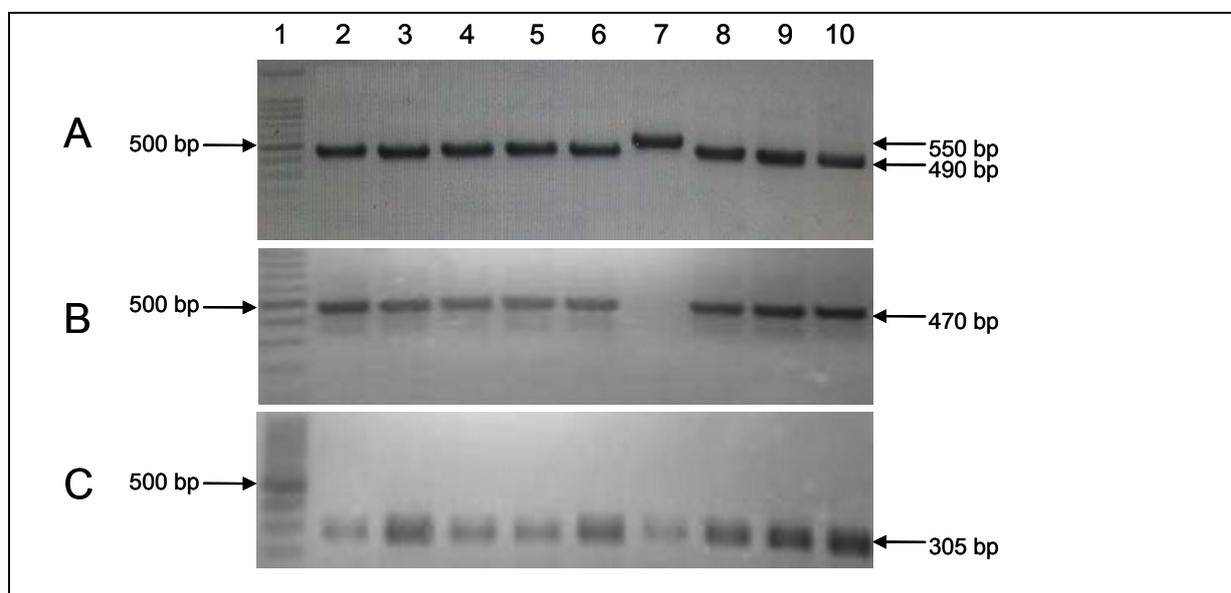
FIGURE 4.1
Identification of *Brettanomyces/Dekkera* isolates by means of the polymerase chain reaction. (A) PCR using the ITS1/ITS4 primer set. (B) PCR using the DB1/DB2 primer set. (C) PCR using the DB90F/DB394R primer set. Lanes: 1, 100 bp DNA Ladder (Marker XIV, Roche); 2, CB3; 3, CB12; 4, CB19; 5, CB28; 6, CB38; 7, CB52; 8, CB40; 9, CB53 and 10, CB68. Arrows on the right indicate the expected band size.

4.3.2 STRAIN DIFFERENTIATION

4.3.2.1 PCR-DGGE

DGGE of PCR-amplified rRNA genes has been described as a direct method to characterise the yeast diversity within wine fermentations (Cocolin *et al.*, 2000; 2001; 2004).

This study evaluated PCR-DGGE for possible application in interspecies and intraspecies discrimination of *Brettanomyces* wine isolates. This technique was performed on 80 isolates within the collection (only the strains shown in Figure 4.2 are listed in Table 4.1). The PCR with the NL1gc/LS2 primers successfully yielded the amplification product of approximately 250 bp for all isolates (data not shown) which served as the necessary template for DGGE analysis. From the DGGE analysis in Figure 4.2 four band profile groups were obtained, two for each species. Results showed that all isolates of *B. bruxellensis* exhibited either a single band or two bands. These formed group 'B' (for *bruxellensis*) and are grouped into B1 (single band) and B2 (two bands). Group 'A' (for *anomalus*) consisted of only single bands obtained with *B. anomalus* and are represented by A1 (single high band) and A2 (single low band) as indicated in Figure 4.2.
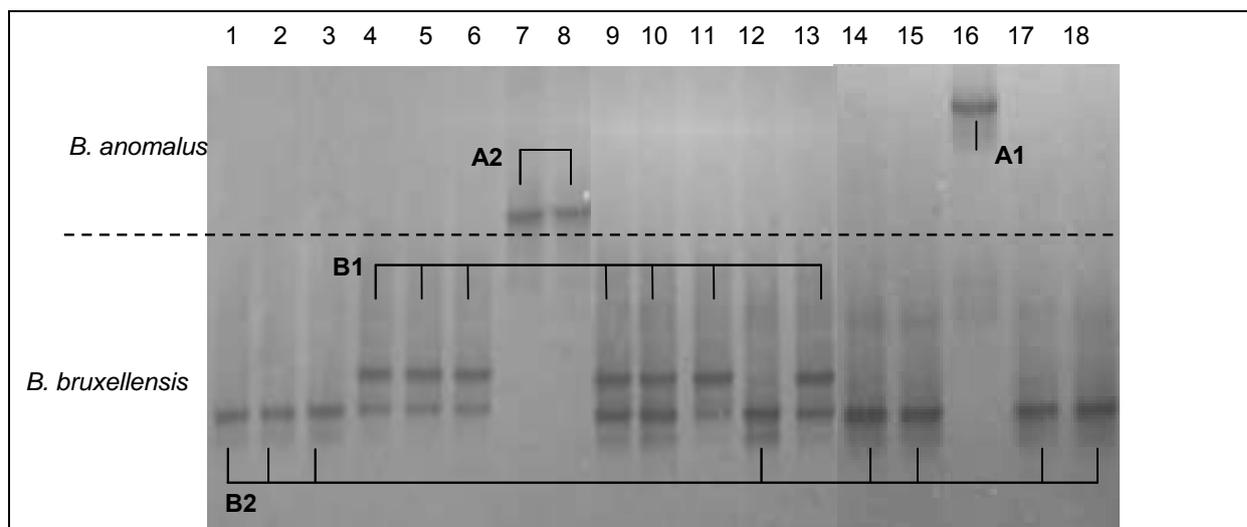

FIGURE 4.2
Grouping of wine isolates of *Brettanomyces/Dekkera* yeasts by means of PCR-DGGE analysis targeting the D1-D2 loop of the 26s rRNA region. Lanes: 1, CB9; 2, CB11; 3, CB12; 4, CB3; 5, CB19; 6, CB20; 7, CB61; 8, CB60; 9, CB21; 10, CB29; 11, CB40; 12, CB38; 13, CB63; 14, CB27; 15, CB28; 16, CB52; 17, CB53 and 18, CB68. The isolates are arranged into four groups: Two groups B1 and B2 belong to *Brettanomyces bruxellensis* and two groups A1 and A2 belong to *Brettanomyces anomalus.*

From the large group of 77 isolates of *B. bruxellensis,* only two different band profiles (B1 and B2) were seen for this dominant species of wine *Brettanomyces,* suggesting only two groups of strains. Likewise, the three isolates of *B. anomalus* showed two different profiles and suggested two possible groups for this species, but too few

isolates were studied. As the bands obtained with *B. anomalus* differed entirely from *B. bruxellensis* it was clear that PCR-DGGE could distinguish between the species. This approach however, did not prove useful for strain discrimination. The PCR-DGGE was evaluated several times and the specific isolates repeatedly gave the same band profile in each case. It was interesting to note that many strains of *B. bruxellensis* yielded two bands of similar intensity (grouping B1), although only single bands were expected. Repeatability of the results suggested that these strains possibly contained specific mutations in copies of the D1-D2 regions. Similar findings in *S. cerevisiae* have been mentioned (Divol *et al.*, 2006).

4.3.2.2 INTRON SPLICE SITE-PCR

A PCR-based method that uses oligonucleotide primers complementary to yeast intron splice sites has previously been used to permit species identification and intraspecies differentiation of *S. cerevisiae* strains (De Barros Lopes *et al.*, 1996; 1998).

In this study the PCR with the single EI1 primer targeting the yeast intron splice site was also evaluated on 14 randomly chosen *Brettanomyces* isolates for the purpose of strain differentiation. In Figure 4.3, although similar specific amplification products in the range of 600 to 1200 bp can be seen among the isolates, differences in specific bands higher than 1500 bp suggested polymorphisms. From the 14 isolates it was possible to distinguish seven groups with similar patterns indicated by the letters a to g. For example, in Figure 4.3 it can be seen that isolates CB3, CB20, CB29 and CB40 (group e) shared a similar banding profile. Isolates SAA, SA82 and CB12 (group b) and CB19, CB21 and CB23 (group f) also shared similar profiles. The other groups all had unique profiles.
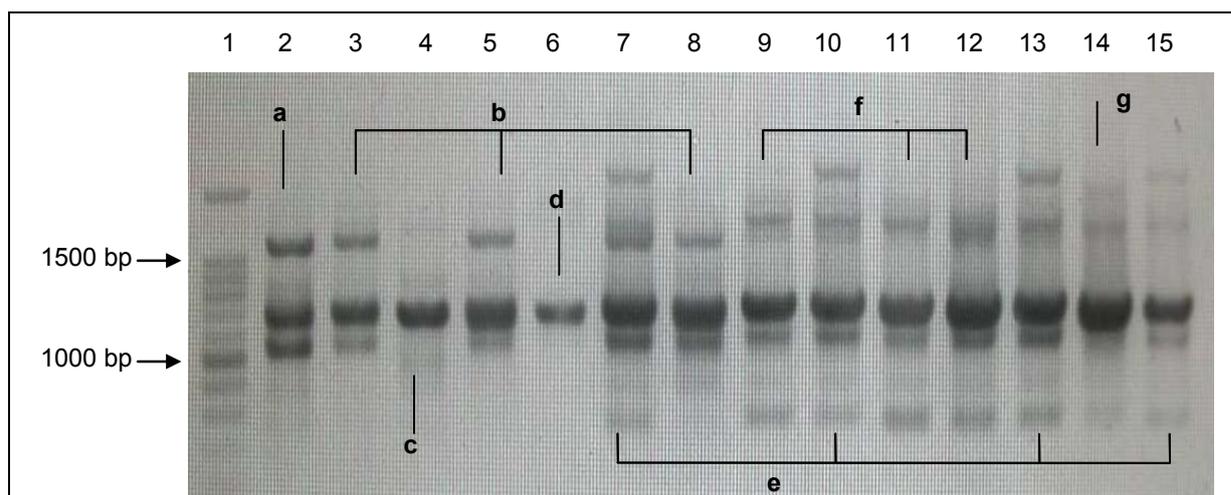
FIGURE 4.3
Differentiation of wine isolates of *Brettanomyces bruxellensis* isolates using PCR with intron primer EI1. Lanes: 1, 100 bp DNA ladder (Marker XIV, Roche); 2, Clib300; 3, SAA; 4, SAF; 5, SA82; 6, CB53; 7, CB3; 8, CB12; 9, CB19; 10, CB20; 11, CB21; 12, CB23; 13, CB29; 14, CB38 and 15, CB40. The isolates are arranged into groups with similar amplification fingerprints indicated by the letters a, b, c, d, e, f, g.

To determine the usefulness of this method for *B. bruxellensis* strain discrimination, tests were performed with randomly selected isolates from the collection. However, it was found that the reproducibility of this PCR method with genomic DNA was poor and it often yielded contradictory profiles. This was not a surprise as PCRs performed with a single primer are known to give results such as variation in amplicon length and band intensities, and occasional unspecific binding (Screaton *et al.*, 1993). It has been indicated that ISS-PCR has not yet revealed significant strain differences, but only species differentiation (De Barros Lopes *et al.*, 1996; 1998). Nevertheless, it was decided to evaluate this method as it was possible to apply to a larger collection of isolates of the same species than normally mentioned in literature.

4.3.2.3 REA-PFGE

Restriction analysis of total DNA was performed on strains selected from the combination of preliminary data. Prior to the restriction digestion with *Not*I, it was important to establish whether differences in the chromosome number occurred between selected wine strains of *Brettanomyces.* The molecular karyotype of all the *B. bruxellensis* isolates evaluated appeared to be similar with three large chromosomes being evident (Fig. 4.4). This corresponded to previous findings by Mitrakul *et al*. (1999) who showed that some wine isolates of *Brettanomyces* contained three large chromosomes (with approximate sizes of 2.2, 3.0 and 3.5 Mb). Ibeas *et al*. (1996) stated that their study had found four chromosomes from *Brettanomyces* yeasts isolated from sherry. The chromosomes from wine isolates differed when compared to reference strains from other origins, and it is known that yeasts belonging to the genus *Brettanomyces* contain between three and seven chromosomes (Mitrakul *et al*. 1999). In Figure 4.4 it can be seen that in all cases, the relative amount of DNA was higher in some bands than in others. Ibeas *et al*. (1996) explained this by showing that different chromosomes are similar in size or that certain chromosomes are present in higher numbers than others, reflecting an aneuploid condition.

The restriction profile obtained from the digestion of the three chromosomes with *Not*I indicated clear differences between wine isolates of yeasts belonging to the genus *Brettanomyces*. Figure 4.5 shows that each of the selected strains had a unique electrophoretic profile and that the major differences could be observed amongst the largest restriction fragments of between 825 kb and 2200 kb. Of all the molecular techniques evaluated in this study, this method allowed for the best discrimination of the strains. Other authors have found REA-PFGE to be very reliable and showed that this technique enabled the highest discrimination in comparison with techniques such as random-amplified polymorphic DNA, PCR fingerprinting with microsatellite oligonucleotide primers and SAU-PCR (Miot-Sertier and Lonvaud-Funel, 2007).

From the combined results obtained with REA-PFGE and the other techniques, eight different strains were selected and evaluated for volatile phenol production in synthetic media and wine.
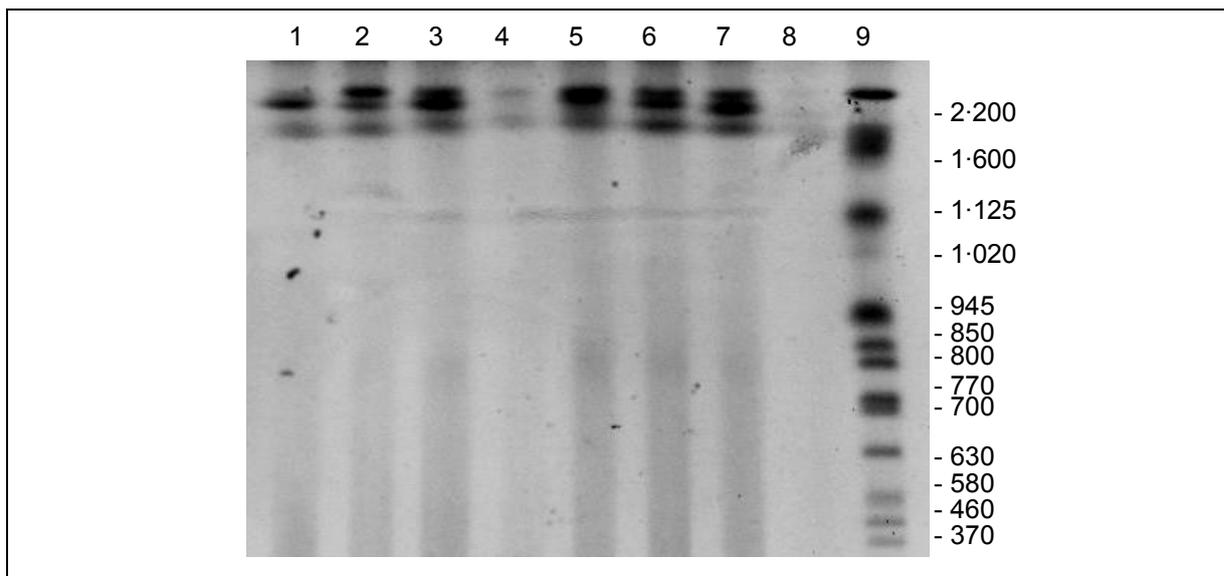
FIGURE 4.4
Chromosomal karyotyping of eight wine isolates of *Brettanomyces bruxellensis* yeasts using pulsed field gel electrophoresis. Lanes 1, CB3; 2, CB12; 3, CB19; 4, CB23; 5, CB28; 6, CB38; 7, CB63; 8, CB68 and 9, *Saccharomyces cerevisiae* YNN295.
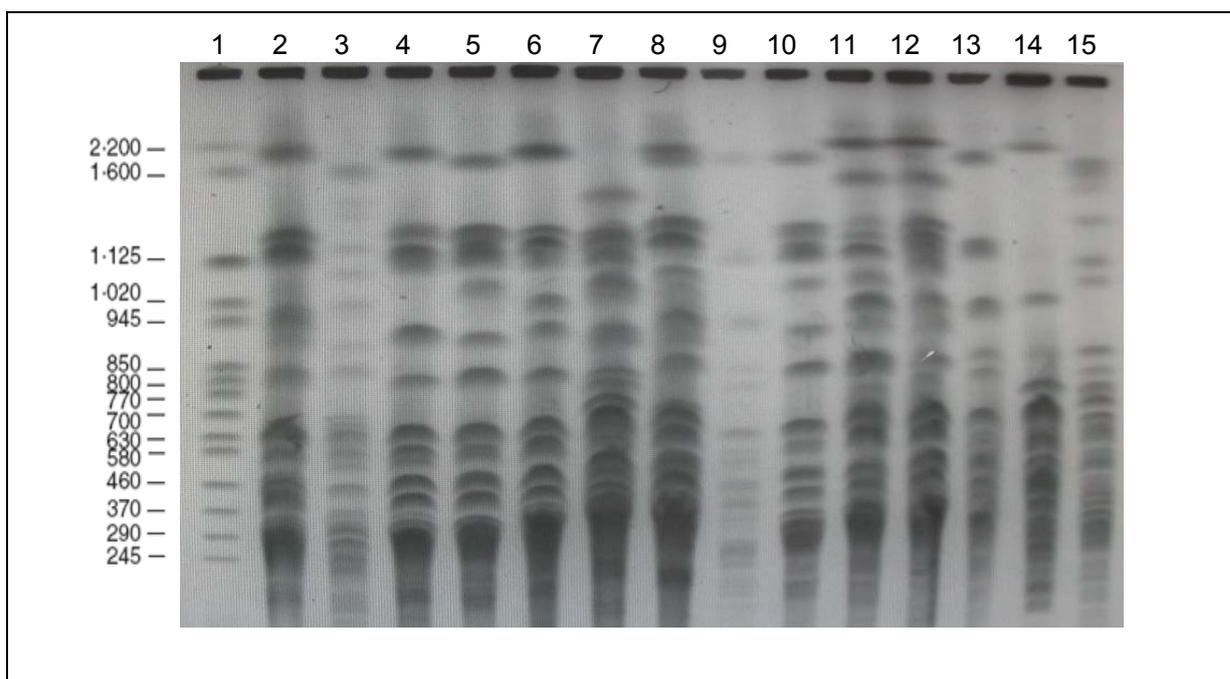
FIGURE 4.5
Pulse field gel electrophoresis of chromosomal DNA from *Brettanomyces bruxellensis* isolates after *Not*I digestion. Lanes: 1, *Saccharomyces cerevisiae* YNN295; 2, CB3; 3, CB12; 4, CB19; 5, CB20; 6, CB23; 7, CB28; 8, CB29; 9, CB30; 10, CB40; 11, CB53; 12, CB63; 13, Clib300; 14, SAF and 15, SA82.

4.3.3 PRODUCTION OF VOLATILE PHENOLS

4.3.3.1 In synthetic media

The eight strains of *B. bruxellensis* selected (strains CB3, CB12, CB19, CB23, CB28, CB38, CB63 and CB68) were first evaluated in the synthetic media containing *p*-CA as precursor. Successful cultivation was obtained in media AI (glucose 20 g/L) and BI (glucose 2 g/L and ethanol 8%), but not in CI (ethanol 12%). Even though the cultures were pre-conditioned for growth in ethanol, they could not be cultivated in a concentration of ethanol above 10%, even in the presence of glucose. This finding supported the data from Dias *et al.* (2003b) who showed that ethanol above 10% drastically influenced the growth of a *B. bruxellensis* strain. However, his study focussed on evaluating strain differences with regards to the production of volatile phenols and the other two media (AI and BI) suited this purpose.

In Figure 4.6 the growth curves and consequent production of the volatile phenols (4-VP and 4-EP) in medium AI and BI can be seen. In AI, the strains exhibited different growth rates with the average highest and lowest optical density ($OD_{600\,nm}$) being 6.7 (strain CB28) and 0.8 (strain CB12), respectively. Strains CB23, CB28 and CB63 had similar growth curves, as did strains CB19 and CB38, as well as strains CB3 and CB68. All the strains reached late exponential phase/beginning stationary phase after approximately 6 to 7 days. The capacity of all the strains to produce 4-EP became evident after the GC-MS analysis. Figure 4.6C clearly shows that all the strains were capable of producing large quantities of 4-EP in the ranges of 40 to 70 mg/L, despite the significant differences in biomass formation between the strains and also between the media AI and BI (Figs 4.6A and 4.6B). Although the concentrations of 4-EP after 7 days differed between the strains, none of them could be classified as weak producers. No correlation could be drawn between the final total biomass and volatile phenol production. For example, comparison of strains CB12 and CB38 revealed similar amounts of 4-EP produced (approximately 43 mg/L) although these strains had significant different growth curves. The OD_{600nm} measurement was ~0.7 and ~5.2 for CB12 and CB38, respectively, after 7 days. Also, the largest quantity of 70.1 mg/L of 4-EP was not produced by the strain (CB28) that reached the highest population but by strain CB68. This suggests that the complete *p*-coumarate transformation may have been achieved in the early growth phase so that no difference could be seen afterwards, irrespective of the final biomass. The maximum conversion from 100 mg/L *p*-CA acid to ~70 mg/L of 4-EP was in accordance with Dias *et al.* (2003b), who obtained similar values in the same glucose containing medium (AI). Additionally, the low levels of 4-VP measured in comparison to the large concentrations of 4-EP indicated that all strains contained an active vinyl phenol reductase enzyme responsible for the formation of 4-EP. The concentrations of 4-VP were too low to be visualised on the graph in Figure 4.6C.
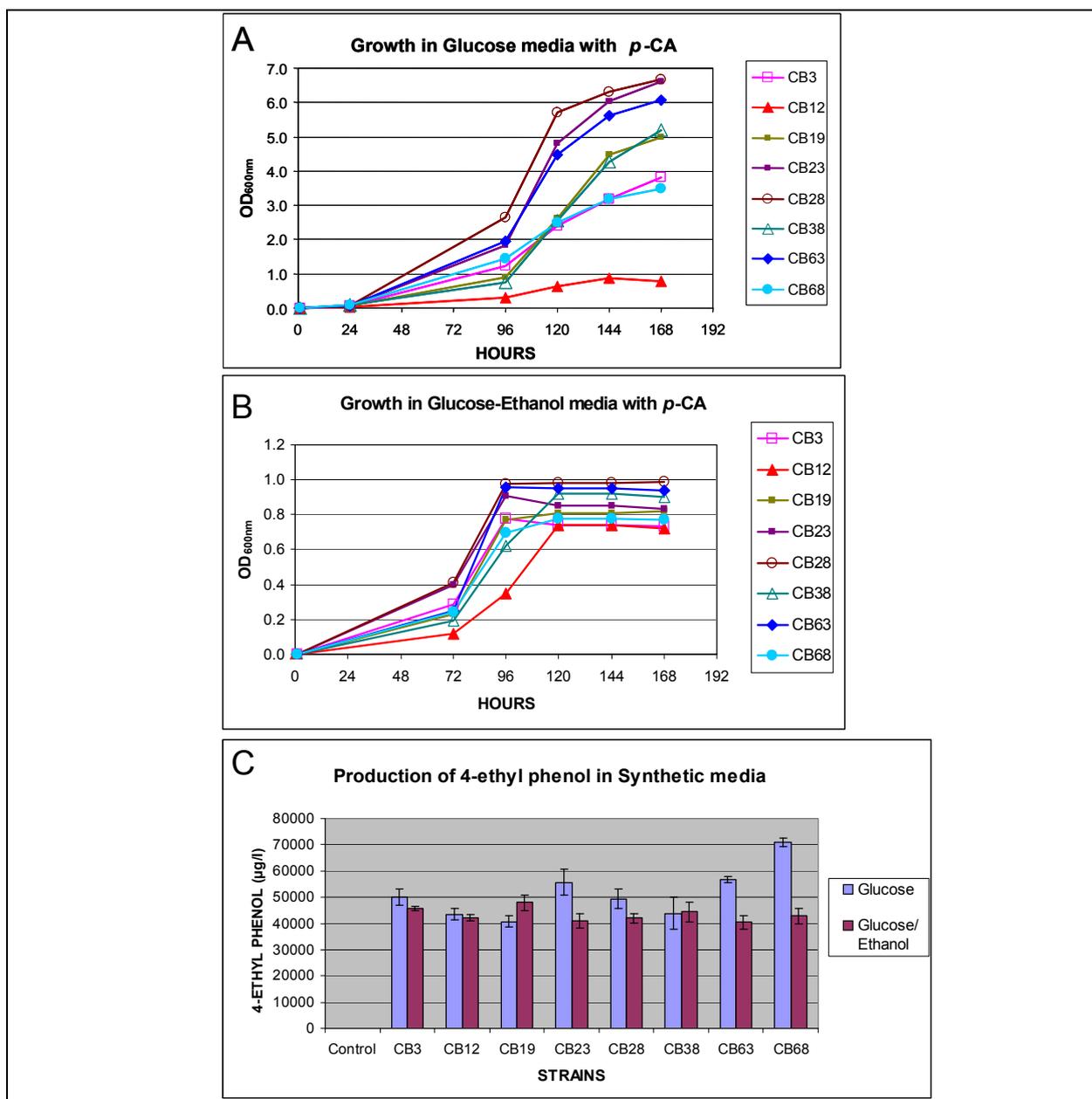
FIGURE 4.6
Evaluation of selected *Brettanomyces bruxellensis* strains in (A) glucose media AI and (B) glucose-ethanol BI media containing 100 mg/L *p*-CA. (C) Comparison of 4-ethyl phenol analysis after 7 days. Control was uninoculated media. Data represents average of duplicate experiments.

The evaluation of the strains in medium BI containing ethanol (Fig. 4.6B) yielded the same general tendency. In this instance, although all the strains showed poor biomass production (OD_{600nm} below 1.0 for all) due to glucose limitation and the presence of ethanol, all the strains were again capable of producing large quantities of 4-EP. However, it was interesting to observe that the differences in 4-EP between strains were less evident (Fig. 4.6C) than in the medium containing glucose as the only carbon source. The maximum values of 4-EP produced in the presence of 8% ethanol were lower and significantly more 4-VP remained (450 – 4417 µg/L). This result again agreed with Dias *et al.* (2003b) who showed that the maximum conversion rates of

p-CA to 4-EP was lower in the ethanol-containing media. The highest conversion rates (close to 90%) from *p*-CA to 4-EP were obtained in media with glucose as sole carbon source (Dias *et al.,* 2003b).

Comparison of the growth curves in the two different media (AI and BI) in Figures 4.6A and 4.6B showed that ethanol greatly influenced the growth rates under conditions of glucose limitation and the occurrence of an extended lag phase necessitated the use of pre-conditioned cultures. The cultures reached stationary phase much sooner in the ethanol containing media (day 4) as a result of weaker growth (lower maximum $OD_{600\ nm}$ <1.0). The shorter time period in which the 4-EP concentrations were obtained from weaker growing strains in the ethanol-containing media, reflects the risk involved with the survival of these yeasts, and thus possible volatile phenol production in wine. The tendencies of the different strains to produce 4-EP of similar concentrations were surprising because it was expected that more significant differences would be seen. This suggests that the total biomass needed to convert the 100 mg/L of *p*-CA in those conditions was reached by all the strains.

The evaluation of the strains for volatile phenol production from different precursors in synthetic media can be seen in Figures 4.7A, B and C. All the strains revealed similar growth patterns with *p*-CA and FA as individual substrates (Figures 4.7A and B), except strain CB19. In comparison with the other strains, CB19 exhibited weaker growth with each of the precursors, with the highest influence coming from ferulic acid that prevented growth almost completely. The combination of precursors at a final concentration of 200 mg/L (100 mg/L of each *p*-CA and FA) exerted a higher inhibitory affect on the growth and biomass formation of the yeasts and none of the strains could reach an OD_{600nm} of 1.0 (Fig. 4.7C). The production of volatile phenols from the different precursors revealed that the strains could produce 4-EG from FA equally as well or in similar concentration ranges (above 45 mg/L) as 4-EP from *p*-CA (Figs. 4.7D and E). This indicated that the *Brettanomyces*-derived enzymes involved in volatile phenol formation were functional towards both hydroxycinnamic acid precursors and their corresponding vinyl derivatives. This agreed with Edlin *et al.* (1995) who isolated a hydroxycinnamic acid decarboxylase from *B. anomalus* that is active toward ferulic acid, *p*-coumaric acid and caffeic acid.

In Figure 4.7D, it can be seen that the precursor for 4-EP, namely 4-VP was detected at higher concentrations in strains CB3 and CB68, which produced lower final concentrations of 4-EP. This result reflected the two consecutive reactions necessary for the production of the ethyl derivatives from the initial substrate and indicated the vinyl derivatives as an intermediate. It is likely that with prolonged incubation the strains would have converted most of the 4-VP also into 4-EP. As the samples were not analysed again, this could not be supported.
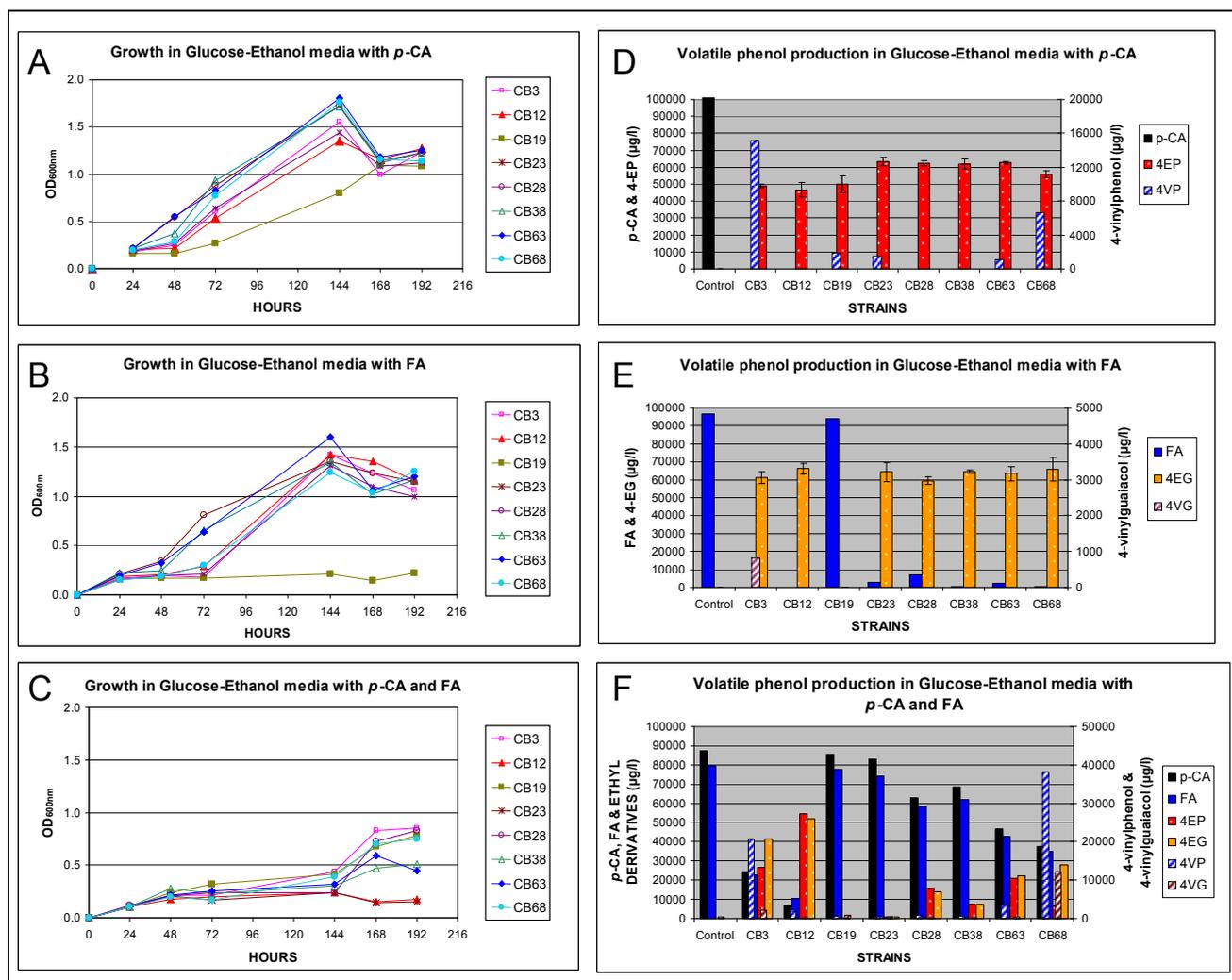
FIGURE 4.7
Evaluation of the growth and volatile phenol analysis of selected *Brettanomyces bruxellensis* strains in glucose-ethanol media containing different precursors: (A) growth with 100 mg/L *p*-coumaric acid, (B) growth with 100 mg/L ferulic acid, (C) growth with 200 mg/L of *p*-coumaric acid and ferulic acid together, (D) volatile phenol analysis from 100 mg/L *p*-coumaric acid, (E) volatile phenol analysis from 100 mg/L ferulic acid and (F) volatile phenol analysis from 200 mg/L of *p*-coumaric acid and ferulic acid together. Data represents average of duplicate experiments.

In Figure 4.7F, it is evident that the combination of both precursors at a total of 200 mg/L had an influence on the final levels of 4-EG and 4-EP produced. Strains CB19 and CB23 barely converted either of the precursors compared to the uninoculated control. All the other strains, however, produced 4-EP and 4-EG within 7 days despite the high concentrations of the precursors. The concentrations of the ethyl derivatives that were produced from the mixture of substrates (*p*-CA and FA) differed significantly between the strains and similar ranges higher than 45 mg/L for the respective ethyl forms, 4-EP and 4-EG, were not obtained as they were from the individual precursors. This could potentially be explained by two reasons. Firstly, both precursors together triggered a greater inhibitory effect on the yeast growth and consequently resulted in much lower levels of enzyme production or, secondly, the

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enzymes could be inhibited by high substrate concentrations. It is unclear whether hydroxycinnamic acids (*p*-CA and FA) have an influence on each other, as with competitive inhibitors, specifically regarding their uptake and conversion, when they occur at similar concentrations. This should be a focus for future studies.

4.3.3.2 In wine

The eight selected strains of *B. bruxellensis* successfully grew in the wine and aroma differences between the different induced assays were noticeable one week after the inoculations. Seven days after the initial *Brettanomyces* inoculations some differences and similarities were noticeable between the samples after an informal aroma assessment. These observations were later confirmed by the analytical data that was obtained. The wines inoculated with strains CB3, CB23 and CB68 showed a similar volatile phenol profile after this time period and were the closest to the control (unspoiled) wine. The analytical data in Table 4.4 and Figure 4.8A supported this result by revealing the lowest amounts of 4-EG and 4-EP (less than 100 µg/L) produced by these three strains after 7 days. Similarly, the wines inoculated with strains CB12, CB19, CB28 and CB63 showed the tendency to group together with a typical spicy, clove-like taint on the nose with sample CB63 being the most prominent. This corresponded with the expectation of significant levels of 4-EG as was confirmed by the GC-MS analysis with sample CB63 revealing the highest level of 4-EG in this group after 7 days (Fig. 4.8A and Table 4.4). The four samples CB12, CB19, CB28 and CB63 had average values of 148, 269, 264 and 390 µg/L 4-EG, respectively, which were above the detection threshold of 110 µg/L (Chatonnet *et al.*, 1995; Coulter *et al.*, 2003). The 4-EP values were very low and insignificant. Finally, sample CB38 completely distinguished itself from the rest with a typical 'Elastoplast/Band-aid and wet-dog' taint. The level of 4-EP at 1013 µg/L was surprising and a much higher level was expected considering the strong phenolic 'Elastoplast' aroma that this wine presented. Nevertheless, it was above the detection threshold of 425 µg/L, as stated by Chatonnet *et al.* (1995), and more generally regarded as a good example of a typically *Brettanomyces* infected wine. The spoilage potential of these yeasts, however, became evident by the fact that a large amount of 4-EG of close to ~15 mg/L was measured in this sample. Considering the capacity of these yeasts to produce large quantities of volatile ethyl derivatives from similar precursor concentrations in synthetic media, such high levels could be anticipated under wine conditions. The concern however, was that these levels were achieved only 7 days after inoculation and this showed the seriousness of a sudden *Brettanomyces* amplification under conditions favouring their growth. It was interesting to notice in Figure 4.8A that in all the samples, higher levels of 4-EG were produced as opposed to 4-EP. This suggested that the conversion pathway from FA as precursor was preferred in this instance. The specific chemical mechanism regarding the substrate affinity is not known at the moment. Two hypotheses might be applicable: Firstly, that FA is slightly more toxic for the yeast cells than *p*-CA, consequently resulting in a faster uptake and

conversion in an attempt to detoxify the environment (Larsson *et al.*, 2000). Secondly, that the added FA is less bound by the wine components and was therefore more freely available. This should be investigated more thoroughly in future.

TABLE 4.4.
Volatile phenols analysis of the inoculated wine after 7 and 30 days.

Samples	[#]4-ethylphenol (µg/l)		[#]4-ethylguaiacol (µg/l)		[#]pH
	7 days	30 days	7 days	30 days	30 days
Control	2	12	0	27	3.70
CB3	26	38	71	170	3.62
CB12	38	4738	148	19467	3.65
CB19	55	174	269	1164	3.56
CB23	26	11	34	24	3.54
CB28	78	5163	264	22098	3.35
CB38	1013	12526	14994	85219	3.42
CB63	89	125	390	573	3.58
CB68	28	42	38	60	3.40

[#]Values are averages of duplicate analysis
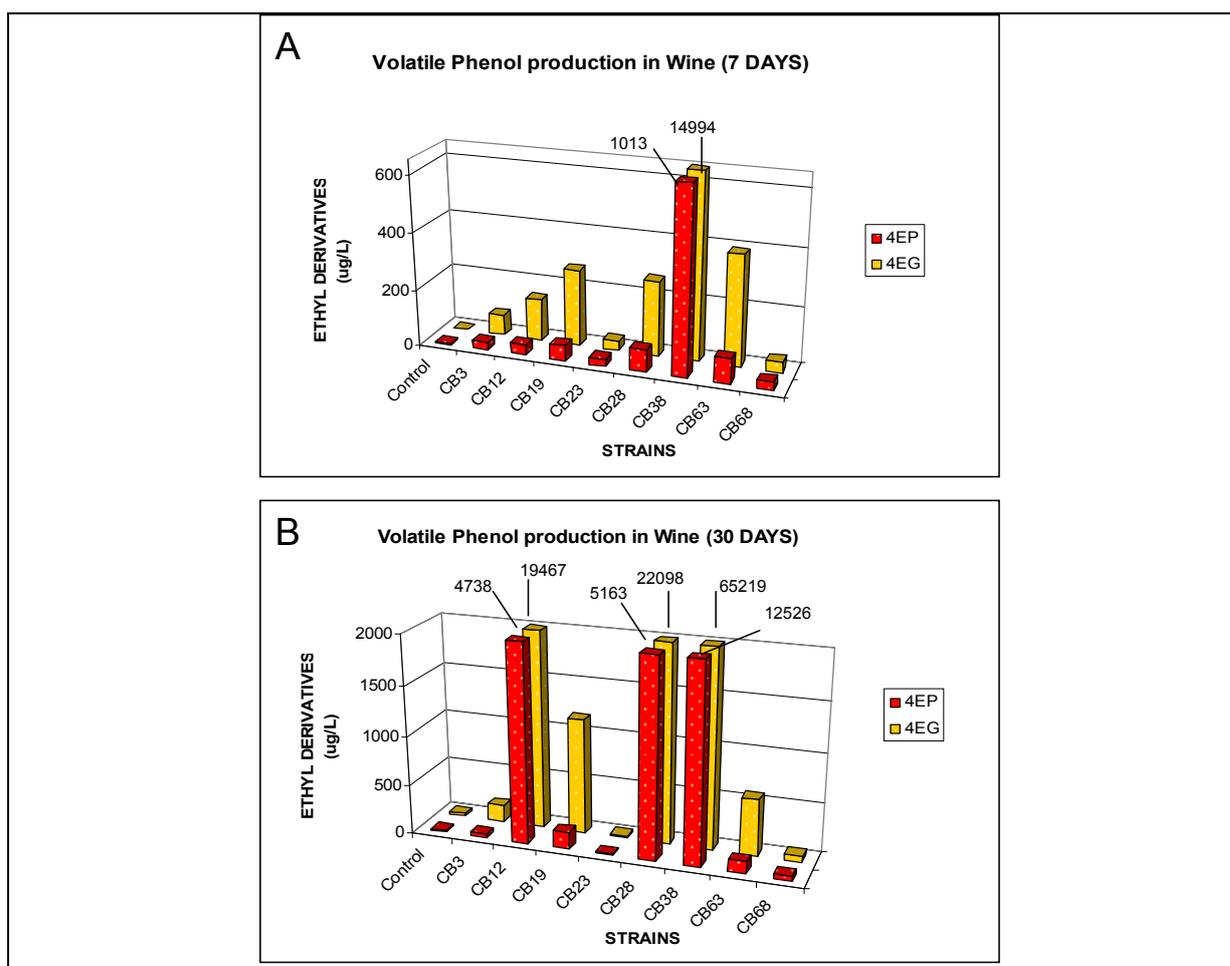
FIGURE 4.8
Wine analysis for volatile phenol after inoculation with selected *Brettanomyces bruxellensis* strains: (A) after 7 days; (B) after 30 days. Control was uninoculated wine.

The extended incubation up to 30 days showed increased levels of volatile phenols indicating continuous *Brettanomyces* activity in all the wine samples (Fig. 4.8B). The specific concentration values of 4-EG and 4-EP can be seen in Table 4.4. Similarly to the analysis after 7 days, samples 3, 23 and 68 were still the weaker group with regards to the levels of 4-EP and 4-EG produced, with no significant increases over the extended incubation time. The low amounts of ethyl derivatives produced by this group of *B. bruxellensis* strains would be regarded by many as favourable as these might positively contribute to the wine aroma profile. Wine samples 19 and 63 showed slightly increased levels of 4-EG after day 30 and now were completely separated from samples 12 and 28, which were previously in the same group according to volatile phenol concentrations. The latter group of samples (12 and 28) developed overwhelming odours of 'elastoplast' and 'barnyard' that corresponded to significant levels of 4-EP and 4-EG. With levels of 4-EP and 4-EG in the ranges of 4.7-5.1 mg/L and 19-20 mg/L respectively, it becomes too difficult to attribute specific characters describing the compounds as these far exceed threshold values and influence sensory perception. It was no surprise that the fruity character was completely distorted in these two wines. Wine sample 38 continued to stand out entirely from all the samples with quantities of 4-EG (~85 mg/L) and 4-EP (~12.5 mg/L).

The analytical data supported the general findings from the informal aroma observations and indicated that the aroma differences originated from the *B. bruxellensis* strains.

In addition to the differences obtained with the volatile phenol analysis, the pH-values were determined for all the wines after 30 days. The pH determinations followed after prominent odours of volatile acidity (VA) were detected among some wine samples. The pH-values between the wines differed significantly and ranged from 3.70 (control wine) to 3.35 (Table 4.4). The pH-values appeared to be completely independent from the degree of spoilage by volatile phenols. For example, wine sample 68 showed a pH decreased to 3.40 despite the fact that insignificant amounts of volatile phenols were produced. *Brettanomyces* yeasts are well known for the production of acetic acid, which contributes to the wine's VA and this could possibly explain the decreased pH levels (Heresztyn, 1986; Loureiro and Malfeito-Ferreira, 2003). However, neither the VA nor the ethanol concentrations of the wines were determined as these could have been indicative as to whether or not acetic acid production resulted from ethanol oxidation.

The precursor concentration of 100 mg/L used for *p*-CA and FA was far above the levels found in wine (2.9 – 19 mg/L) (De Beer *et al.*, 2002). The concentrations were selected in accordance with the evaluation in synthetic media as previously performed by Dias *et al.* (2003b). Even so, this concentration suited the purpose of strain evaluation and the initial tests showed that all the strains could produce high levels of both 4-EP and 4-EG. However, the combination of precursors (*p*-CA and FA) at a final concentration of 200 mg/L appeared to have an effect on the maximum levels

of volatile phenols produced in both the synthetic media and in wine. This effect is likely to be due to yeast growth inhibition resulting in an extended lag-phase or otherwise, saturation effect as the maximum conversion rate from the available enzymes involved, was obtained. It is worthy investigating if higher concentrations of hydroxycinnamic acids could induce a 'viable but non-culturable' state consequently resulting in low cell numbers followed by a residual enzymatic activity. Nevertheless, the higher concentration of hydroxycinnamic acids used in combination allowed more prominent discrimination between the strains with greater differences in the levels of ethyl derivatives produced being evident.

4.4 CONCLUSIONS

The results obtained from the work in this study indicated that diversity exists even among a limited number of *B. bruxellensis* strains. By combining molecular techniques that allowed for strain identification followed by the use of analytical techniques for the evaluation of the selected strains, it was possible to discriminate between the strains.

The most significant finding was that the tendencies observed regarding the volatile phenol production between the strains in synthetic media did not mimic the tendencies in the wine matrix. The behaviour of *Brettanomyces* strains in wine is poorly understood and therefore future studies should incorporate more wine matrices to obtain a better understanding of these contentious yeasts in such a complex medium. The influence of wine constituents, such as phenolic compounds, organic acids, etc., on *B. bruxellensis* growth and consequent volatile phenol production is not well understood and remains an area that needs further investigation. In particular, 4-EP production should be studied at the protein level including the transport and conversion of the substrates and their regulation according to environmental factors.

From the preliminary results presented in this chapter, it is evident that a study on the intraspecific diversity of *B. bruxellensis* species is needed. It should be based on a wider collection of strains typed by different molecular and analytic methods and grouped by both phylogenetic and phenotype analysis. From such studies, tools that could be used for risk prediction could be expected to be gained.

4.5 ACKNOWLEDGEMENTS

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Research Results

Differentiation of *Brettanomyces bruxellensis* strains isolated from red wines by infrared spectroscopy

5. RESEARCH RESULTS

Differentiation of *Brettanomyces bruxellensis* strains isolated from red wines by infrared spectroscopy

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ABSTRACT

The objective of this study was to evaluate different infrared spectroscopy methods in combination with chemometrics for the differentiation between *Brettanomyces bruxellensis* strains. These methods of discrimination were applied to intact yeast cells of *B. bruxellensis* strains and on wines spoiled by the same strains. Eleven wine isolates of *B. bruxellensis* were evaluated for volatile phenol production in red wine and their genetic diversity was determined by Restriction Endonuclease Analysis - Pulsed Field Gel Electrophoresis (REA-PFGE). Fourier transform mid-infrared (FTMIR) spectroscopy was used to obtain spectral fingerprints of the spoiled wines. Attenuated total reflectance (ATR) was used to obtain spectral fingerprints from the intact cells of the 11 *B. bruxellensis* strains. The groupings from the genetic fingerprints obtained with REA-PFGE were used as reference firstly for comparison with the groupings observed with the FTMIR spectral fingerprint of the wines and secondly for the FTIR-ATR spectral fingerprints from the whole cells. Results indicated that ATR-IR spectra obtained by scanning whole cells of *B. bruxellensis* could be useful for rapid strain typing in comparison or complementary to molecular techniques. In addition, FTMIR spectra from the wine samples allowed for the discrimination of the wines even though no differences could be seen with the standard wine parameters that were tested.

Keywords: *Brettanomyces, Dekkera, red wine, spoilage, volatile phenols, discrimination, FTMIR, ATR, spectral fingerprints.*

Abbreviations
p-**CA** *p*-coumaric acid, **FA** ferulic acid, **4-EP** 4-ethylphenol, **4-EG** 4-ethylguaiacol, **4-VP** 4-vinylphenol, **4-VG** 4-vinylguaiacol, **MVDA** multivariate data analysis, **FTMIR** fourier transform mid-infrared, **ATR** attenuated total reflectance, **REA-PFGE** restriction endonuclease analysis - pulsed field gel electrophoresis

5.1 INTRODUCTION

Under poorly controlled winemaking conditions, a large variety of spoilage yeasts can produce unfavourable compounds that could potentially decrease wine quality (Loureiro and Malfeito-Ferreira, 2003). One such group of spoilage yeasts belongs to the genus *Brettanomyces* (or *Dekkera* the sporogenous teleomorph). These species are highly adapted to proliferate in red wine and can have detrimental effects on the organoleptic quality of wines. Amongst a range of spoilage compounds produced by this genus (Fugelsang *et al.,* 1993; Aguilar-Uscanga *et al.,* 2003; Mansfield *et al.,* 2002; Snowdon *et al.,* 2006), the formation of volatile phenols, specifically 4-ethylphenol and 4-ethylguaiacol, is seen as the most important indicator of *Brettanomyces* activity in red wine (Chatonnet *et al.,* 1992, 1995, 1997; Joseph and Bisson, 2004; Loureiro and Malfeito-Ferreira, 2003). The concentrations of volatile phenols vary in wine and the phenolic off-flavours perceived are typically described by terms such as 'medicinal', 'barnyard-like', 'inky', 'sweaty leather' and 'Band-aid' (Chatonnet *et al.,* 1992; Rodrigues *et al.,* 2001). These yeasts are particularly well adapted to survive the winemaking process due to their relative resistance to the sulfur dioxide (SO_2) concentrations normally used in wine, superior ethanol tolerance and growth in nitrogen limited conditions (Licker *et al.,* 1998; Woolfit *et al.,* 2007).

Recent years have seen higher incidences of *Brettanomyces* contaminated wines and consequently numerous identification methods for this slow-growing yeast followed (Ibeas *et al.,* 1996; Mitrakul *et al.*, 1999; Egli and Henick-Kling, 2001; Cocolin *et al.,* 2004; Renouf *et al.,* 2006). With the molecular identification techniques that allow for inter- and intraspecies identification of *Brettanomyces* (Curtin *et al.*, 2005; Miot-Sertier and Lonvaud-Funel, 2007), it became evident that more information is required regarding the diversity of *Brettanomyces* strains that occur during winemaking.

Preliminary studies focusing on strain diversity regarding volatile phenol production by different strains of *B. bruxellensis* isolated from South African red wines indicated that an alternative, rapid and reliable approach to strain differentiation was needed. In order to detect possible variation in standard wine parameters of wines spoiled by isolates of *B. bruxellensis*, Fourier transform infrared (FTIR) spectroscopy, which facilitates the quantification of important wine parameters, was applied. FTIR spectroscopy relies on the principle of detecting molecular vibrational frequencies in the mid infrared (MIR) region of the electromagnetic spectrum. Each organic molecule has a characteristic frequency in the mid infrared region. The region 929 cm^{-1} to 1600 cm^{-1} captures a substantial amount of characteristic chemical information and is referred to as the 'fingerprint' region (Smith, 1999). Since the MIR absorbance spectrum contains information about the chemical composition of a substance, calibrations to quantify specific individual components have to be developed for each. The global calibrations for the FTMIR currently include the standard wine parameters (e.g. pH, VA, ethanol, malic

acid, lactic acid etc.) as quantifiable compounds. In addition to the quantified wine parameters obtained from such spectral analysis, FTMIR spectra produced from scanning a wine could also serve as a spectral fingerprint of a wine at a given time. This has already been used as a rapid screening tool of the fermentation profiles of wine yeasts and shown to differentiate between wines made with different commercial *S. cerevisiae* strains (Nieuwoudt *et al.*, 2006; Osborne, 2007). Additionally, FTIR spectroscopy has also been investigated as a tool for rapid differentiation of bacterial and yeast strains (Naumann *et al.*, 1991; Wenning *et al.*, 2002; Ngo-Thi *et al.*, 2003; Essendoubi *et al.*, 2005).

The primary goal of this study was to evaluate the potential of infrared spectroscopy as a discriminating tool for *B. bruxellensis* wine isolates. The first objective was to investigate FTIR transmission spectroscopy on spoiled wines from 11 *Brettanomyces* isolates. The second objective was to determine if FTIR-attenuated total reflectance (FTIR-ATR) when applied to whole cells could discriminate between the *Brettanomyces* isolates. Multivariate data analysis was applied on all generated data.

5.2 MATERIALS AND METHODS

5.2.1 YEAST STRAINS AND MAINTENANCE

The *B. bruxellensis* strains used in this study are listed in Table 5.1. These were all from the IWBT laboratory culture collection (Institute for Wine Biotechnology, Stellenbosch University, South Africa) and were isolated from various wines from the Stellenbosch wine-producing region. Yeasts were maintained on YPD agar medium (glucose 20 g/L, yeast extract 10 g/L, peptone 10 g/L, agar 25 g/L) containing cycloheximide 50 mg/L (Sigma-Aldrich, Steinheim, Germany), chloramphenicol 30 mg/L (Roche, Mannheim, Germany) and kanamycin 30 mg/L (Roche). Incubation occurred over a period of 5-11 days at 30ºC.

TABLE 5.1.
List of *Brettanomyces/Dekkera* strains used in this study.

Strain	Species	Sample ID	Isolated from	Source
1	*Brettanomyces bruxellensis*	SA1	Red wine	IWBT
2	*Brettanomyces bruxellensis*	SA29	Red wine	IWBT
3	*Brettanomyces bruxellensis*	SA33	Red wine	IWBT
4	*Brettanomyces bruxellensis*	SA35	Red wine	IWBT
5	*Brettanomyces bruxellensis*	SA36	Red wine	IWBT
6	*Brettanomyces bruxellensis*	SA39	Red wine	IWBT
7	*Brettanomyces bruxellensis*	SA45	Red wine	IWBT
8	*Brettanomyces bruxellensis*	SA47	Red wine	IWBT
9	*Brettanomyces bruxellensis*	SA59	Red wine	IWBT
10	*Brettanomyces bruxellensis*	SA60	Red wine	IWBT
11	*Brettanomyces bruxellensis*	SA61	Red wine	IWBT

IWBT, Institute for Wine Biotechnology, Stellenbosch University, South Africa.

5.2.2 STRAIN IDENTIFICATION AND MOLECULAR DIFFERENTIATION

Two molecular techniques were applied to differentiate the strains among the 11 isolates of *Brettanomyces*, namely Pulsed Field Gel Electrophoresis (PFGE) and Restriction Endonuclease Analysis - Pulsed Field Gel Electrophoresis (REA-PFGE). The latter method was performed as previously described by Miot-Sertier and Lonvaud-Funel (2007).

5.2.3 SMALL-SCALE WINE FERMENTATIONS

All strains used in this study (Table 5.1) were evaluated for volatile phenol production in a Shiraz red wine. The wine was sampled after alcoholic fermentation (AF). Prior to inoculation, the wine was pre-treated. The natural microbial flora was inhibited with the addition of 200 mg/L dimethyldicarbonate (DMDC, Velcorin®) where after the wine was kept at 8°C for one week before filtration (0.45-µm filters; Millipore, USA). The wine was additionally diluted with sterile distilled water to lower the final ethanol concentration from 14.5% to approximately 10%. Standard wine analyses were done on the wine before inoculation with the various *B. bruxellensis* isolates (Table 5.2). The concentrations of volatile phenols of the wine are also given in Table 5.2. The free measurable hydroxycinnamic acid precursors were measured at 1.6 mg/L and 1.7 mg/L for *p*-coumaric acid (*p*-CA) and ferulic acid (FA), respectively. The *p*-CA concentration was adjusted by spiking 10 mg/L *p*-CA to the wine. All *Brettanomyces* strains were pre-cultured in YPD media containing 10 mg/L *p*-CA for 5 days at 30°C. Wines were inoculated with ~10^4 cells/mL in 100 mL of wine and were incubated (without agitation) at 30°C. Samples were drawn for microbial analysis after 7, 14 and 21 days, respectively. Analysis of volatile phenols and standard wine parameters were performed at day 0 and day 21. Experiments were done in triplicate.

TABLE 5.2.
Standard wine measurements and volatile phenol concentrations of the wine before inoculation with *Brettanomyces bruxellensis* isolates.

Standard parameters		unit	Volatile phenols		unit
pH	3.69		4-VP	327	µg/L
Volatile acidity	0.43	g/L	4-VG	nd	
Total acidity	4.54	g/L	4-EP	nd	
Malic acid	0.07	g/L	4-EG	nd	
Lactic acid	0.56	g/L			
Residual sugar	0.60	g/L			
Ethanol	9.79	%(v/v)			
Glycerol	10.42	g/L			

nd – not detected

5.2.3.1 FTMIR spectral analyses of the wine

FTMIR (transmission) spectra were generated by using a Winescan FT120 instrument (software version 2.2.1) equipped with a purpose built Michelson interferometer (FOSS Electric A/S, Hillerød, Denmark). Wine samples were first centrifuged and then filtered by vacuum pump prior to analysis. Duplicate spectra were acquired in the spectral range of 4992.25 to 929.778 cm^{-1} for each sample and the spectra were averaged for data processing. Samples were preheated to 40°C in a heater block before analysis. Each spectrum is based on an average of 20 repeat scans at 4 cm^{-1} intervals under fixed instrument settings as described by the supplier of the spectrometer (Winescan FT120 Type 77110 and 77310 Reference manual, FOSS Electric, Denmark, 2001). The background absorbance of water was corrected by doing a zero setting using the FOSS Zero liquid S-6060 prior to the sample and so obtaining a zero-beam spectrum. FTIR data are recorded as absorbencies.

5.2.3.2 Standard wine analysis

Quantified chemical data were obtained from the FTMIR analysis to indicate the distribution of standard wine measurements (Table 5.3). These components are important to monitor as they can indicate major variation between samples originating from the different *Brettanomyces* strains. The conversion of the linearised absorbance spectra into quantifiable results was achieved with the commercially available calibrations referred to as global calibrations (provided with the Winescan FT120 instrument). These global calibrations are constructed on the basis of a partial least squares (PLS) regression. Absorbance at selected wavenumbers (cm^{-1}) or groups of wavenumbers are used to generate a regression algorithm which best fit the reference values in the data set (Patz *et al.*, 2004).

TABLE 5.3.
Standard wine measurements obtained from FTMIR analysis.

Chemical analysis	
pH	Ethanol
Total acidity (TA)	Acetic acid
Malic acid	Volatile acidity (VA)
Glycerol	Lactic acid
Residual sugar (RS)	

5.2.3.3 Volatile phenol analysis

The method used for volatile phenol analysis entailed sample preparation by means of ether extraction followed by GC-FID (Gas Chromatography – Flame Ionisation Detection) analysis. A volume of 50 µL of the internal standard (200 mg/L 3,4-dimethylphenol in absolute ethanol) was added to 15 mL of the sample and extracted with 2 mL of diethyl ether (BDH, Lancashire, UK) for 30 min on a rotary mixer. A volume of 2 µL of the organic phase was injected into a Hewlett Packard HP

5890 Series II Gas Chromatograph (Avondale, PA, USA) fitted with a flame ionisation detector (FID). The column employed was a HP-INNO Wax (60 m x 0.25 mm x 0.5 μm). The carrier gas was hydrogen (pressure: 115 kPa). The injector (split/splitless) was heated to 250°C with a split ratio of 1:20. The oven temperature was held at 50°C for 2 min, increased to 150°C at a rate of 10°C/min, where after the temperature was increased to 160°C at a rate of 5°C/min. A final ramp up to 220°C was performed at which the temperature was held for 10 min. Volatile phenols were determined quantitatively after a calibration had been set up using standard compounds and the Chemstation chromatography software package (Agilent Technologies, Wilmington, USA). The standard compounds 3, 4-dimethylphenol, 4-ethylguaiacol, 4-ethylphenol and 4-vinylphenol were purchased from Sigma-Aldrich (Steinheim, Germany). The 4-vinylguaiacol standard was purchased from Lancaster Synthesis (Lancashire, UK).

5.2.4 FTIR-ATR SPECTROSCOPY

The cell biomass was removed directly from the YPD media with the use of a sterile tip and presented to the FTIR-ATR instrument for scanning. Infrared analysis of the introduced biomass was performed on a Nexus 670 FTIR apparatus containing the Smart Golden Gate Single Reflection Diamond ATR sampling accessory (Thermo Electron, San Jose, CA, USA). The accessory contained a type IIa diamond crystal. The spectrometer equipped with a Ge-on-KBr beamsplitter and DTGS/CsI detector, was continuously purged with UHP nitrogen gas from AFROX (Cape Town, South Africa). The Smart Golden Gate accessory requires minimal sample preparation and only necessitate that the sample must be in contact with the diamond under uniform pressure. The latter is achieved by a self-levelling sapphire anvil that can exert pressures up to 17.6 kg/cm^2. Each sample scan was an average of a composite of 32 scans after being fourier-transformed. Spectra were collected in the mid-infrared range (4000-650 cm^{-1}) at a resolution of 4 cm^{-1}. Residual CO_2 or water absorptions were removed by spectral subtraction with the OMNIC spectroscopy software (Thermo Electron). Five independently cultured repeats of each strain were performed and served as technical repeats.

5.2.5 MULTIVARIATE DATA ANALYSIS (MVDA)

Principal component analysis (PCA) and partial least squares discrimination (PLS2) were performed with *The Unscrambler* 9.2 software (Camo, 2005) on the FTIR spectra of the spoiled wines and whole yeast cells. The FTIR-ATR spectra were subjected to pre-processing by means of second order derivatives (Savitsky-Golay algorithm) prior to multivariate data analysis (Savitzky and Golay, 1964).

5.2.5.1 Principal component analysis (PCA)

PCA was used to extract information from multivariate data (Kettanah *et al.*, 2005) and summarise the data (Wold, 1982; Wold *et al.*, 1987). PCA is a multivariate

technique that reduces the dimensionality of the original data matrix containing numerous variables to a more visually understandable model while retaining the maximum amount of variation in the data set. This permits an overview of the data structure by revealing relationships between the objects, as well as the detection of deviating objects. This mathematical procedure resolves sets of data into orthogonal components of which linear combinations approximate the original data to any desired degree of accuracy (Adams, 1995; Otto, 1999; Martens and Martens, 2001; Naes *et al.*, 2002). These models therefore enable the identification of possible groupings of samples (Mardia *et al.*, 1979) with similarities and relate these groupings to specific variables or groups of variables (Wold *et al.*, 1982; 2001; Martens and Naes, 1989; Tenenhaus, 1998; Wold and Josefson, 2000).

5.2.5.2 Discriminant partial least squares regression (PLS-Discrim)

Discriminant analysis was performed by developing PLS-Discrim models using a non-metric 'dummy' Y-variable as a reference value. The 'dummy' variable is an arbitrary number for a sample which belongs to a particular group. PLS2-discriminant analysis was used for discriminating between the different yeast strains. A 'dummy' Y-variable is created representing each of the yeast strains used. A value of +1 is assigned to a sample when it belongs to a specific yeast class and -1 if it does not belong to the specific yeast class.

The PLS-discrim model is then developed by regression of the spectral data (X-variables/matrix) against the assigned reference value (dummy variable). This approach is referred to as a discriminant PLS (DPLS or PLS-Discrim) (Osborne *et al.*, 1993; Ding *et al.*, 1999; Naes *et al.*, 2002) and has wide application (Cozzolino *et al.*, 2002, 2003).

The second derivatives of the original ATR-IR spectra were calculated by using the Savitzky-Golay algorithm (Savitzky and Golay, 1964) with five left and right data points and a third-order polynomial fit. The resulting PLS2-discrimination models were used to analyse the data structure. The FTIR-ATR spectra of a biological independent repeat was used as a cross validation of the calibration set to give an indication of the usefulness of any future prediction type models.

Data analysis on spectra from the FTIR-ATR work was done after an initial survey of data of the full spectral range (650 to 4000 cm^{-1}). Hereafter, a reduced spectral region (720 to 1440 cm^{-1}) was selected as this included the mixed region, the polysaccharide region and the 'true' chemical 'fingerprint region' (Naumann *et al.*, 1991). Two water absorbance regions, 1582 to 1698 cm^{-1} and 2971 to 3627 cm^{-1} were deselected along with the region from 3627 to 4992 cm^{-1} as this contains very little information as it is close to the near infrared region (Naumann *et al.*, 1991).

PLS2-discriminant analysis was also used with the FTMIR-transmission data where different *Brettanomyces* isolates were evaluated for volatile phenol production in wine. More detail regarding the description of PCA and PLS-regression can be found in chemometric textbooks (Martens and Naes, 1989; Esbensen, 2002).

5.3 RESULTS AND DISCUSSION

5.3.1 STRAIN DIFFERENTIATION BY MOLECULAR FINGERPRINTING

All isolates were positively identified as strains belonging to the species *B. bruxellensis* by means of PCR prior to molecular fingerprinting techniques (see section 3.2.6.2). The intraspecies identification of the *B. bruxellensis* isolates by a molecular technique was necessary for comparison of strain-specific discrimination by FTIR spectroscopy.

5.3.1.1 PFGE

PFGE was used to determine the number and size of chromosomal DNA bands for the selected *Brettanomyces* isolates. The chromosome banding patterns gave a preliminary indication of the genetic diversity that existed among the selected yeast isolates. PFGE analysis showed that all the isolates of *B. bruxellensis* evaluated contained either three or four large chromosomal DNA bands of varying sizes (Fig. 5.1A). Other authors have had similar findings and showed that yeast of the genus *Brettanomyces/Dekkera* isolated from a winemaking environment contained three to four large chromosomes (Ibeas *et al.*, 1996; Mitrakul *et al.*, 1999.) In Figure 5.1A, four chromosome bands can be seen for isolates 2, 3, 8 and 9 while the chromosome banding pattern of isolate 3 is different. Similarly, isolates 1, 4, 5, 6, 7, 10 and 11 showed three chromosome bands while isolates 1 and 5 appeared to have different banding patterns. In addition to the chromosome length polymorphism (CLP) that was observed among the isolates, the relative amount of DNA was also higher in some bands. A similar observation by Ibeas *et al.* (1996) attributed to the fact that some chromosomes of similar lengths could increase the intensity of the DNA bands on the gel and to the fact that certain chromosomes could be present in higher numbers than others, reflecting an aneuploid condition. Therefore, the number of chromosomal DNA bands on a gel does not necessarily correlate with the actual number of chromosomes. Thus, it might well be that the *B. bruxellensis* isolates analysed in this study contain more than three or four chromosomes. Despite this uncertainty, the chromosome banding patterns (the number and size of the gel bands) suggested four possible strain groups among the 11 isolates.

5.3.1.2 REA-PFGE

Restriction patterns obtained after chromosomal DNA digestion with *Not*I showed clear differences among the *B. bruxellensis* wine isolates (Fig. 5.1B). A unique electrophoretic fingerprint was observed for each of the 11 isolates, suggesting each isolate represents a different strain of *B. bruxellensis*. The largest variation was for restriction fragments between 825 and 2200 kb and this gave the clearest distinctions. Previous authors have found REA-PFGE to be very reliable and also that this technique allowed higher discrimination in comparison with techniques such

as random-amplified polymorphic DNA, PCR fingerprinting with microsatellite oligonucleotide primers and the SAU-PCR method (DNA is first digested with the *Sau*3A restriction endonuclease and then amplified with a primer designed on the restriction site) (Miot-Sertier and Lonvaud-Funel, 2007).
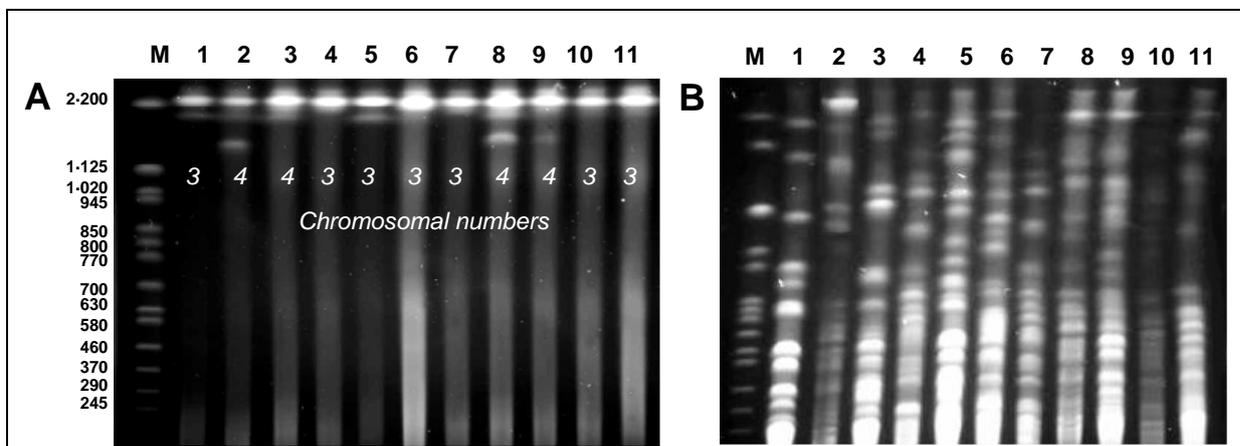

FIGURE 5.1.
Differentiation of 11 *Brettanomyces bruxellensis* wine isolates. (A) Chromosomal DNA banding patterns as resolved by pulsed field gel electrophoresis (PFGE). (B) Chromosomal DNA banding patterns as resolved a combination of Restriction Endonuclease Analysis and PFGE (termed REA-PFGE) after digestion with *Not*I. Chromosomal DNA from *Saccharomyces cerevisiae* was used as marker (lane M) while the chromosomal DNA of the *B. bruxellensis* isolates was loaded in lanes 1-11.

5.3.2 STANDARD WINE ANALYSIS

No major differences were observed among the different *Brettanomyces* spoiled wines after comparing the standard wine parameters measured with the FTMIR (measured by Foss Winescan). PCA performed with the spectral data representing the standard wine parameters did not show any apparent grouping tendencies as can be seen on the scores plot in Figure 5.2. The triplicate repeats were widely scattered between the different principal components (PCs) and overlapping of the different isolates occurred. This rendered the use of the standard wine parameters in the PCA insufficient for strains groupings.
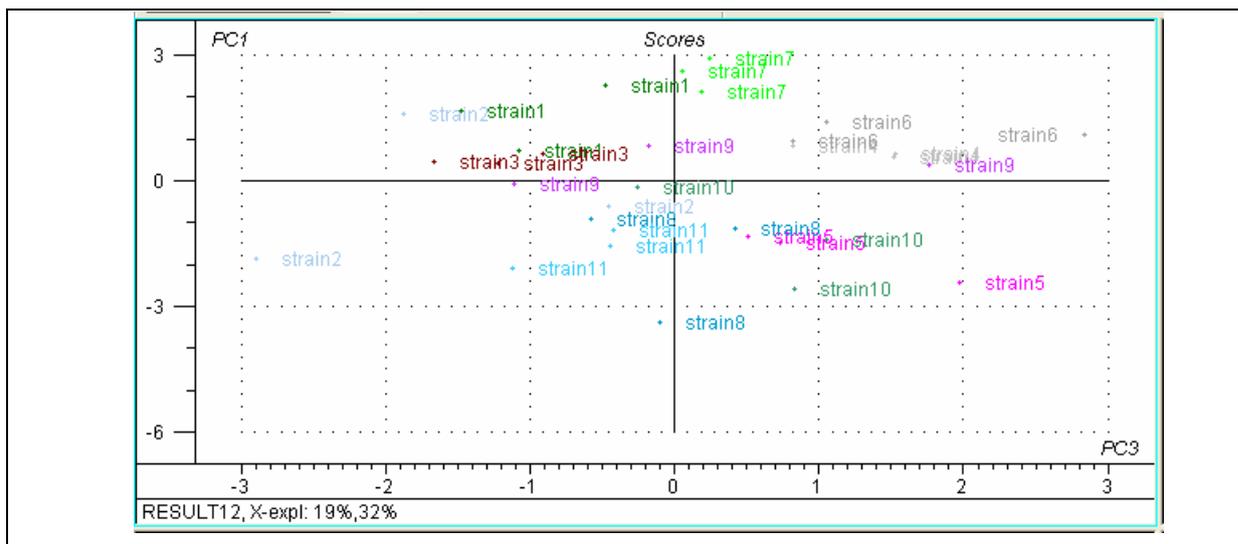
FIGURE 5.2.
Differentiation of wines inoculated with different isolates of *Brettanomyces bruxellensis* by Fourier transform mid-infrared (FTMIR) spectroscopy. Principal component analysis (PCA) scores plot performed on the quantitative data shows no significant groupings or clustering.

5.3.3 FTMIR SPECTRA FROM WINES

To further investigate the use of FTMIR spectroscopy for differentiation between the isolates, the 'fingerprint region' of IR spectra from the spoiled wines was used for data analysis. Contrary to the quantitative spectral data, this region can contain additional undefined chemical information or 'hidden' information not necessarily captured by the quantified parameters that can be more informative.

PCA performed on the 'fingerprint region' of the spoiled wines showed distinct groupings (Fig. 5.3). These grouping tendencies were found with different PC comparisons and it was evident that isolates 4, 6 and 7 clustered separately from the remaining group of isolates. Within the remaining group, a sub-grouping was visible as isolates 2 and 3 appeared to separate away from isolates 1, 5, 8, 9, 10 and 11.
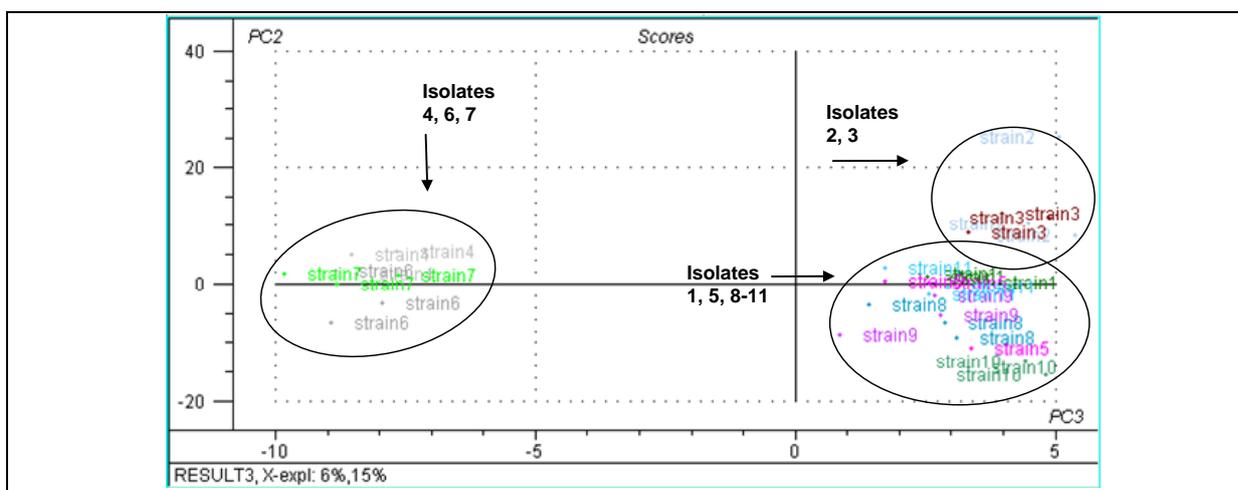

FIGURE 5.3.
Differentiation of wines inoculated with different isolates of *Brettanomyces bruxellensis* by Fourier transform mid-infrared (FTMIR) spectroscopy. Principal component analysis (PCA) scores plot performed on the 'fingerprint region' within the spectra.

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This result indicated differences among closely related strains. Consequently, a more discriminatory method was required and therefore PLS-2 discriminant analysis was performed on the spoiled wines containing the different *Brettanomyces* isolates and excluding the control. Although the score plots could not clearly demonstrate the discriminatory power of this technique, correlation coefficients ranging from 0.79-0.92 were obtained (Table 5.4). Considering the robustness of the approach together with the low RMSEC (root means squares error of calibration) values obtained, these correlations were acceptable and suggested that was possible to discriminate between the strains of *B. bruxellensis* used in the small-scale winemaking to evaluate volatile phenol production. The discriminatory power was however reflected in the regression plot (data not shown).

TABLE 5.4.
PLS-2 discriminant analysis of the wines from the different *B. bruxellensis* strains determined by FTMIR spectroscopy.

Wines*	Correlation	RMSEC[#]
Brett 1	0.850	0.303
Brett 2	0.905	0.245
Brett 3	0.815	0.334
Brett 4	0.871	0.282
Brett 5	0.791	0.352
Brett 6	0.803	0.343
Brett 7	0.923	0.222
Brett 8	0.919	0.227
Brett 9	0.913	0.235
Brett 10	0.912	0.236
Brett 11	0.787	0.355

*Wines named "Brett" refer to the wines contaminated with the different *Brettanomyces* "strains" as indicated in the other figures.
[#]The correlation coefficients and RMSEC values of the calibration set was obtained with leverage correction.

The IR spectral profiles of the spoiled wines after 21 days were subjected to PLS2-discrim and compared to the control wines at day 0 and day 21. The scores plot in Figure 5.4 shows the separate groups that were formed. Differentiation of the 11 wines inoculated with the different *Brettanomyces* strains were clear in comparison with the control wines that were not inoculated. The position of the control wine I at day 0 (original wine) in comparison to the control wine II is largely the result of chemical changes that occurred over the duration of 21 days. This might have been the result of the wine's exposure to air when samples were drawn for weekly microbiological analysis (oxidation). The separation of the spoiled wines (III) from the control wines at day 21 (II) is due to the wine's spectral fingerprint that was altered by the metabolic activity of the *B. bruxellensis* strains in terms of the production of the distinctive metabolites in the wine. The spoilage of the wines by the *Brettanomyces* strains was successful under our tested conditions after comparing the volatile phenol levels (ethyl derivatives).
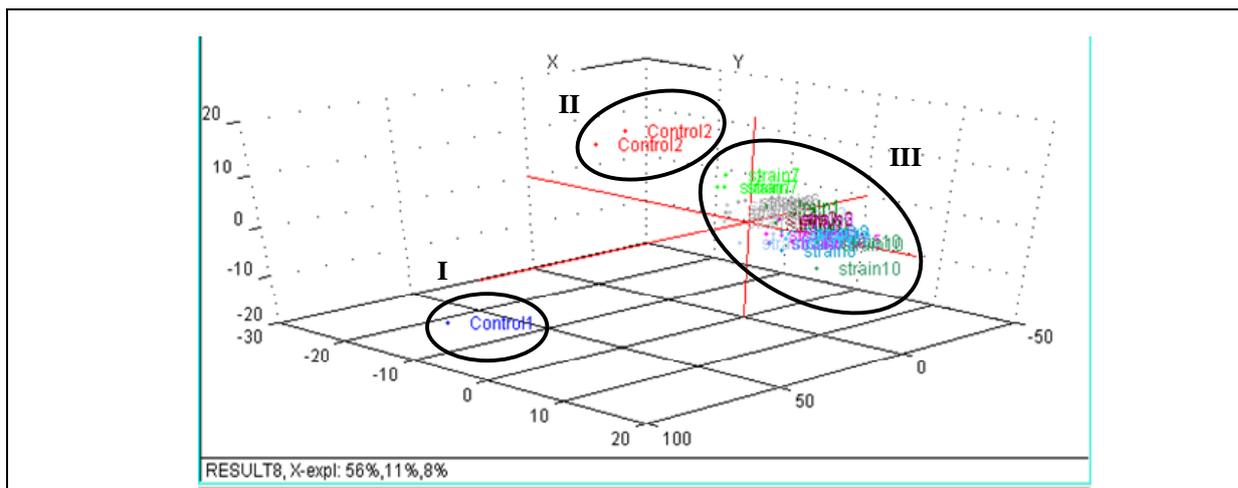
FIGURE 5.4.
Discrimination potential of Fourier transform mid-infrared (FTMIR) spectroscopy on spoiled wines by *Brettanomyces bruxellensis* isolates. Principal Component Analysis (PCA) scores plot (3-D) showing clustering of the different wine groups. I – Control 1 (Original wine, uninoculated at day 0); II – Control 2 (Uninoculated wine after 21 days); III – all the *Brettanomyces* spoiled wines.

The application of FTMIR spectroscopy followed by MVDA techniques showed great potential for the evaluation of *Brettanomyces* strains in wines. Various metabolites produced by the different yeast strains could result in the detection of unique chemical fingerprints with the use of FTMIR spectroscopy. This phenomenon was successfully implemented as a first screening method for differentiating between yeast strains in wine and could definitely be of use in future research.

5.3.4 VOLATILE PHENOL PRODUCTION IN WINE

The Shiraz wine tested negative for any microbial growth prior to inoculation with the selected *B. bruxellensis* strains (day 0). All the isolates grew successfully in the wine during the 21 day period and increased to similar cell numbers, except for isolate 1 and 3, which were the highest and lowest respectively, in comparison (Fig. 5.5). After an informal aroma assessment, minor aroma differences were noticeable among the different contaminated samples one week after the inoculations. As anticipated, typical 'elastoplast' and 'sweaty leather'-like aromas were produced. These are indicative of 4-ethylphenol formation from the conversion of *p*-coumaric acid precursors present in wine by yeasts of the genera *Brettanomyces/Dekkera* (Suárez *et al.*, 2007). After 21 days, the formation of 4-ethylphenol and 4-ethylguaiacol was evident in all the wines, except for the un-inoculated control and ranged from 5336-7979 µg/L and 101-140 µg/L, respectively (Fig. 5.6). The differences in the levels of 4-EP produced suggested that strain diversity existed among the isolates. The results obtained in this study correlate well with the findings reported in other studies of (Joseph and Bisson, 2004) which also showed large variation in volatile phenol production for different strains of *Brettanomyces/Dekkera*.

Comparisons of the grouping tendencies obtained with PLS-2 discrimination of the wines did not correlate with the differences obtained with the original cell number counts or volatile phenol levels obtained at day 21 (data not shown). Although the molecular fingerprinting indicated that all the strains were genetically different, their metabolic contributions to the wines were only slightly different (Fig. 5.6). The IR spectra of the spoiled wines showed similarities as indicated by the spectral groupings (Fig. 5.3).
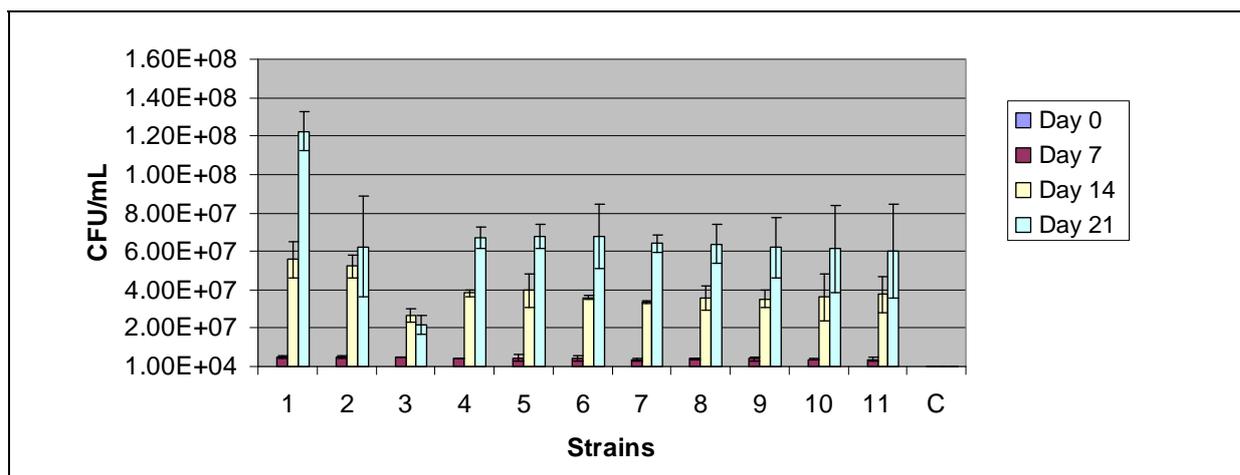

FIGURE 5.5.
Growth of the 11 *Brettanomyces bruxellensis* strains in the wine over 21 days. Each bar represents the average of triplicates. Cell counts are presented as colony forming units (CFU) per mL. The standard deviation is indicated by error bars.
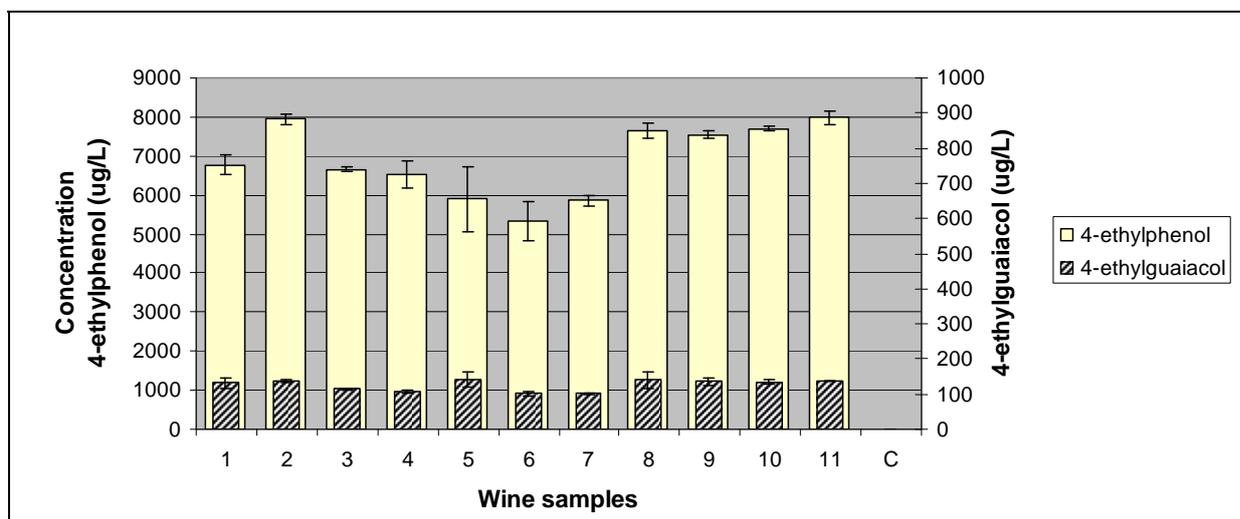

FIGURE 5.6.
The production of volatile phenols (ethyl derivatives) in wines by the different strains of *Brettanomyces bruxellensis* determined by GC-FID. Each bar represents the average of triplicates. The standard deviation is indicated by error bars.

5.3.5 FTIR-ATR SPECTRA OF CELLS

The FTIR-ATR spectra generated from pure cultures of different *B. bruxellensis* strains were evaluated as a rapid screening tool for discrimination among the strains. Five independently cultured technical repeats of each of the 11 strains were scanned and their spectral fingerprints were subjected to data analysis. The first set of spectral data was used for the construction of a calibration set (with leverage correction) on which the discriminant analysis (PLS-2 Discrim) was performed. It was found that the spectra from the calibration set could be used to distinguish between the different strains. The 3-D scores plot in Figure 5.7 is a visual representation (cluster analysis) of the strains after PLS-2 Discrim was performed and clearly shows how the strains differed. The repeats of each strain clustered closely and showed the repeatability of the method and instrumentation. Only triplicate repeats are shown on the scores plot for better visualisation purposes. The discriminatory power is more characteristically presented on the predicted versus measured plot as shown in Figure 5.8. The specific 'predicted' plot versus the 'measured' plot shows that the model could differentiate strain 2 (all five repeats) from the rest of the strains with high correlation (0.93). Similarly, each of the strains was analysed and these could individually be discriminated from the rest of the strains with high correlation coefficients and low calibration errors. The comparative correlation coefficients and the corresponding RMSEC values of all the strains are listed in Table 5.5A.
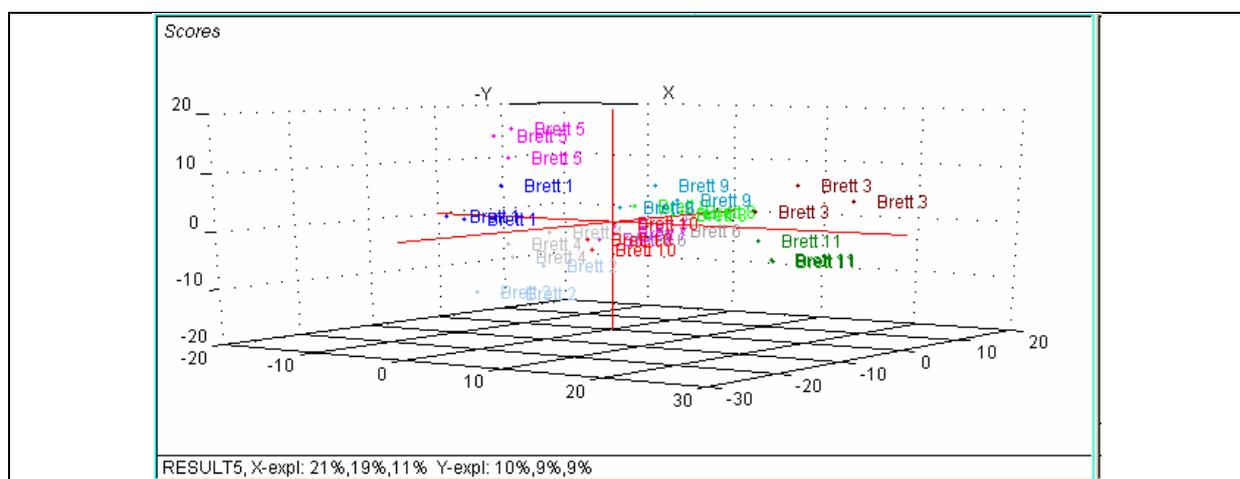

FIGURE 5.7.
Discriminant analysis of different *Brettanomyces bruxellensis* strains using FTIR-ATR spectroscopy. PLS-2 discrimination (3-D scores plot) was performed with calibration set (leverage correction).
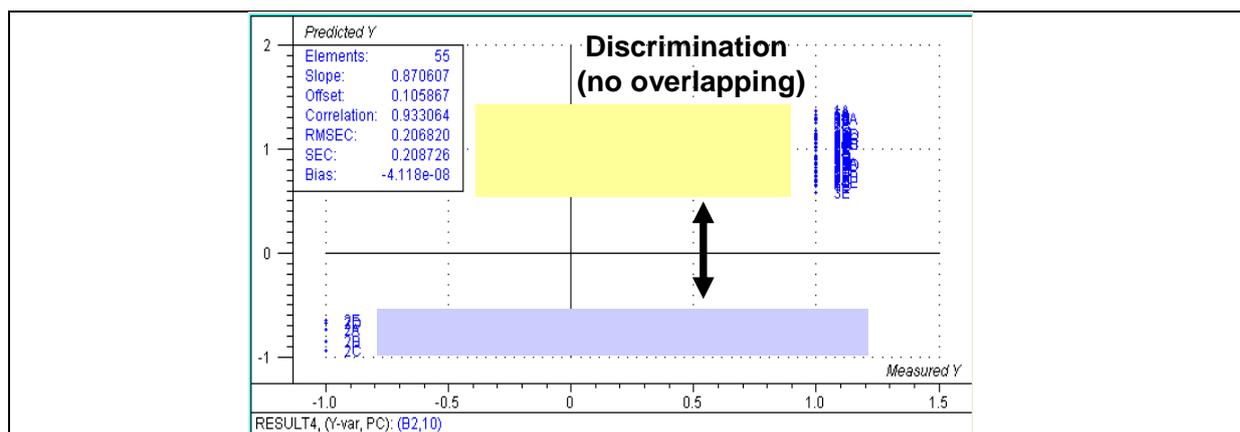
FIGURE 5.8.
PLS-2 discriminant analysis of different *Brettanomyces bruxellensis* strains using FTIR-ATR spectroscopy. The predicted vs. measured plot shows that an individual strain (blue block) could be distinguished from the other strains (yellow block) with high correlation.

In order to validate the calibration model described above, a biologically independent data set was required. This was performed by re-scanning all the strains at a later date and the spectral data generated was used for a cross-validation of the calibration set. All the strains were analysed by FTIR-ATR spectroscopy after they were cultured on the same media and under the same growth conditions as before. This time, however, the independently cultured technical repeats were performed in triplicate. PLS-2 discriminant analysis was performed with the calibration set as before and cross-validated with the experimental repeat set or second data set.

This time the predicted versus measured plots showed significant differences as seen in Figure 5.9. It was evident that not all the strains could be individually differentiated from the rest and the strains that could be discriminated, exhibited lower correlation coefficients. The representations of the discriminatory and non-discriminatory predicted versus measured plots with cross-validation can be seen in Figures 5.9A and 5.9B, respectively. Figure 5.9A shows that the model could discriminate strain 5 from the rest of the strains, with a correlation coefficient of 0.8. Figure 5.9B shows that the model could not precisely differentiate strain 8 from the rest of the strains and this was supported by a low correlation coefficient of 0.17. Again, each of the strains was similarly analysed and individually compared to the rest of the strains. The comparative correlation coefficients and the corresponding RMSEP (root means squares error of prediction) values of all the strains are listed in Table 5.5B. The correlation coefficients of the calibration set after cross-validation (Table 5.5B) were acceptable for the majority of the 11 strains (correlations above 0.5). These correlations are especially good considering the small model that was tested and the low average RMSEP values (~0.45) obtained. These results suggest that the model holds potential for future predictive modelling purposes and more biological independent repeat sets should be incorporated to improve the model.

TABLE 5.5.
PLS-2 discriminant analysis of different *B. bruxellensis* strains by FTIR-ATR spectroscopy.

A	**Strains**	**Correlation**	**RMSEC**	**B**	**Strains**	**Correlation**	**RMSEP**
	Brett 1	0.933	0.207		Brett 1	0.707	0.410
	Brett 2	0.933	0.207		Brett 2	0.681	0.424
	Brett 3	0.933	0.207		Brett 3	0.710	0.407
	Brett 4	0.900	0.250		Brett 4	0.533	0.493
	Brett 5	0.958	0.165		Brett 5	0.808	0.340
	Brett 6	0.899	0.251		Brett 6	0.515	0.502
	Brett 7	0.929	0.213		Brett 7	0.501	0.510
	Brett 8	0.915	0.232		Brett 8	0.171	0.603
	Brett 9	0.937	0.210		Brett 9	0.417	0.506
	Brett 10	0.906	0.243		Brett 10	0.506	0.505
	Brett 11	0.915	0.232		Brett 11	0.754	0.380

A The correlation coefficients and *RMSEC values of the calibration set with leverage correction.
B The correlation coefficients and *RMSEP values of the calibration set with cross-validation.
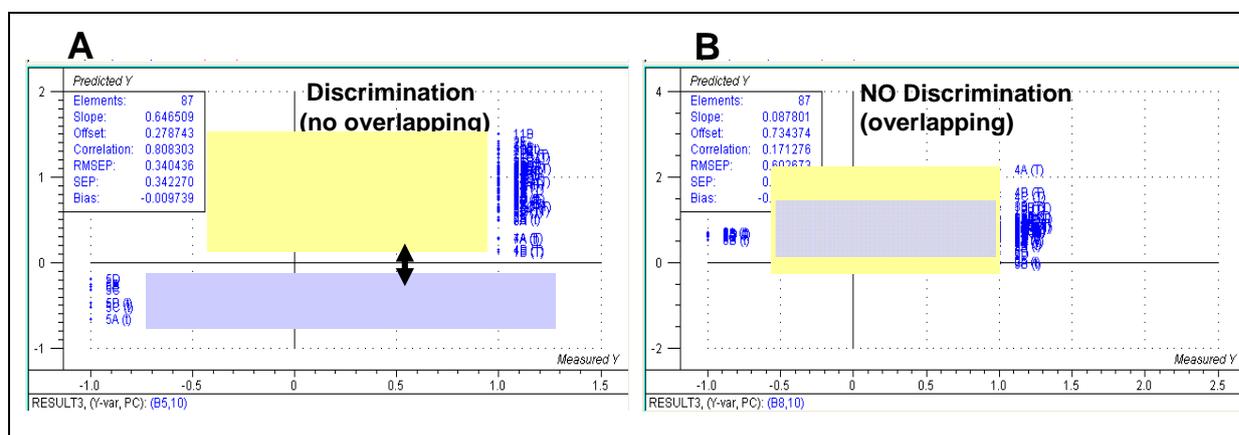
FIGURE 5.9.
PLS-2 discriminant analysis of different *Brettanomyces bruxellensis* strains using FTIR-ATR spectroscopy. (A)The predicted vs. measured plot shows that an individual strain (blue block) could be distinguished from the other strains (yellow block) with high correlation. (B) The predicted vs. measured plot shows that an individual strain (blue block) could not be distinguished from the other strains (yellow block) with poor correlation.

5.4 CONCLUSIONS

This feasibility study focused on evaluating FTIR spectroscopy as an alternative approach for the rapid identification of strains of *B. bruxellensis* from oenological origin. Traditional methods based on morphological and physiological tests are not suitable for strain discrimination and molecular techniques are often inconclusive and necessitate various extensive preparation steps. Based on its strain identification and differentiation potential, FTIR spectroscopy could become a reliable alternative to molecular based methods for the typing of *B. bruxellensis* strains. Considering the two approaches that were taken in this study, by capturing spectral fingerprints from wines spoiled by different strains of *B. bruxellensis* and also directly from pure cultures, FTIR spectroscopy shows endless possibilities of differentiation.

5.5 ACKNOWLEDGEMENTS

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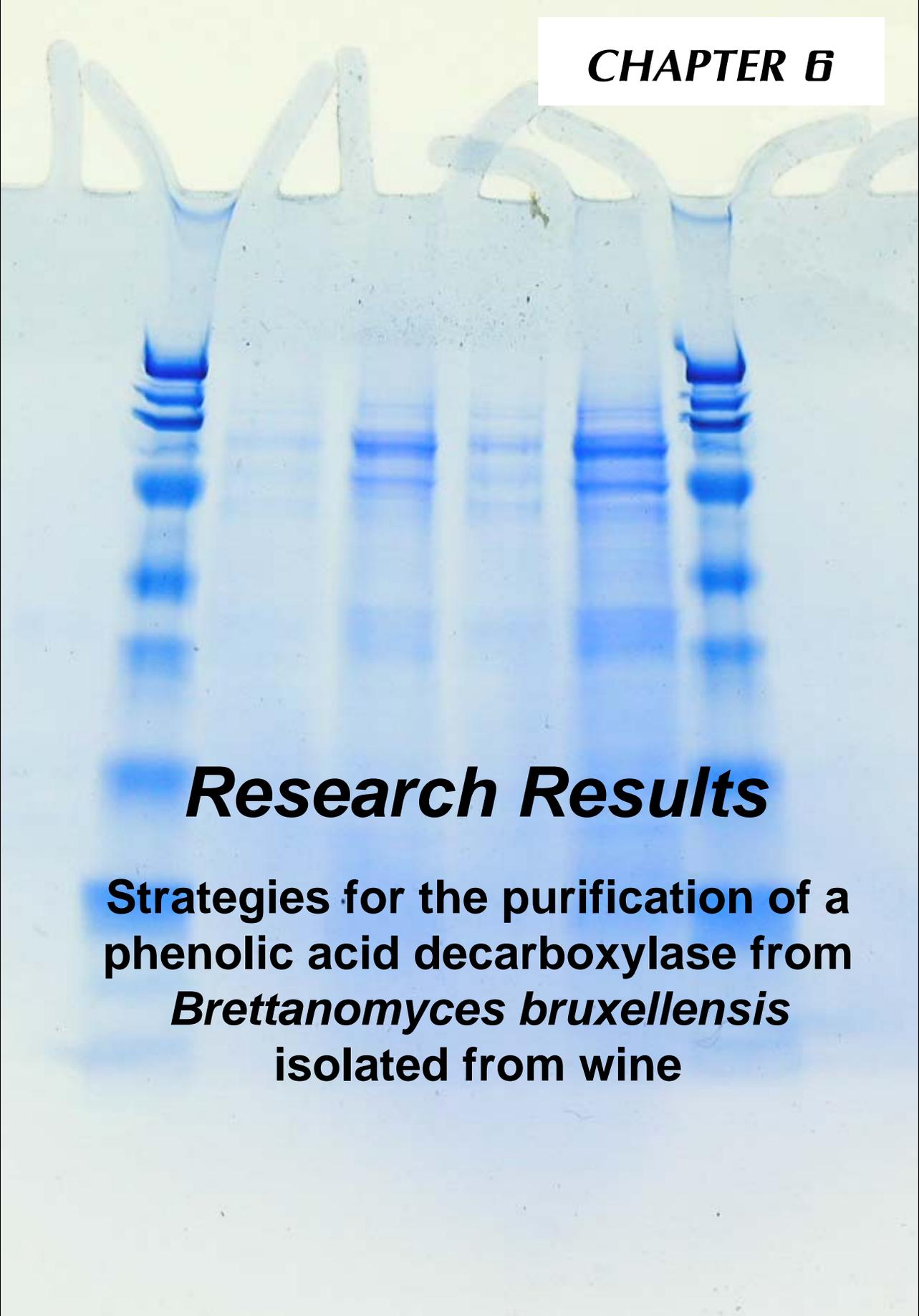
Research Results

Strategies for the purification of a phenolic acid decarboxylase from *Brettanomyces bruxellensis* isolated from wine

6. RESEARCH RESULTS

Strategies for the purification of a phenolic acid decarboxylase from *Brettanomyces bruxellensis* isolated from wine

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ABSTRACT

The formation of volatile phenols by species of the genera *Brettanomyces* and *Dekkera* present a real spoilage risk to winemakers. These yeasts can metabolise the phenolic (hydroxycinnamic) precursors originating from the grapes resulting in the sequential formation of vinyl phenols and ethyl phenols that are detrimental to the wine's organoleptic quality. The enzymatic activities of this reaction are well known and involve a phenyl acrylic (phenolic) acid decarboxylase (PAD) and a vinyl phenol reductase (VPR). However, there is very little information available about these enzymes in *Brettanomyces/Dekkera* yeasts. This investigation therefore describes attempts of purification and isolation of the *PAD* gene using different molecular strategies. The PAD enzyme from a *Brettanomyces bruxellensis* strain was purified 23-fold and two short peptide sequences were obtained. Degenerate primers designed from PAD gene orthologs from other yeast yielded a partial sequence of 464 bp with 97% homology to the PAD1 gene of *Saccharomyces cerevisiae*.

Keywords: *Brettanomyces, Dekkera, volatile phenols, phenolic, phenylacrylic, hydroxycinnamic, decarboxylase, PCR, protein purification*

Abbreviations
PA phenolic acid, **PCA** *p*-coumaric acid, **FA** ferulic acid, **4-EP** 4-ethylphenol, **4-EG** 4-ethylguaiacol, **4-VP** 4-vinylphenol, **4-VG** 4-vinylguaiacol
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6.1 INTRODUCTION

Yeast of the genus *Brettanomyces* or its teleomorph *Dekkera* has been identified as one of the most controversial spoilage microorganisms in wine. These yeasts can produce several compounds that are detrimental to the organoleptic quality of wine, including volatile phenols (Fugelsang *et al.*, 1993; Ibeas *et al.*, 1996; Chatonnet *et al.*, 1997; Licker *et al.*, 1998; Mansfield *et al.*, 2002; Aguilar-Uscanga *et al.*, 2003; Snowdon *et al.*, 2006). These phenolic off-flavours impart characters that have been described as 'medicinal', 'elastoplast', 'sweaty leather', 'barnyard', 'spicy' and 'clove-like' and can consequently ruin the aroma profile of red wines at high concentrations (Chatonnet *et al.*, 1992, 1995).

The formation of volatile phenols is the result of the sequential conversion of the hydroxycinnamic acid precursors (*p*-coumaric, ferulic and caffeic acid) originating from the grapes into hydroxystyrenes (4-vinylphenol, 4-vinylguaiacol and 4-vinylcatechol respectively), which are further reduced into ethyl derivatives (4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol) (Heresztyn, 1986; Chatonnet *et al.*, 1992; Hesford *et al.*, 2004).

Hydroxycinnamic acids are natural constituents of grape juice and wine and are released from phenolic compounds (cinnamates) in the grapes, where they are generally esterified with tartaric acid (Dugelay *et al.*, 1993). Particularly in the free form, such as *p*-coumaric and ferulic acid, phenolic acids can have an inhibitory effect on the growth of most microorganisms (Zaldivar and Ingram, 1999; Barthelmebs *et al.*, 2001). However, one of the methods by which many microorganisms achieve resistance against phenolic acids, is by enzymatic conversion into a less toxic form such as volatile phenols (Barthelmebs *et al.*, 2000a).

The two enzymes that facilitate the biotransformation of phenolic acids involve a phenolic acid decarboxylase for the formation of the vinyl derivatives and a vinyl phenol reductase for the formation of the ethyl derivatives thereafter. The decarboxylation step resulting in the formation of vinyl phenols exists in numerous bacteria, fungi and yeast species of which some are present during the winemaking process (Heresztyn, 1986; Chatonnet *et al.*, 1992; Cavin *et al.*, 1993, 1997; Clausen *et al.*, 1994; Edlin *et al.*, 1995; Shinohara *et al.*, 2000; Van Beek and Priest, 2000). However, the reduction step and ethyl phenol formation occur less frequently in microorganisms (Chatonnet *et al.*, 1995; Barthelmebs *et al.*, 2001; Dias *et al.*, 2003). A number of phenolic acid decarboxylases have been reported from bacteria, such as for the genera *Lactobacillus* (Barthelmebs *et al.*, 2001), *Pediococcus* (Barthelmebs *et al.*, 2000b), *Bacillus* (Degrassi *et al.*, 1995; Cavin *et al.*, 1998), *Pseudomonas* (Huang *et al.* 1994) and *Streptomyces* (Chow *et al.*, 1999). In yeasts, the phenolic acid decarboxylase (PAD) enzyme from *Saccharomyces cerevisiae* and a hydroxycinnamate decarboxylase from *Brettanomyces anomalus* have been characterised (Clausen *et al.*, 1994; Edlin *et al.*, 1998). The latter is the only published attempt aimed at the purification of the PAD enzyme from

Brettanomyces/Dekkera species. This report, however, did not reveal any genetic or peptide sequence data.

The objective of the present study was to devise strategies for the purification of the phenolic acid decarboxylase from *Brettanomyces bruxellensis* isolated from wine and to obtain sequence data of both the enzyme and the phenolic acid decarboxylase gene (*PAD*).

6.2 MATERIALS AND METHODS

6.2.1 YEAST STRAINS AND MAINTENANCE

The yeast strains used in this study are listed in Table 6.1. The strains of *B. bruxellensis* (*D. bruxellensis*) from the IWBT laboratory culture collection (Institute for Wine Biotechnology, Stellenbosch University, South Africa) were isolated from various wines from the Stellenbosch grape-growing region. Yeasts were maintained on YPD agar medium (glucose 20 g/L, yeast extract 10 g/L, peptone 10 g/L, agar 25 g/L) containing cycloheximide 50 mg/L (Sigma-Aldrich, St Louis, USA), chloramphenicol 30 mg/L (Roche, Mannheim, Germany) and kanamycin 30 mg/L (Roche). Incubation occurred over a period of 5 to 11 days at 30ºC.

TABLE 6.1.
List of *Brettanomyces/Dekkera* strains used in this study.

No.	Yeast species	Sample ID	Isolated from	Source
1	*Saccharomyces cerevisiae*	VIN13		Anchor yeast
2	*Saccharomyces cerevisiae*	YNN295		FOEB
3	*Brettanomyces bruxellensis*	TFref	red wine	TF
4	*Brettanomyces bruxellensis*	CB 63	red wine	FOEB
5	*Brettanomyces bruxellensis*	SA18	red wine	IWBT
6	*Brettanomyces bruxellensis*	SA29	red wine	IWBT
7	*Brettanomyces bruxellensis*	SA33	red wine	IWBT
8	*Brettanomyces bruxellensis*	SA36	red wine	IWBT
9	*Brettanomyces bruxellensis*	SA38	red wine	IWBT
10	*Brettanomyces bruxellensis*	SA47	red wine	IWBT

IWBT - Institute for Wine Biotechnology, Stellenbosch University, South Africa.
FOEB - Faculté d'Oenologie de Bordeaux, Bordeaux, France.
TF - Thalès (member of Chène and Cie), France.

6.2.2 PREPARATION OF CELL EXTRACTS

The yeast *B. bruxellensis* CB63 was cultivated in 1 L of a modified YNB-based media consisting of (yeast nitrogen base 6.7 g/L, glucose 2 g/L, biotin 0.6 g/L, thiamin hydrochloride 1.6 g/L and ethanol 8% v/v). After autoclaving, the substrate *p*-coumaric acid (PCA) (Sigma-Aldrich) was added to a final concentration of 100 mg/L (100 mg was pre-dissolved in 1 mL ethanol 96% v/v). The cells were allowed to grow for 6 days until late exponential phase from a pre-conditioned culture

in the same media. The culture was harvested by centrifugation at 5000 g for 15 min. Thereafter, the pellet (~3 g) was washed twice with 8 mL 100 mM sodium phosphate buffer (pH 6) and resuspended in 5 mL 50 mM sodium phosphate buffer (pH 6). Cell extracts were obtained by disrupting concentrated cell suspensions by means of a French press at 1.2 x 10^8 Pa. The supernatant obtained was centrifuged for 30 min at 12 000 rpm at 4°C to remove any cellular debris. The cell extract was stored at -20°C prior to enzyme analysis.

6.2.3 PHENOLIC ACID DECARBOXYLASE ASSAY

Phenolic acid (PA) degradation and derivative production were measured by UV spectrophotometry as previously described (Barthelmebs *et al.* 2000a) with some modifications. The assay mixture for the phenolic acid decarboxylase activity consisted of 50 mM sodium phosphate buffer (pH 6) containing 100 mg/L of *p*-coumaric acid. Reactions were started by adding cell extract (10% v/v) to the assay mixture followed by incubation at 37°C for 1 to 24 h (the times of incubation varied depending on the stage of protein purification). Before analysis, samples were diluted 20-fold in Stop buffer [25 mM Tris-HCl, 0.3% sodium dodecyl sulfate (SDS), pH 6] to stop activity. Residual PCA concentrations were determined by direct UV spectrophotometry (250-340 nm) in quartz cuvettes by monitoring the disappearance of the absorption peak of the substrate tested (286 nm for PCA) and the appearance of a new peak corresponding to vinyl derivatives (258 nm for 4-VP).

6.2.4 PROTEIN DETERMINATION

The total protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, USA).

6.2.5 MONO Q SEPARATION

Mono Q separation was performed as described by Lucas and Lonvaud-Funel (2002) with some changes. Four mL crude extract was loaded onto a Mono-Q (HR10/10) column (Amersham-Pharmacia Biotech Inc., Piscataway, USA) equilibrated in buffer A (Tris-HCl 20 mM pH 7.5, glycerol 5%, PLP 0.1 mM, EDTA 0.1 M, 2-mercaptoethanol 0.1 mM). Bound proteins were eluted at a flow rate of 1.0 mL/min with a 40 mL linear gradient of 0-0.5 M NaCl in buffer A. Fractions of 0.5 mL were collected and assayed for PA decarboxylase activity. Hereafter, the fractions containing the activity were pooled, diluted to double the volume with buffer A and again loaded onto the Mono-Q column equilibrated in the same buffer. Bound proteins were eluted with a 0-1.0 M linear salt gradient of 40 mL. The collected fractions were assayed for decarboxylase activity.

6.2.6 PHENYL SUPEROSE SEPARATION

Phenyl Superose column chromatography was performed with the pooled samples from the Mono Q separation. The Phenyl Superose (HR5/5) column was from

Amersham-Pharmacia Biotech. A volume of the active fractions were diluted with an equal volume of buffer A containing $(NH_4)_2SO_4$ (3.4 M) to obtain a final concentration of 1.7 M. The sample was loaded onto the column that was equilibrated in buffer A containing $(NH_4)_2SO_4$ (1.7 M). Bound proteins were eluted with a 10 mL linear gradient of 1.7-0.0 M $(NH_4)_2SO_4$ at a flow rate of 0.5 mL/min. The fractions obtained were assayed for decarboxylase activity and active fractions were kept at -20°C for SDS-PAGE analysis.

6.2.7 PAGE ANALYSIS

The protein extracts from the various purification steps containing PAD activity were resolved by denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (15% resolving gel) as previously described (Sambrook *et al.*, 1989) on Mini-PROTEAN II electrophoresis cell (Bio-Rad, Hercules, CA, USA). A broad range molecular weight marker (6-175 kDa) (New England Biolabs, Ipswich, USA) was used as standard. The samples for the SDS-PAGE were diluted with one volume of sample buffer (Sigma-Aldrich) and boiled for 3 min. The molecular weight marker was diluted 1:20 with 1x sample buffer and heated for 2 min at 65°C. The protein bands and the protein standards on the gel were visualised after electrophoresis with Coomassie Blue R-250, or where necessary, by using a silver staining kit (Amersham).

6.2.8 SEQUENCING OF PROTEIN FRAGMENTS

The protein bands of interest were excised from the SDS-PAGE gel (after Coomassie blue staining) and submitted to microsequencing. Peptide sequences were determined by reverse phase chromatography at the Pasteur Institute (Plateforme d'analyse et microséquençage des proteines, Paris, France).

6.2.9 PRIMER DESIGN AND POLYMERASE CHAIN REACTIONS (PCR)

The polymerase chain reaction (PCR) method was used for the detection of possible PAD genes. All the primers used in this study are listed in Table 6.2. Degenerate primers were designed from sequences aligned with BioEdit (software version 7.0.3) that utilises an automated ClustalW algorithm (www.mbio.ncsu.edu/BioEdit/bioedit). All primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA), except for the PADGEN primers that were synthesised by Eurobio (Paris, France). PCR reactions were performed with genomic DNA from the *B. bruxellensis* strains. DNA from a commercial yeast *S. cerevisiae* (VIN13) was used as template for *PAD1* amplification and served as control for the reactions. Amplifications were performed on a TRIO-Thermoblock cycler (Biometra, Göttingen, Germany). The thermal cycler parameters were as follows: initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 48°C-56°C (depending on the primer pairs used; see Table 6.2) for 40 s, and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. PCR amplifications were performed in a 50 µL (final volume)

reaction mixture with Takara ExTaq and 10x ExTaq buffer (containing 20 mM MgCl$_2$) (Takara Bio Inc., Japan). The reaction mixture consisted of 1.25 U of *Taq* polymerase, 0.2 mM dNTPs (Takara), 0.3 µM of each primer, 1.5 mM - 2.mM MgCl$_2$ and approximately 100 ng DNA template. All PCR products were analysed by 1% (w/v) agarose gel electrophoresis and visualized under UV illumination after ethidium bromide staining.

TABLE 6.2.
List of primers used in this study.

Primer	Sequence 5' - 3'	Tm[a]	Reference
PAD1 (Forward)	GAATTCATGCTCCTATTTCCA	50	Smit *et al.*, 2003
PAD1 (Reverse)	GATCCTCGAGTTCAATTAGAATGATAT	53	Smit *et al.*, 2003
PADGEN 1 (Forward)	[b] SUIGSIAUIACIGGIGCIACIGG	60	This study
PADGEN 2 (Reverse)	[b] TRTARAAIGCIGGIAYIGGIGG	57	This study
PADAO 1 (Forward)	[b] CCWAAGAGAATWGTYKTVGCAATHACYGG	62	This study
PADAO 2 (Reverse)	[b] GGTYTTGTATAAAAYGCWGGYACHGGDGG	65	This study
PADAO 3 (Reverse)	CACGTCATGCGGTTCCCAATCTGTTTC	62	This study
PADAO 4 (Forward)	CATGTTGTCTTTATGCAGGGCAGGTG	60	This study
PADAO 5 (Forward)	GTTGTCGCAATTACTGGTGCGAC	59	This study
PADAO 6 (Reverse)	CACCTGCCCTGCATAAAGACAACATG	60	This study
PADAO 7 (Reverse)	CTACGCTCAACTCTTTTAGCACTTGTAGA	58	This study
PADAO 8 (Forward)	GAGAATCGTAAGTTACTACTGGTTACTCG	56	This study
PADAO 9 (Forward)	[b] CATGATGGWATGATTGTT	44	This study
Primer PCD 489F	AACGGCTGGGAATACGA	52	Van Beek & Priest, 2000
Primer PCD 813rR	GCAAATTCGGGTACAAC	48	Van Beek & Priest, 2000
LABPAD 1 (Forward)	[b] AARAAYGAYCAYACYRTTGATTAC	53	This study
LABPAD 2 (Reverse)	[b] TTCTTCWACCCAYTTHGGGAAGAA	65	This study

Degenerate code: S=G or C; R=G or A; Y=C or T; W=A or T; K= G, T or A, V=A, C or G; I=Inosine.
[a] Melting temperatures as indicated by manufacturer
[b] Degenerate primers

6.2.10 SOUTHERN BLOT ANALYSIS

Southern blot analysis was performed as described in sections 2.9 and 2.10 of Current Protocols (Ausubel *et al.*, 2000) by using a digoxigenin non-radioactive nucleic acid labelling and detection system. Standard methods for yeast genomic DNA isolations were used (Sambrook *et al.*, 1989). Genomic DNA (10 µg) was digested with numerous restriction enymes (*Nde*I, *Xho*I, *Sca*I, *Eco*RI, *Spe*I, *Bgl*I, *Eco*RV, *Hind*III and *Bam*HI) and separated on a 0.8% (w/v) agarose gel. All the restriction endonucleases were purchased from Roche Diagnostics (Johannesburg, South Africa). Gels were transferred to positively charged Hybond-N nylon membranes and prehybridisation (3-5 h) and hybridisation (overnight) reactions were carried out at 40°C. Probes were designed by amplifying the *PAD*1 gene of *S. cerevisiae* or part thereof using the PAD1 (F/R) and PADAO 5/6 (F/R) primers respectively. The probes were labelled with digoxigenin by means of the PCR DIG

labelling kit from Roche. After hybridisation, membranes were washed twice at room temperature with 2x SSC and 0.1% SDS (w/v) for 20 min followed by another two washes at 42°C with 0.2x SSC and 0.1% SDS (w/v) for 20 min each (for details refer to the DIG System User's Guide (Roche Diagnostics, Mannheim, Germany). Chemiluminescent detection was done using CSPD as substrate (Roche).

6.2.11 INVERSE PCR PROCEDURE

Genomic DNA was digested with *Eco*RI at 37°C for 16 h. The reaction mixture contained 10 µg genomic DNA, 6 µL of reaction buffer, 5 µl (10 U/µL) of enzyme, and made up to a final volume of 60 µL with Milli-Q water. Digested fragments were separated by electrophoresis on a 1% (w/v) agarose gel using *BstE*II digested lambda DNA as molecular marker. The fragment sizes indicated by the Southern Blot analysis were isolated and purified using the QIAquick gel extraction kit (Qiagen). Purified fragments were re-ligated on itself in a 50 µL reaction (final volume) comprising of 20 µL *Eco*RI digested products (~ 5 ng/µL), 5 µL of 10x ligase buffer, and 0.7 µL T4 DNA ligase (3 U/µL) (Promega, Madison, USA). Reaction volume was filled up with Milli-Q water and incubated at 4°C overnight.

Inverse PCR amplifications were carried out with divergent primers to determine the 5' and 3' flanking regions of the already known DNA sequence. For this purpose a set of four non-degenerate perfectly matching primers were synthesised. The primers PADAO 3, PADAO 4, PADAO 7 and PADAO 8 (see Table 6.2) were used in different combinations in an amplification mixture consisting of Elongase Enzyme Mix (Invitrogen, Carlsbad, CA, USA) and 10 µL of the religation mixture (~ 20 ng template DNA). The preparation of the reaction mixture was according to the manufacturer specifications (Invitrogen). The reaction conditions were as follows: initial denaturation at 95°C for 1 min, 30 s of melting at 95°C, 30 s of annealing at 54°C, and 3 min of extension at 68°C. The reaction was performed for 35 cycles and a final extension of 5 min at 68°C was performed. Inverse PCR products were purified using a QIAquick gel extraction kit (Qiagen) after agarose gel electrophoresis.

6.2.12 SEQUENCING OF PCR PRODUCTS

PCR products of interest were excised from the gel and purified by means of a QIAquick PCR purification kit (Qiagen). Hereafter, products were cloned into pGEM-T-easy (Promega) and positive (white) colonies were sent away for sequencing.

6.3 RESULTS AND DISCUSSION

6.3.1 PHENOLIC ACID DECARBOXYLASE ASSAY

The ability of a *B. bruxellensis* strain to decarboxylate a hydroxycinnamic acid was determined by monitoring the changes in UV absorbance and included the disappearance of PCA resulting in the accumulation of 4-VP. Typical results can be seen in Figure 6.1. The UV spectra showed that *p*-coumaric acid had a maximum absorbance peak at approximately 286 nm in the phosphate buffer (0 h) and this was in agreement with other authors (Barthelmebs *et al.,* 2000a; Van Beek and Priest, 2000). The supernatant obtained from the *B. bruxellensis* CB63 cell extracts revealed PAD enzyme activity after substrate conversion was measured. The loss of the absorbance peak at 286 nm accompanied by the formation of an absorbance peak at 258 nm (representing 4-VP) formed the basis of all the PCA decarboxylase assays that followed.
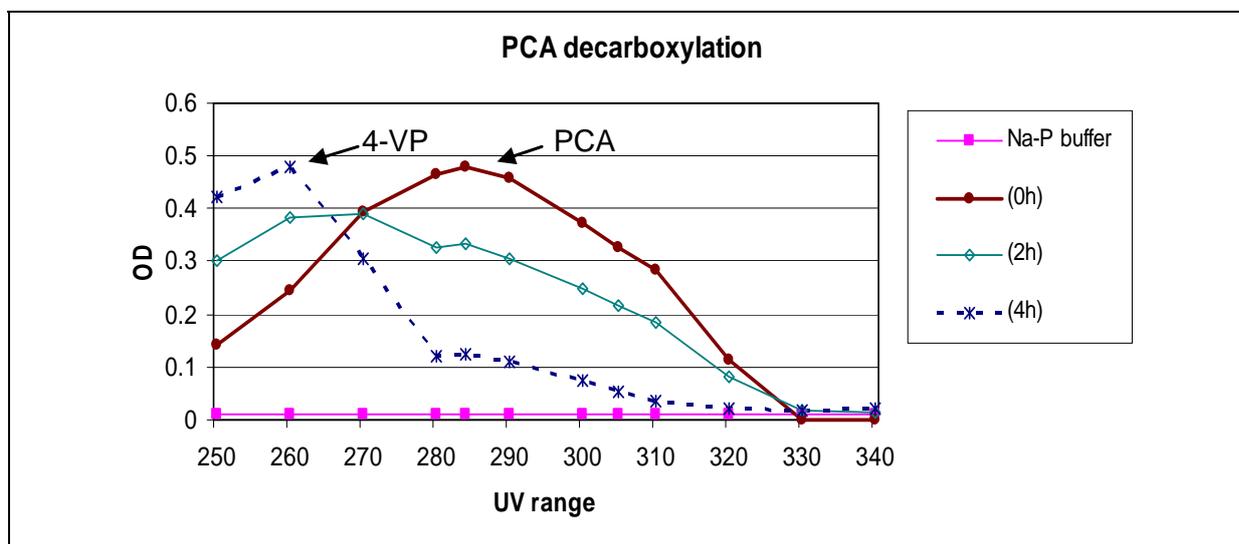

FIGURE 6.1.
PCA decarboxylation assay performed with the cell extract of *Brettanomyces bruxellensis* CB63 in 50 mM sodium phosphate buffer (pH 6) containing 100 mg/L *p*-coumaric acid.

The assay was also tested with the YNB supernatant in which the yeast strain was cultured. No activity could be detected and it was unsure if the specific enzyme activity in the supernatant was too low or if the enzyme contained any extracellular characteristics. For this purpose, whole cells of *B. bruxellensis* CB63 were evaluated in the sodium phosphate buffer. Cells were harvested from the culture and resuspended to an optical denisity (measured at 600 nm; OD_{600}) of 1.0 in 50 mM sodium phosphate buffer (pH 6) containing 100 mg/L PCA. Incubation occurred for up to 72 h at 30°C after which supernatants were assayed for decarboxylation activity. No activity was detected after a period of 72 h as none of the assays showed the formation of a new absorbance peak at 258 nm corresponding to 4-VP (Fig. 6.2). It was assumed that the PCA transport into the cells was not possible in the buffer

resulting in no or minimal substrate degradation.
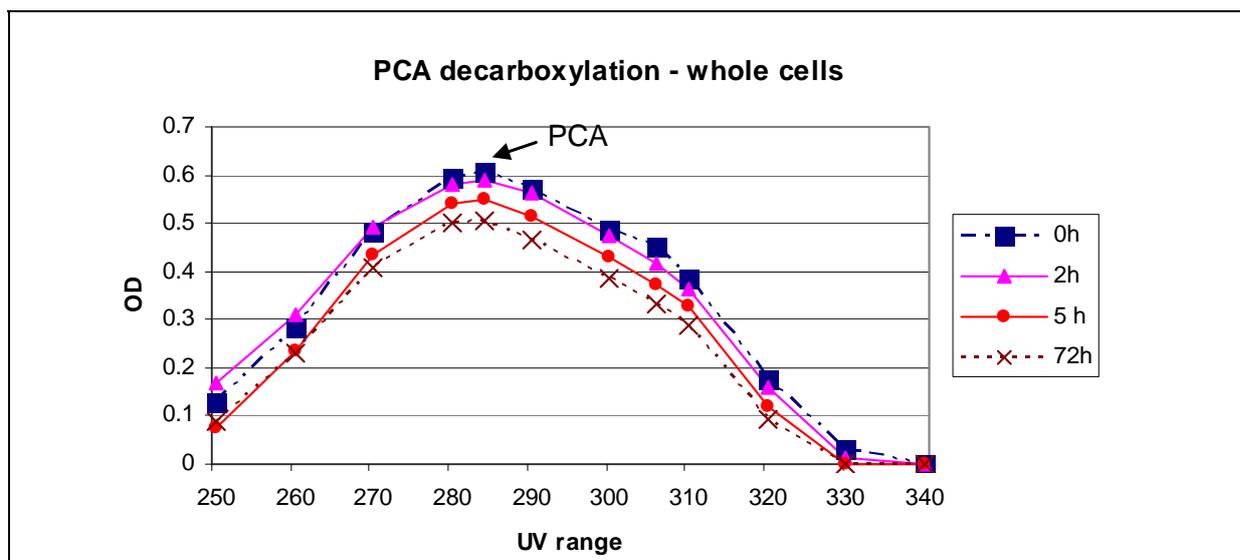
FIGURE 6.2.
PCA decarboxylation assay performed with whole cells of *Brettanomyces bruxellensis* CB63 in 50 mM sodium phosphate buffer (pH 6) containing 100 mg/L *p*-coumaric acid.

6.3.2 PURIFICATION OF PHENOLIC ACID DECARBOXYLASE

PA decarboxylase activity was detected in the intracellular fractions of the yeast *B. bruxellensis* CB63. The cell extracts were subjected to different purification steps of which results are shown in Figures 6.3, 6.4 and 6.5. The first Mono Q purification yielded two large peaks consisting of most of the recoverable proteins from the cellular extract. A large amount of unbound proteins was also collected. However, these did not reveal any activity (data not showed). The first purification stage mainly attempted to recover the active proteins from cellular debris and other possible contaminants. In Figure 6.3A it can be seen that fractions containing PA decarboxylase activity was eluted at a NaCl concentration of 0.15-0.22 M. The activities measured with the PA decarboxylase assay can be seen in Figure 6.3B (only some fractions showed). All active fractions were pooled and subjected to a second Mono Q run with a higher salt gradient to minimise the dilution factor of the samples. The active fractions similarly eluted at the same salt concentration range as before (Fig. 6.4A) and only one large peak was obtained (excluding the sharp peak as a result of artifactual reading) and this confirmed the efficiency of the first purification. Again, all the fractions containing activity (Fig. 6.4B) were pooled and subjected to the hydrophobic column. The third purification on the Phenyl superose column showed four distinct peaks (Fig. 6.5A). The decarboxylase assay performed on the fractions from these peaks showed that only the last peak (35), which eluted near 0 M $(NH4)_2SO_4$, contained activity (Fig. 6.5B). After the whole purification procedure a 23-fold purification and a final yield of 3.2% was obtained (Table 6.3). The low purification fold obtained was largely ascribed to the low initial protein concentration from which the purification procedure was started. In this regard, it

must be mentioned that many difficulties were experienced with obtaining enough total proteins from the cellular extract.

Additional to this section, alternative ways of obtaining the intracellular proteins were investigated that were not discussed. These included the use of sonification (ultrasonic waves) and a homogenizer with 0.45 mm glass beads with different protocols. However, the use of a French press resulted in the highest amount of total proteins extracted (data not shown).

TABLE 6.3.
Purification of phenolic acid decarboxylase from *Brettanomyces bruxellensis*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	% Yield
Crude extract	57	416.7	7.3	1.0	100.00
Mono Q (1st)	1.44	92.1	64.0	8.8	22.12
Mono Q (2nd)	0.6	42.1	70.1	9.6	10.10
Phenyl superose	0.08	13.4	166.9	22.8	3.21

One unit of enzyme activity (U) is defined as the amount of enzyme that causes 1 μmol of product to be formed per min.
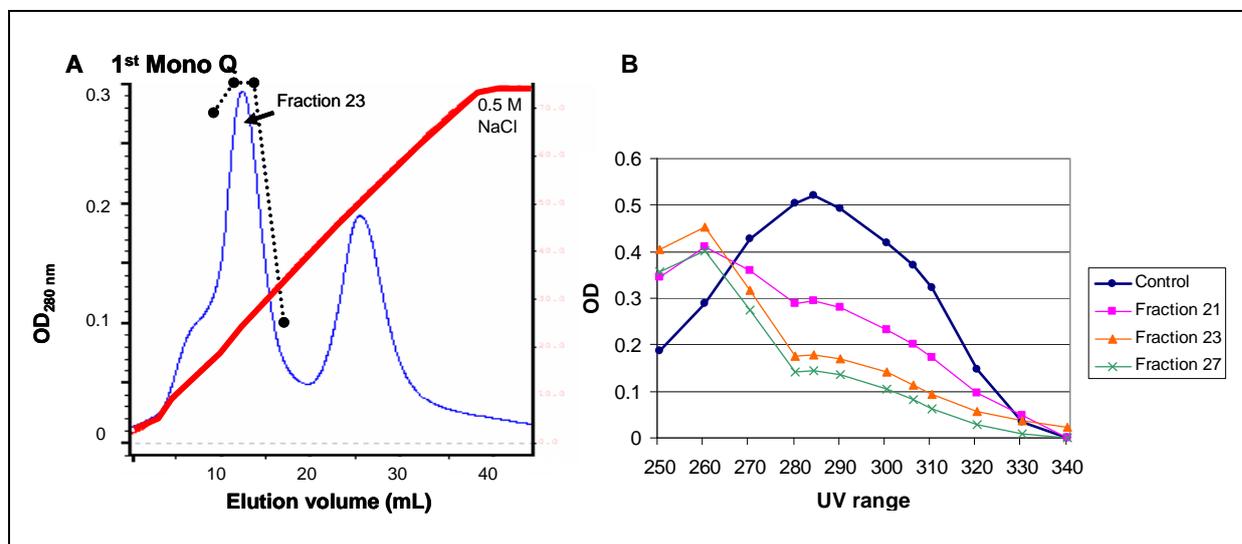

FIGURE 6.3.
First purification stage of the phenolic acid decarboxylase from *Brettanomyces bruxellensis* CB63 by column chromatography. (A) Purification on the Mono Q anion exchange column. The black dashed line represents the area of collected fractions that contained activity. The optical density (OD) measurement at 280 nm is represented by the blue line and the salt gradient is indicated by the thick red line. (B) PCA decarboxylation assay showing activity (after 8 h) in the fractions that were collected. It can be seen that fraction 23 contained the highest activity.
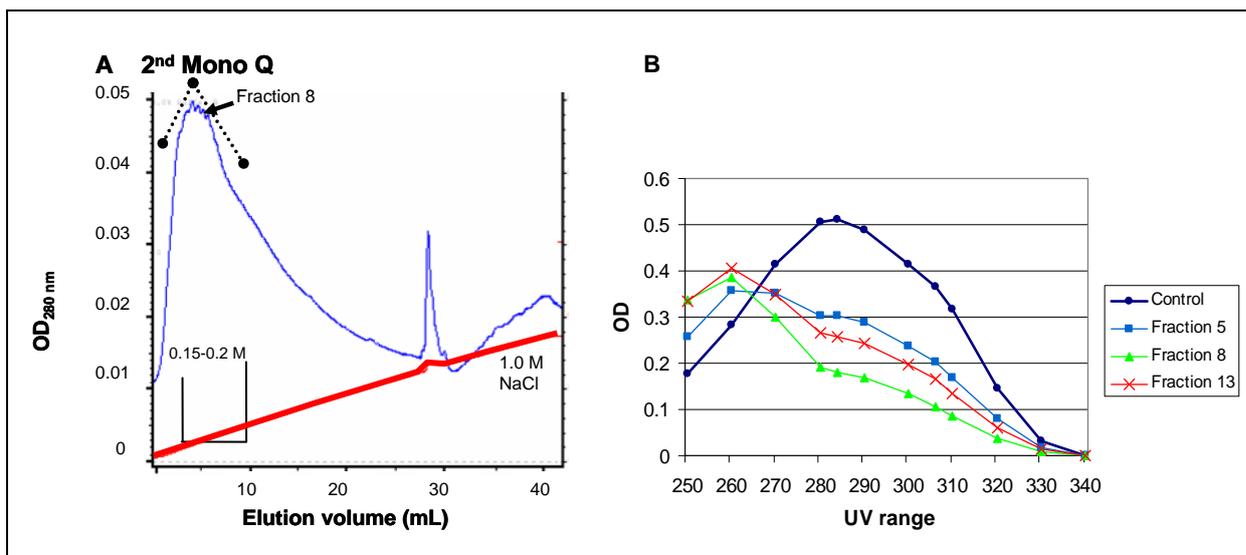
FIGURE 6.4.
Second purification stage of the phenolic acid decarboxylase from *Brettanomyces bruxellensis* CB63 by column chromatography. (A) Purification on the Mono Q anion exchange column. The black dashed line represents the area of collected fractions that contained activity. The optical denisty (OD) measurement at 280 nm is represented by the blue line and the salt gradient is indicated by the thick red line. (B) *p*-Coumaric acid decarboxylation assay showing activity (after 16 hrs) in the fractions that were collected. It can be seen that fraction 13 contained the highest activity (red line).
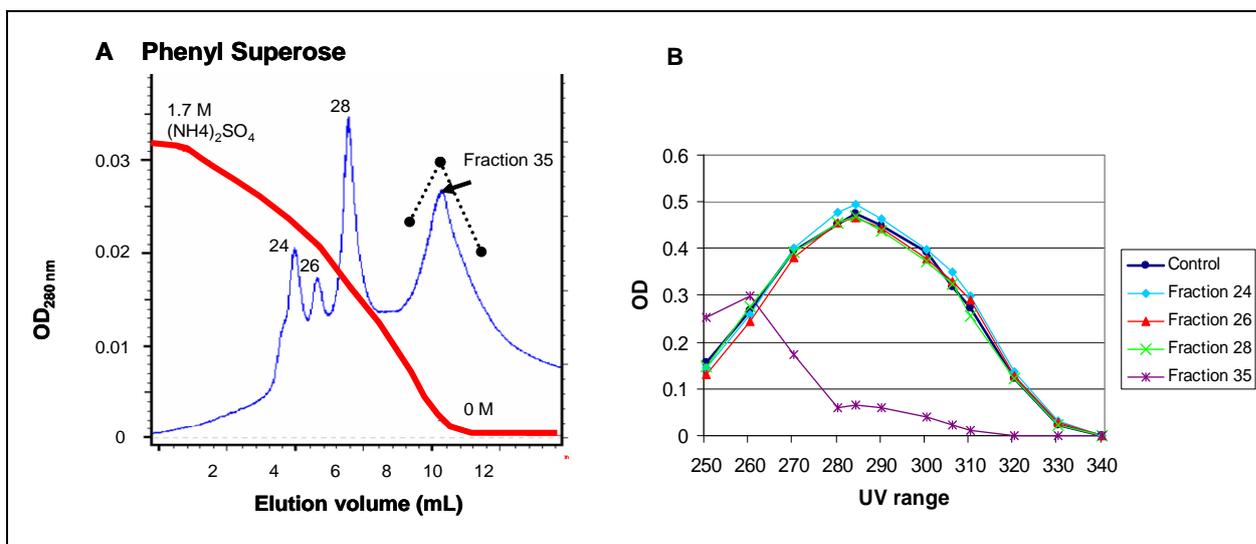

FIGURE 6.5.
Third purification stage of the phenolic acid decarboxylase from *Brettanomyces bruxellensis* CB63 by column chromatography. (A) Purification on the Phenyl superose hydrophobic column. The black dashed line represents the area of collected fractions that contained activity. The optical denisity (OD) measurement at 280 nm is represented by the blue line and the salt gradient is indicated by the thick red line. (B) *p*-Coumaric acid decarboxylation assay showing activity (after 48 h) in the fractions that were collected. It can be seen that from the fractions of the four different peaks obtained (24, 26, 28 and 35) only fraction 35 contained activity.

The SDS-PAGE analysis revealed three distinct protein bands of approximately 22, 23 and 24 kDa after the Phenyl superose purification. These bands were present in all the active fractions, but not in the inactive fractions (Fig. 6.6). The molecular

masses of the proteins were determined by comparison to the electrophoretic mobilities of the protein standard used as marker.

The smallest of the three protein bands had an approximate size of 22 kDa and corresponded with the findings of Edlin *et al.* (1998) that purified and characterised a hydroxycinnamate decarboxylase from a strain of *B. anomalus* with a size of 21.8 kDa*.* Therefore, these three bands were excised from the gel (after Coomassie blue staining) and sent for sequencing.
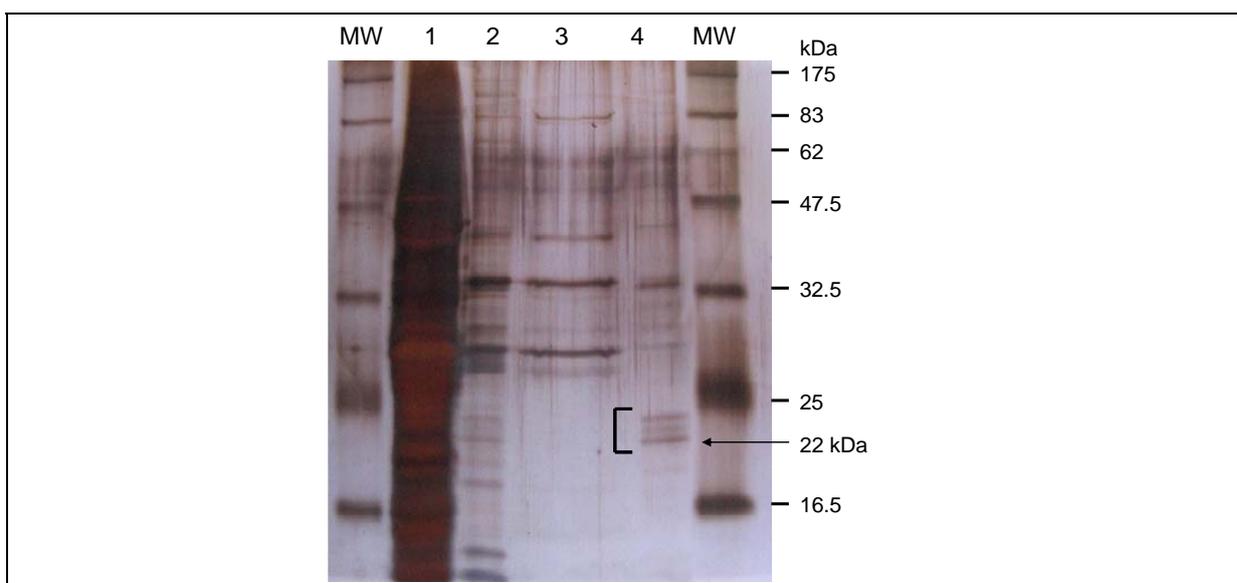

FIGURE 6.6.
SDS-PAGE analysis of the phenolic acid decarboxylase fractions from the different stages of purification. Lanes: (MW) molecular weight marker, (1) crude cell extract (with activity),   (2) 2nd Mono Q (with activity), (3) Phenyl superose – peak 28 (no activity) and (4) Phenyl superose – peak 35 (with activity).

6.3.3 IDENTIFICATION OF PEPTIDE SEQUENCES

Two short peptide sequences were obtained after sequencing (Table 6.4). Insights about these sequences were gained by performing a Blast (NCBI) through the BioEdit software. Neither of the two sequences shared similarities to any cinnamate decarboxylase genes or more specific, phenyl acrylic acid decarboxylase (*PAD*) genes from other microorganisms. Blast hits of peptide sequence 1 compared with thioredoxin peroxidases from other yeasts and peptide sequence 2 yielded no significant relevance other than very low homology with oxidoreductases from different microorganisms.

TABLE 6.4.
Sequences of two peptides

Peptide no.	Peptide sequences
1	DYGVLIPEEGIDFX
2	TLTIEKPAPAFSK

6.3.4 PRIMER DESIGN AND EVALUATION

Different PCR primers were designed following the PCR results of the *S. cerevisiae PAD1* gene-specific primers. The amplification of the PAD1 primer pair with different strains of *B. bruxellensis* (*D. bruxellensis*) did not yield the expected products after several attempts under different PCR conditions (data not shown). Therefore the encoded protein of the *S. cerevisiae* YDR538W phenylacrylic acid decarboxylase (*PAD1*) gene, Pad1p (242 amino acids), was used in a NCBI BLAST for the detection of similar peptides in other yeast species. The BLAST (blastp) results found about 100 hits from a variety of microorgansims and indicated that the Pad1p had charateristics of a Flavoprotein, UbiX (3-polyprenyl-4-hydroxybenzoate decarboxylase involved in Coenzyme metabolism) and also 57%-53% homology with 3-octaprenyl-4-hydroxybenzoate carboxy-lyases).

The most relevant species with the highest homologies were selected for alignments with *S. cerevisiae.* These included a hypothetical protein DEHA0G00935g from *Debaryomyces hansenii* CBS767 (73% identity), a phenylacrylic acid decarboxylase from *Candida albicans* SC5314 (72% identity), a phenylacrylic acid decarboxylase *Aspergillus terreus* NIH2624 (57% identity), and a hypothetical protein FG06435.1 from *Gibberella zeae* PH-1 (62% identity). The alignments in Figure 6.7 show the most homologous regions between the different species from which areas were targetted for the design of the degenerate primers, PADGEN 1 and PADGEN 2.
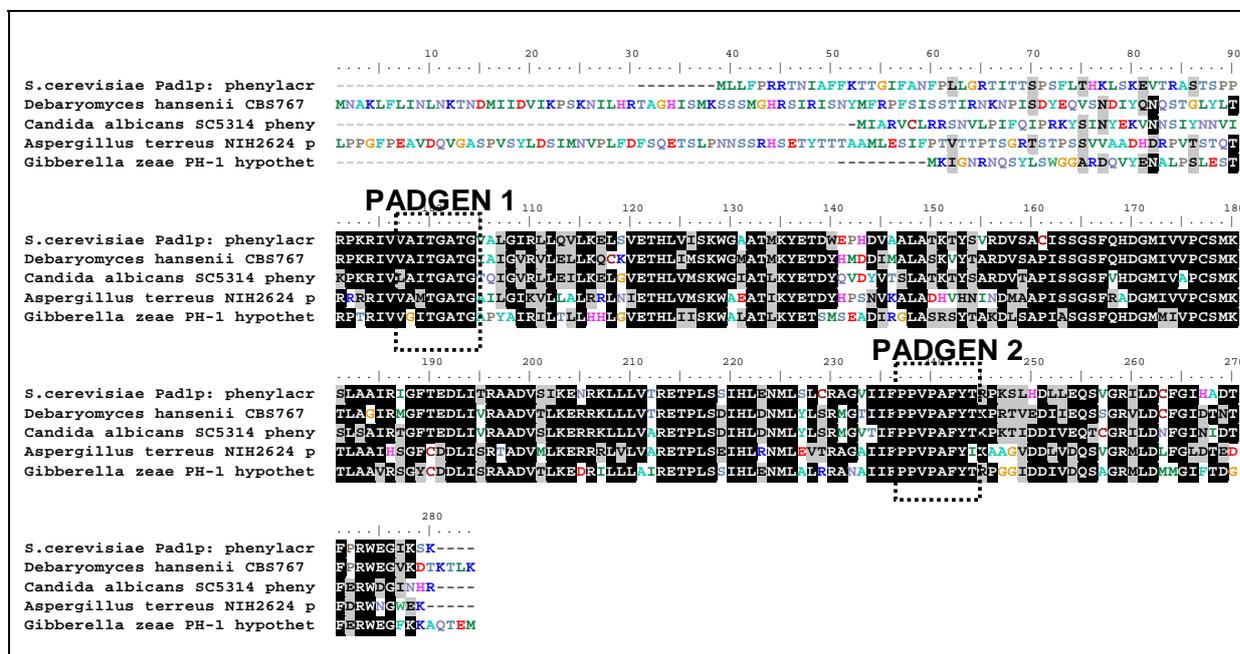

FIGURE 6.7.
Alignment of phenylacrylic acid decarboxylase (PAD) protein sequences of *Saccharomyces cerevisiae* and other species. Areas of degenerate primer design are indicated.

Amplifications with these primers were expected to yield a product of approximately 440 bp. These primers (PADGEN 1/2) and the PAD1 gene-specific primers (PAD1 F/R) were tested as pairs and in combinations on *S. cerevisiae* YNN295 and *B. bruxellensis* CB63. In Figure 6.8 it can be seen that no amplification was obtained for *B. bruxellensis* with any of the primer combinations. The PAD1 F/R primer yielded the expected product of ~900 bp for *S. cerevisiae* (lane 4 control) and also a product of ~750 bp for the PADGEN 1/ PAD1 R combination. This proved the functionality of PADGEN 1, however the reverse degenerate primer was discarded as no amplification was possible when it was used (lanes 1, 3 and 5).
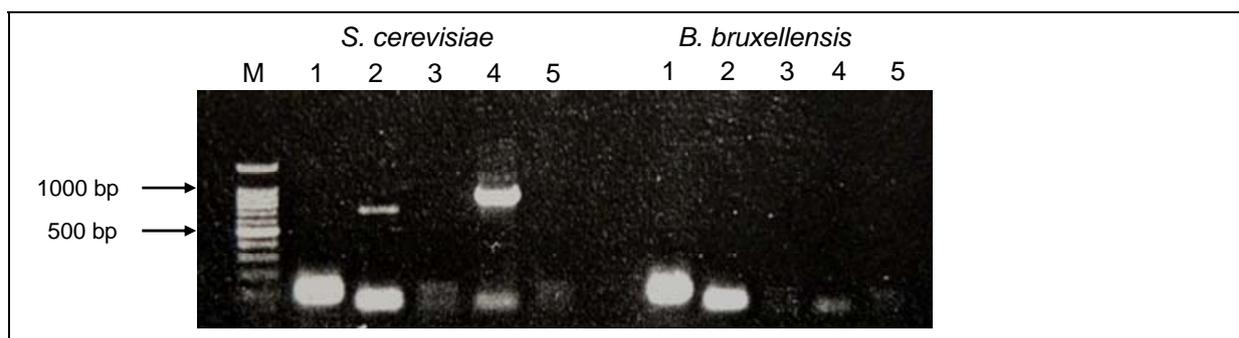

FIGURE 6.8.
PCR products generated with primer sets PADGEN 1/2 and PAD1 F/R on *Saccharomyces cerevisiae* YNN295 and *Brettanomyces bruxellensis* CB63. Lanes (M) 100 bp DNA ladder (Roche marker XIV), (1) PADGEN 1/2, (2) PADGEN 1/ PAD1 R, (3) PADGEN 2/ PAD1 F, (4) PAD1 F/R, (5) PAD1 F/ PADGEN 2.

Additional to the primers designed from the yeast protein sequences, two different bacterial primer sets were evaluated (Table 6.2). The first set PCD 489F and PCD 813R was obtained from Van Beek and Priest (2000) and derived from the alignment of three decarboxylase genes from *Lactobacillus plantarum* (accession no. U63827), *Bacillus pumilus* (accession no. X84815) and *Bacillus subtilis* (accession no. AF017117). The second primer set, LABPAD 1 and LABPAD 2, was designed in this study after a BLAST with the nucleotide sequence of *p*-coumaric acid decarboxylase (PDC) from *Lactobacillus plantarum* showed an alignment with a wider range of LAB species than as described above. From the alignment in Figure 6.9, which included the species of *L. pentosus, L. paracasei, L. fermentum, L. brevis, L. hilgardii, Lactococcus lactis* and *Pediococcus pentosaceus,* two areas were selected for primer design that represented the lowest amount of degeneracy. Both of the primer sets PCD 489F/PCD 813R and LABPAD 1/LABPAD 2, could amplify products of approximately 320 bp and 200 bp respectively from LAB. However, no amplification was obtained for the yeast species of *S. cerevisiae* and *B. bruxellensis* (data not shown). Although previous authors have mentioned that they could not see homology between decarboxylases of LAB and the phenylacrylic acid (cinnamic acid) decarboxylase (PAD1) cloned from *S. cerevisiae,* it was nevertheless evaluated on *Brettanomyces* sp. from a point of interest.
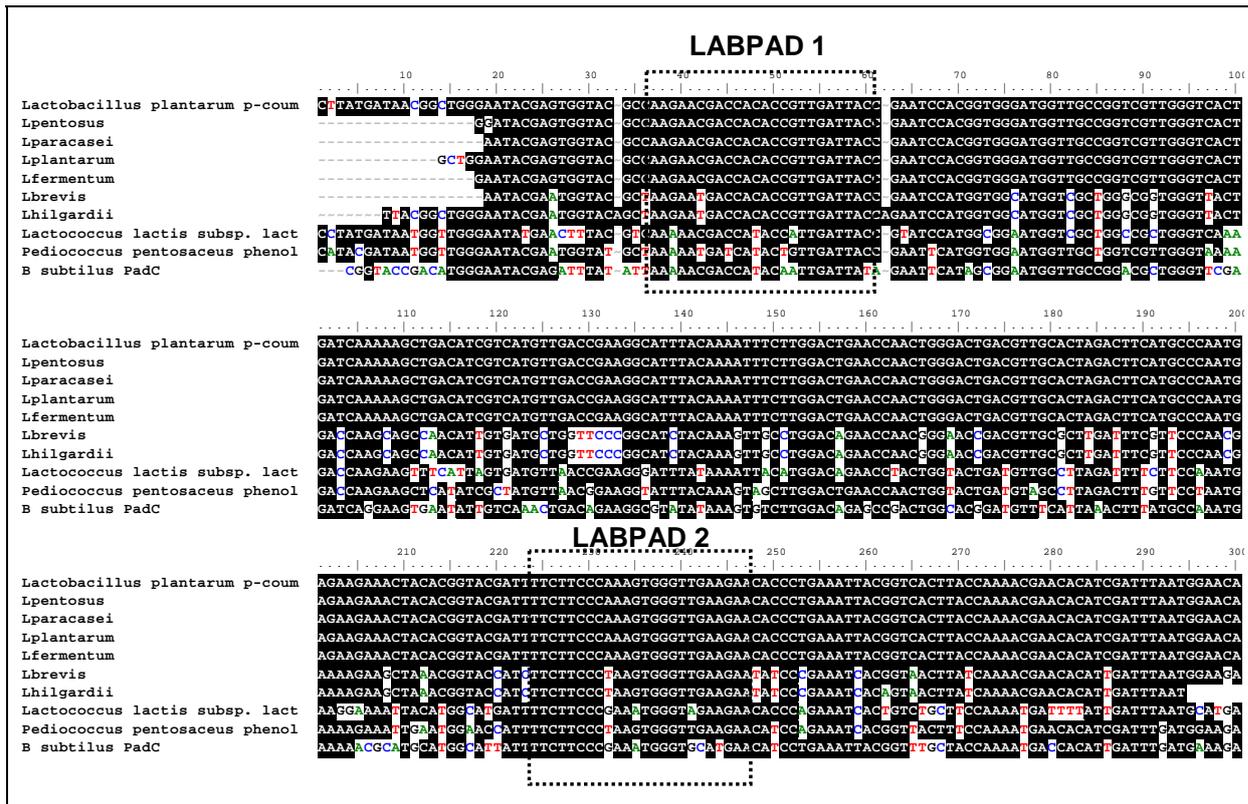
FIGURE 6.9.
Alignment of phenolic acid decarboxylase (PDC) nucleotide sequences of different lactic acid bacteria and a *Bacillus* sp. Areas of primer design are indicated.

With the results from the previous attempts being inconclusive, an alternative approach was investigated. The alignment of the PAD nucleotide sequences from the same yeast genera used in the protein alignments were used to design new degenerate primers (Fig. 6.10). However, in an attempt to decrease the degeneracy of the primers only the sequences from *S. cerevisiae, D. hansenii* (68% identity) and *C. albicans* (68% identity) were used for the primer design. Additionally, primers PADAO 1 and PADAO 2 were selected from the homologous region that would allow for the largest amplification fragment. When these primers were evaluated on gDNA of two *B. bruxellensis* strains, multiple bands were obtained including the expected size of ~465 bp. These primers could also successfully amplify the expected band size in *S. cerevisiae* (Fig. 6.11). Attempts of adjusting the primer annealing temperatures (54-60°C) could not resolve the problem of multiple bands and at higher temperatures the expected band disappeared from *B. bruxellensis* DNA. After several PCRs attempts with adjustments of temperature and time in the cycles and other DNA *Taq* polymerases that were also investigated, the expected bands were obtained in the two strains of *B. bruxellensis* (Fig. 6.12A). The bands were isolated from the gel and purified. The PCR clean-up process lead to a poor DNA yield and concentrations of 3.4 ng/μL and 9.3 ng/μL were obtained for *B. bruxellensis* SA18 and *B. bruxellensis* CB63 respectively. The PCR was repeated using the purified fragments to ensure that the correct products were amplified and to increase the

amount of DNA. In Figure 6.12B it can be seen that amplification was successful and the same band sizes was obtained for *B. bruxellensis* CB63 and *S. cerevisiae* VIN13 but not for *B. bruxellensis* SA18. The latter could have been the result of poor and low DNA quality after the PCR clean-up. Nevertheless, the purified fragment obtained from *B. bruxellensis* CB63 was cloned with pGEM-T-easy and sequenced.


FIGURE 6.10.
Alignment of phenylacrylic acid decarboxylase (PAD) nucleotide sequences of *Saccharomyces cerevisiae* and other species. Areas (positions) of degenerate primer design are indicated. PADAO 1 (277-306), PADAO 2 (712-740). Expected product size of 465 bp.
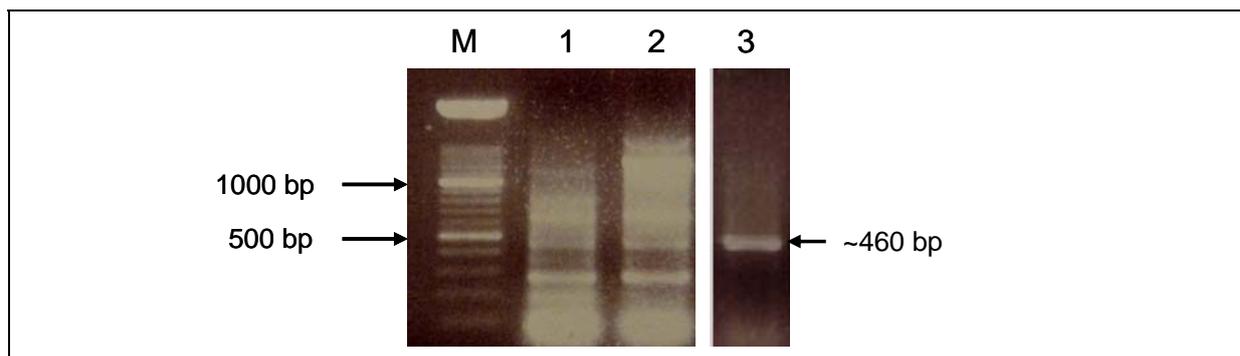
FIGURE 6.11.
PCR products generated with PADAO 1/2 degenerate primers. Lanes (M) 100 bp DNA ladder (Roche marker XIV), (1) *Brettanomyces bruxellensis* SA18, (2) *Brettanomyces bruxellensis* CB63 (3) *Saccharomyces cerevisiae* VIN13.
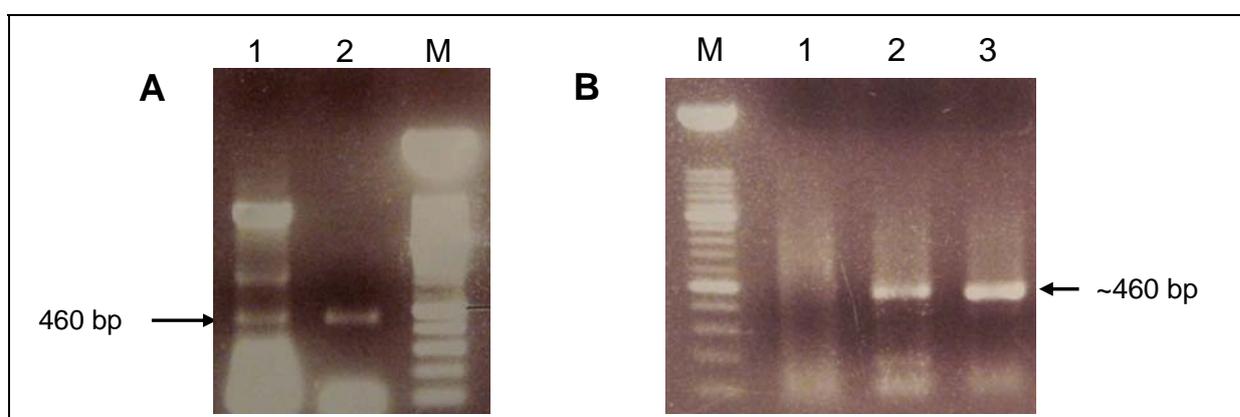
FIGURE 6.12.
PCR products generated with PADAO 1/2 primers. A – PCR showing expected product sizes from gDNA as indicated. B – Repeated PCR performed on purified fragments. Lanes (M) 100 bp DNA ladder (Roche marker XIV), (1) *Brettanomyces bruxellensis* SA18, (2) *Brettanomyces bruxellensis* CB63 (3) *Saccharomyces cerevisiae* VIN13.

6.3.5 PARTIAL *PAD* NUCLEOTIDE SEQUENCE FROM *BRETTANOMYCES*

A sequence of 464 bp was obtained with the PADAO 1/2 primers and aligned with the *PAD* sequences of *S. cerevisiae, D. hansenii* and *C. albicans* (Fig. 6.13). The sequence analysis showed 97% homology with the *PAD*1 gene of *S. cerevisiae.* Although such high sequence homology between genetically divergent species is unlikely, it can not be neglected. From the nucleotide alignments it was evident that this 464 bp segment accounted for the largest homologous domain between the different yeast species. The regions flanking this segment, specifically the 5' regions or initial part of the genes show almost no homology between the yeast species (Figs. 6.7 and 6.13). It is unsure if these regions encode for secretion signals or binding domains of the protein product.

Considering that the short peptide sequences obtained from the protein purification (section 6.2.11) could not be linked with the homologous region that existed between the yeasts, a possibility exists that they form part of the initial 5' flanking region.
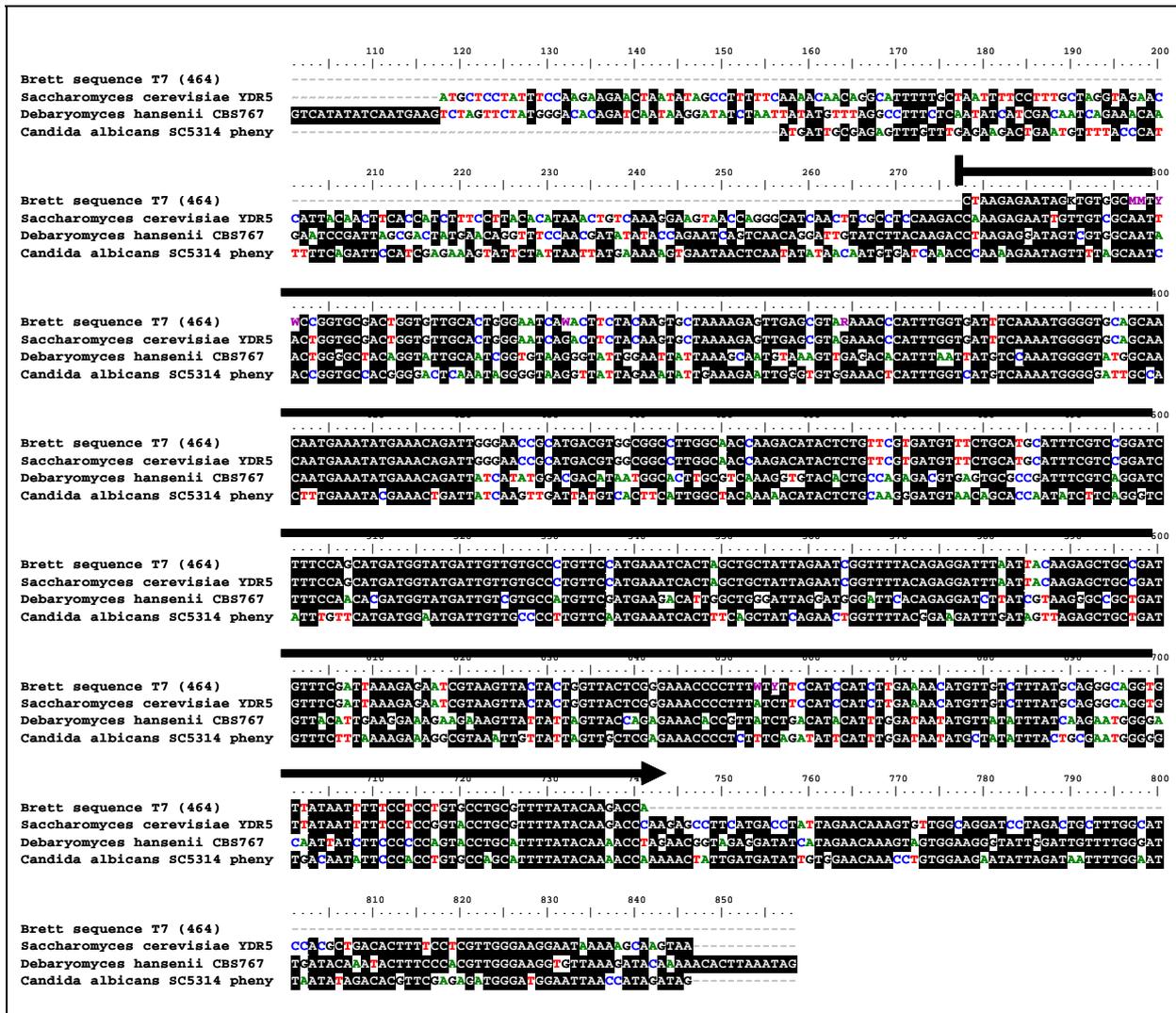
FIGURE 6.13.
Alignment of partial PAD nucleotide sequences of *Brettanomyces bruxellensis* CB63 with other yeasts. The arrow represents the 464 bp product that was obtained which also represented the region with the most homology between the different yeasts PAD sequences.

6.3.6 SOUTHERN BLOT ANALYSIS

Southern blot analysis was performed to identify DNA fragments possibly containing a larger part of or the complete PAD gene sequence. This also served as an independent confirmation of the partial PAD sequence that was obtained.

In Figure 6.14 it can be seen that multiple signals were obtained with the hybridisation of the PAD1 F/R probe on the digested gDNA of *B. bruxellensis* CB63. All the restriction digests yielded bands above the 8.0 kb range, except for *Eco*RI that showed a band estimated at about 3.0 kb. The smaller band from *Eco*RI was targeted as it was more preferred for cloning and inverse PCR purposes and fragments in the range of 3.4 – 2.8 kb were isolated.
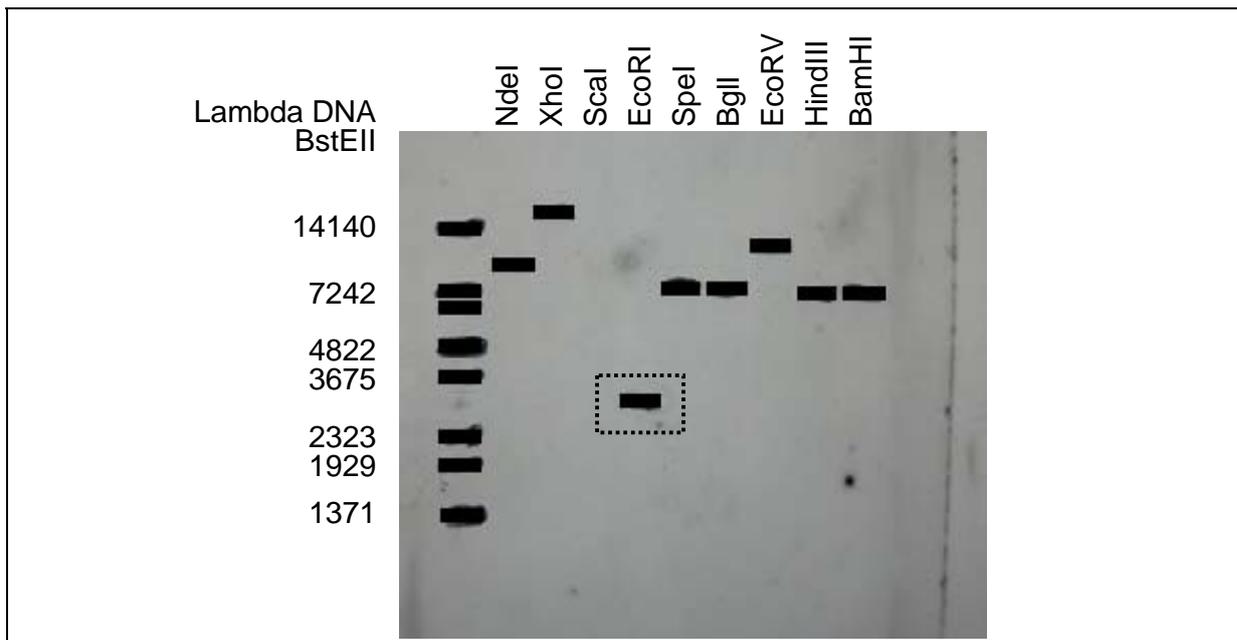
FIGURE 6.14.
Southern Blot autoradiograph. Multiple signals obtained of a possible PAD gene in
Brettanomyces bruxellensis CB63. Lanes indicate the different restriction enymes used.

6.3.7 INVERSE PCR AND CLONING

Inverse PCR (IPCR) is a rapid technique that allows the in vitro amplification of DNA
sequences that flank a region of known sequence. The method uses PCR with the
primers oriented in the reverse direction of the usual orientation. The template for the
reverse primers is a restriction fragment that has been ligated upon itself to form a
circle.

For the purpose of the IPCR, the fragments indicated by the Southern blot (*Eco*RI
digest) ranging from 2.8-3.4 kb were isolated and re-ligated on themselves. These
circled fragments served as template for the IPCR reaction. The IPCR primers listed
in Table 6.2 (PADAO 3R, PADAO 4F, PADAO 7R and PADAO 8F) were designed
from the partial *Brettanomyces* PAD sequence described in section 6.3.5 (Fig. 6.15).
These four primers were evaluated in different combinations. The results showed that
the primer pairs, PADAO 7/8 and PADAO 3/8, successfully amplified products of the
correct sizes with the PADAO 7/8 product being slightly smaller (Fig. 6.16). The
PADAO 3/4 primers yielded no product. The two large fragments of ~3 kb were
isolated and cloned into pGEM-T-easy. Only a few positive integrants were obtained
from the PADAO 3/8 cloning which were verified by enzymatic digestion using *Eco*RI
(cuts pGEM-T-easy 2x), *Sca*I (cuts pGEM-T-easy 1x) and *Nde*I (cuts pGEM-T-easy
1x). Only one of the PADAO 3/8 integrants had a combined size (pGEM-T-easy and
cloned fragment) exceeding 6 kb for each of the three different restriction analysis
(Fig. 6.17). *Eco*RI digest yielded three bands: 1 fragment of 3 kb (possibly pGEM-T-
easy) and two fragments possibly of similar size around 1.7 kb (possibly the cloned

fragment as this also contained an *Eco*RI site). *Sca*I digest yielded two bands: 1 fragment of 3.7 kb and 1 fragment of 2.5 kb (both bands possibly mixture of pGEM-T-easy and cloned fragment and suggested that the cloned fragment contained a *Sca*I restriction site) (3.7 kb + 2.5 kb = 6.3 kb). *Nde*I digest yielded only one band: one fragment of 6.3 kb that suggested that the cloned fragment did not contain a *Nde*I restriction site (3 kb + 3.3 kb = 6.3 kb).
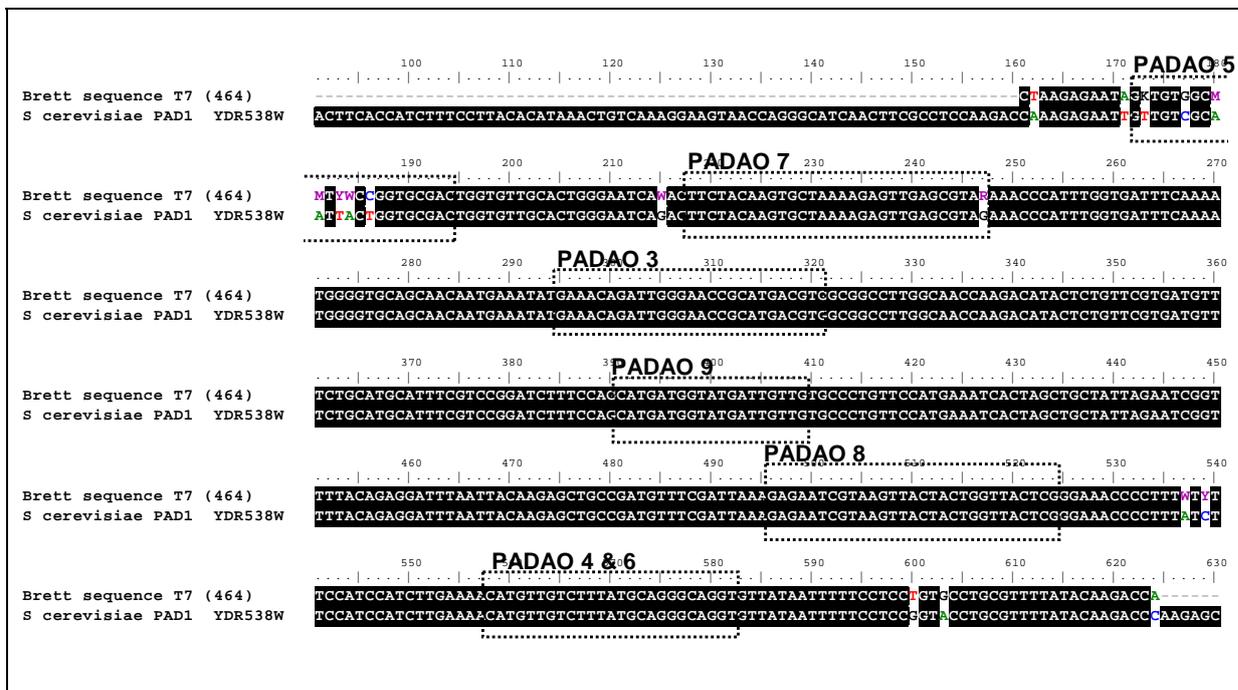
FIGURE 6.15.
Alignment of partial *Brettanomyces* PAD sequence with *Saccharomyces cerevisiae*. Areas of primer design are indicated. PADAO 3 (295-321), PADAO 4 (557-582), PADAO 5 (172-194), PADAO 6 (557-582), PADAO 7 (217-247), PADAO 8 (496-524) and PADAO 9 (391-409).
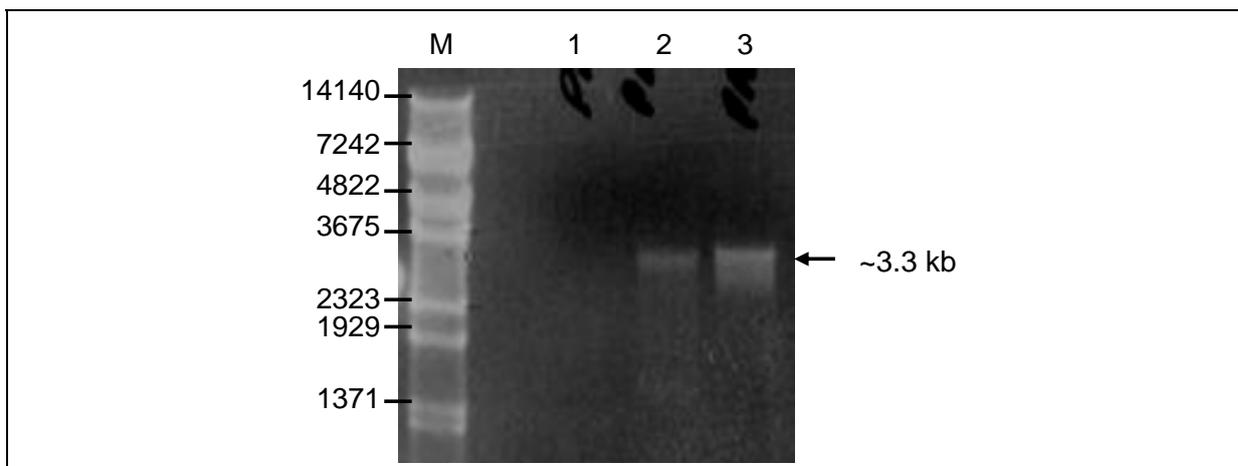

FIGURE 6.16.
IPCR performed on re-ligated fragments. Lanes indicate the primer combinations used. (M) Marker *BstE*II digested lambda DNA (1) PADAO 3/4, (2) PADAO 7/8, (3) PADAO 3/8.
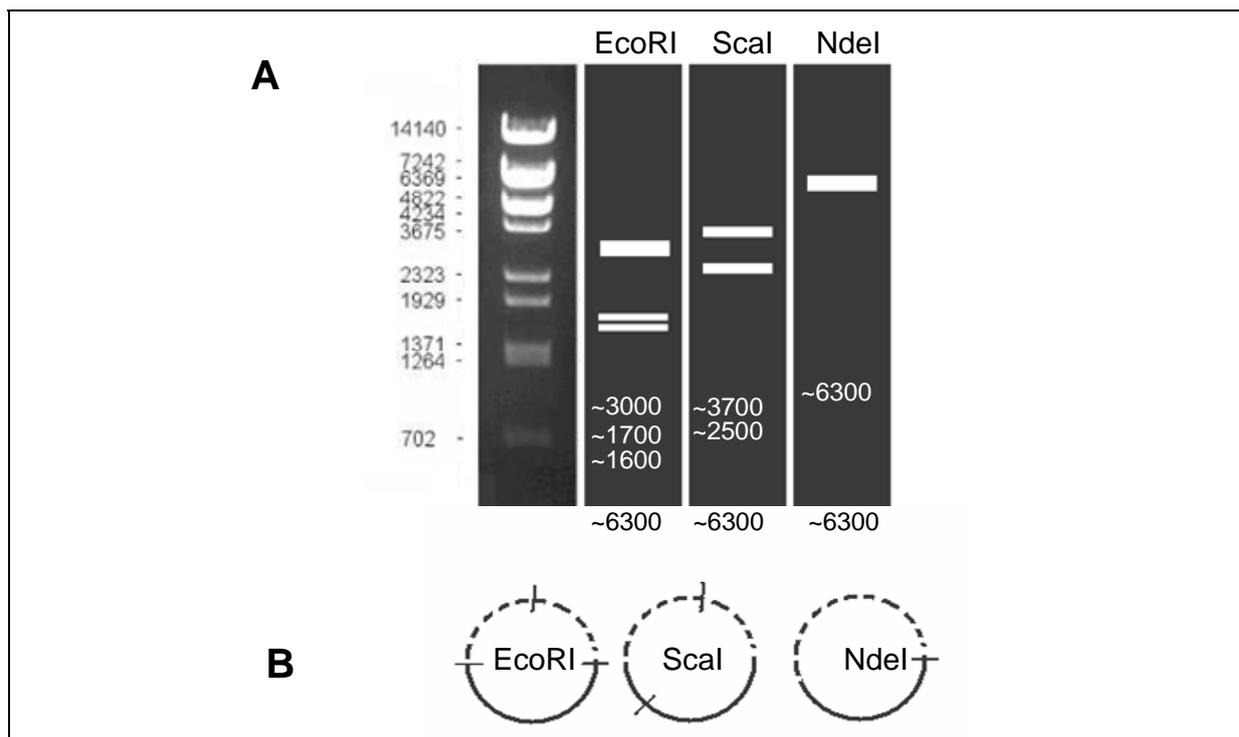
FIGURE 6.17.
Verification of cloned IPCR product in pGEM-T-easy by means of restriction analysis. (A)
The lanes represent the different profiles obtained with the restriction enzymes. (B)
Illustration of the different restriction scenarios from the combination of pGEM-T-easy (solid
line) and cloned IPCR fragment (dashed line).

The results obtained after sequencing the cloned IPCR fragment in the pGEM-T-easy vector posed numerous questions and uncertainties. Based on the results, it was assumed that the correct fragment was cloned (containing the PAD gene flanking regions). However, difficulties with the sequencing suggested otherwise and no sensible sequence could be aquired. When integration of the IPCR product into pGEM-T-easy was tested, multiple bands were obtained including the expected band (data not shown). It was evident that the isolated vectors (plasmids) contained other DNA contaminants. The original plasmids were again transformed and plated under selective pressure (ampillicin resistance) to perform a second plasmid isolation. However, no transformants could be obtained.

After this, none of the results could be repeated by using the exact same procedures. Therefore, various attempts followed that included Southern blots with shorter probes (PADAO 5/6) designed from a partial region of the *S. cerevisiae PAD1* gene, and IPCR with other *Brettanomyces* strains and using other long template *Taq* polymerases. None of these yielded more concrete findings.

6.3.8 PCR SCREENING OF OTHER *BRETTANOMYCES* STRAINS.

Other primers were also derived from the partial *PAD* gene from *B. bruxellensis* CB63 and evaluated. These primers, PADAO 5F, PADAO 6R and PADAO 9F, could succesfully amplify single bands corresponding to the expected sizes from *S. cerevisiae* strains. However, when PCRs were performed on gDNA of *B. bruxellensis* strains, single bands could never be obtained and multiple bands would typically be amplified, including the expected bands (Fig. 6.18). Similar results were also found with PADAO 1/2 primers. These primers are therefore not optimal.
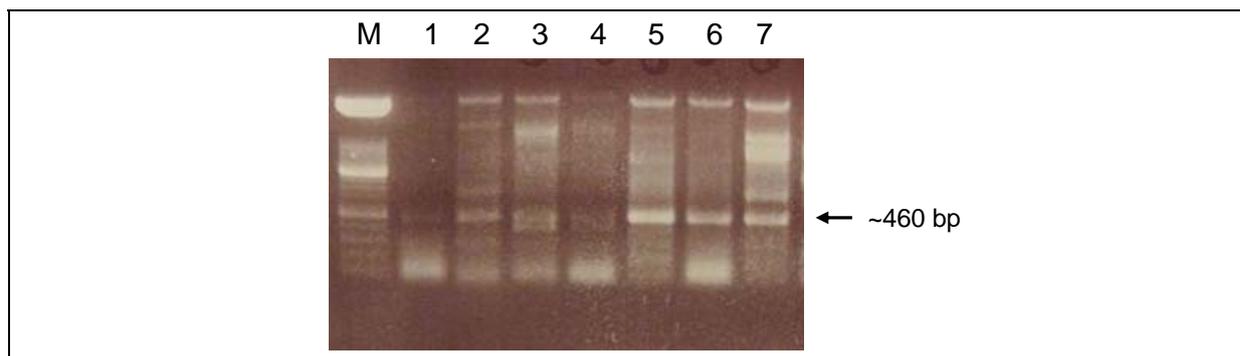

FIGURE 6.18.
PCR with PADAO 5/6 primers. Lanes (M) 100 bp DNA ladder (Roche marker XIV), (1) *S. cerevisiae* VIN13, (2) *B. bruxellensis* TF*,* (3) *B. bruxellensis* SA36, (4) *B. bruxellensis* SA33*,* (5) *B. bruxellensis* SA47, (6) *B. bruxellensis* SA29*,* (7) *B. bruxellensis* SA38.

Additionally in this study, a few attempts at creating and screening sub genomic DNA libraries have also been made. DNA fragments from *Brettanomyces* sp. in the range of 3.4-2.8 kb, as indicated by the Southern blot analysis, have been cloned with the use of different vector systems (GeneJet, pBluescript SKplus). Transformation efficiency has however been very low and success is yet to be obtained.

6.4 CONCLUSIONS

This study focussed on gaining more information about a phenylacrylic (hydroxycinnamic) acid decarboxylase from the species *B. bruxellensis* isolated from red wine. Various strategies were investigated which included protein purification and numerous molecular DNA techniques in an attempt to obtain the genetic sequences coding for the enzyme. The decarboxylase activity of this yeast species towards *p*-coumaric acid was demonstrated in the enzymatic assays during the purification steps. The relevance of the peptide sequences that were isolated remain unknown and must be determined. Furthermore, the partial PAD nucleotide sequence obtained from a wine species of *B. bruxellensis* showed very high homology with the *S. cerevisiae PAD*1 gene and therefore warrants for more intrinsic investigations.

Future research will continue with some of these strategies untill the complete phenolic acid decarboxylase (*PAD*) gene sequence have been obtained for *Brettanomyces* species.

6.5 ACKNOWLEDGEMENTS

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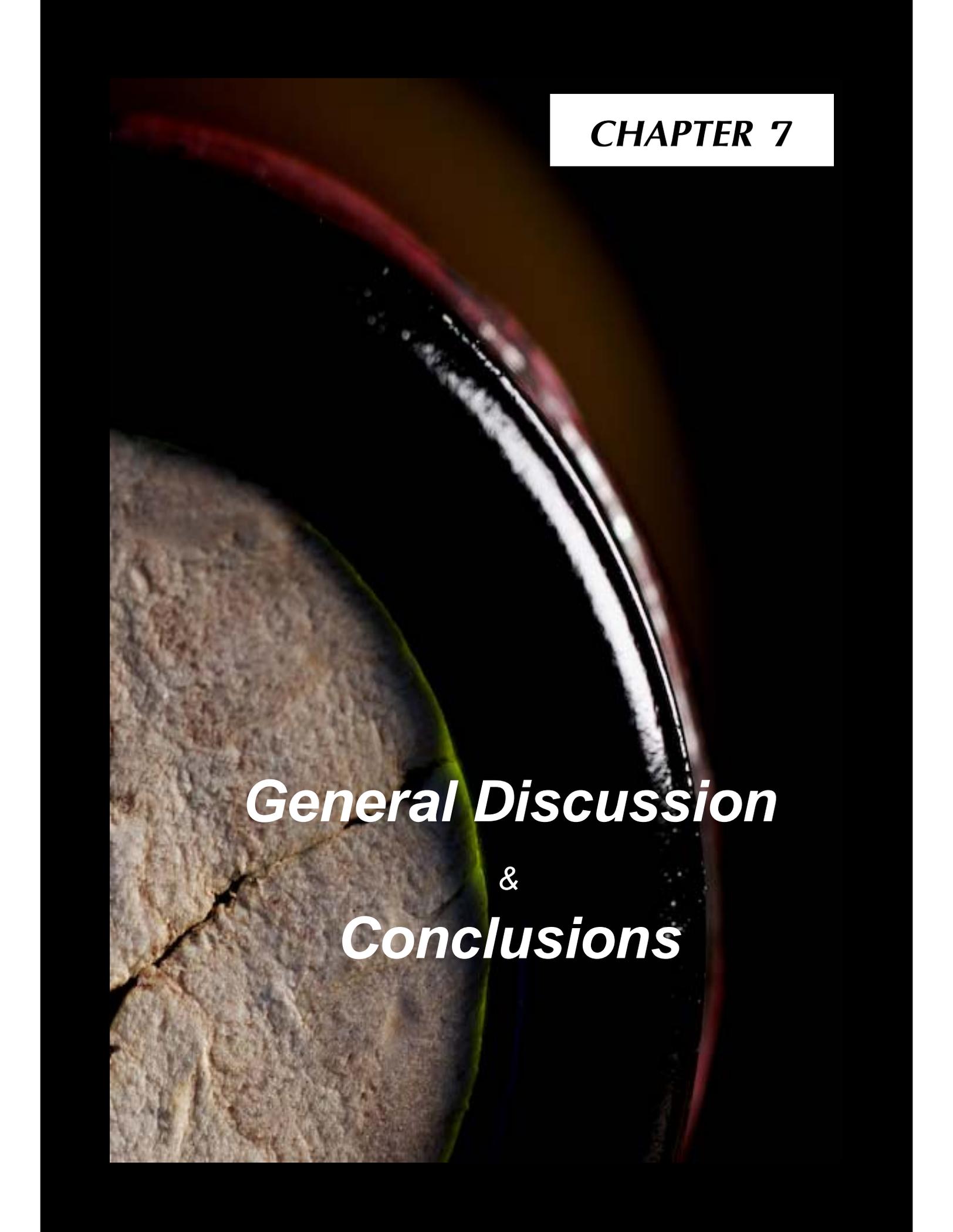
General Discussion

&

Conclusions

7. GENERAL DISCUSSION AND CONCLUSIONS

7.1 GENERAL DISCUSSION

Over the last two decades many research investigations have been performed in an attempt to improve quality, strengthen risk management and decrease the processing costs of winemaking. Along with developments such as optimising starter cultures for alcoholic and malolactic fermentation, also came a better general understanding of the influence of viticultural practices, winemaking procedures and the impact of global climate changes. In addition, the more knowledgeable consumers and the increasing consumer expectation for higher quality products have initiated stricter regulations concerning modern winemaking. This has brought about trends such as producing lower alcohol wine styles and using less sulfur dioxide (SO_2) during the winemaking process. In the latter case, the consequent risk lies in the lower levels of free SO_2 that are available to act as antimicrobial agent, especially under conditions of high pH. These conditions generate a favourable environment for opportunistic spoilage microbes. It is for this reason imperative that the microorganisms already presented from the grapes are managed as these determine the nutritional and sensorial value of the final product.

The main spoilage microbes that cause great economical losses in the wine industry belong to the non-*Saccharomyces* yeasts and lactic acid bacteria. This dissertation focussed on one particular yeast species that is classified in the non-*Saccharomyces* or wild yeast group and is regarded to be detrimental to wine quality.

Yeast of the genus *Brettanomyces* or its teleomorph *Dekkera* has been identified as one of the most controversial spoilage microorganisms in wine largely due to the production of several compounds that are detrimental to the organoleptic quality of wine, of which volatile phenols is the most important (Fugelsang *et al.*, 1993; Ibeas *et al.*, 1996; Chatonnet *et al.*, 1997; Licker *et al.*, 1998; Mansfield *et al.*, 2002; Aguilar-Uscanga *et al.*, 2003; Snowdon *et al.,* 2006). The limited available knowledge about *Brettanomyces* and the potential high impact that this yeast could have on wine quality has attracted much attention in recent years.

The first objective of this study was the optimisation of the isolation media for *Brettanomyces* as we detected growth of other wine microbes on the media previously described (Rodriques *et al.*, 2001). The necessity of accurate isolation and identification methods was of utmost importance as wine isolates were needed to base the rest of the study on. Results showed that DBDM and WLN medium with the supplements described was the most optimal (Chapter 3). This aspect is also of importance as traditional identification methods such as microbiological plating are used in laboratories for the screening of wine samples for viable *Brettanomyces* cells. However, the limitations of this approach lies in the long incubation periods that are required and false results that can be obtained due to the viable but non-culturable (VBNC) state of these yeasts. From previous studies, it became evident that molecular techniques (PCR)

should be applied alongside traditional methods to ensure that more accurate and reliable results can be presented to the industry. This approach, although still time-consuming as DNA isolation procedures are dependant on colony formation on agar media, is very reliable for identification purposes. Further molecular based approaches have been investigated by research groups world-wide (see Chapter 2) in attempts to minimise the time-constraints associated with extensive incubation periods. For this purpose, the use of quantitative real-time PCR directly on total DNA extracted from wine holds great promise for future routine detection systems of this yeast species. This rapid and very sensitive approach is currently still in developmental stages although several authors have reported detection levels of 10 cells per millilitre of wine (Phister and Mills, 2003; Delaherche *et al.*, 2004). However, the viability of this technique (qRT-PCR) and other molecular methods for routine analysis in industry currently have drawbacks due to high input costs.

Focusing on *Brettanomyces* identification, this study also investigated methods of strain identification and differentiation. This followed after questions were raised regarding strain variability and the production of volatile phenols in wine. This is important as under certain conditions winemakers believe that *Brettanomyces* contribute to complexity. In this study evidence was provided on the existence of different strains of *B. bruxellensis* during winemaking and it was found that genetically dissimilar strains produced variable levels of volatile phenols and showed different growth rates under the same conditions (see Chapters 4 and 5). The different techniques that were evaluated for the purpose of intraspecies identification and differentiation made an original contribution as very little knowledge was available on *Brettanomyces* strain identification at the time. However, since this study commenced several other studies have been initiated to gain a more comprehensive understanding on the genetic diversity of *Brettanomyces* wine isolates (Bellon *et al.*, 2003; Conterno *et al.*, 2006 ; Martorell *et al.*, 2006; Curtin *et al.*, 2007; Miot-Sertier and Lonvaud-Funel, 2007; Renouf *et al.*, 2006). These studies were in agreement with this work and indicated that a large degree of diversity exists among wine strains of *Brettanomyces* and that further investigations are eminent.

As this study largely focused on specific methods and techniques that allow for strain identification, little attention was given to characterisation studies apart from the evaluation of some of the different strains for the production of volatile phenols. Further characterisation studies can now follow on the different strains that have been identified and future focuses should include the variable resistance of *B. bruxellensis* strains towards SO_2 and ethanol concentrations. The unpredictable behaviour of this yeast to resume growth from a VBNC state and the factors that lead to the induction of this state should be of particular interest. The factors that influence the viability of *B. bruxellensis* populations in wines and the physiological mechanisms underlying their survival are not clearly understood and should therefore be considered for future research initiatives.

7.2 SOME PERSPECTIVES

FTIR spectroscopy.

The use of FTIR spectroscopy combined with chemometrics which was included in this dissertation (Chapter 5) holds good promise as an alternative approach for the differentiation of *B. bruxellensis* strains. Traditional methods based on morphological and physiological tests are not suitable for strain discrimination and molecular techniques are often inconclusive and necessitate various extensive preparation steps. FTIR spectroscopy (FTMIR and FTIR-ATR) provides a reliable alternative to molecular based methods for the typing of *B. bruxellensis* strains as the constructed models showed classification potential and consequent discriminating capabilities.

The application of FTIR spectroscopic techniques for the differentiation and classification of microorganisms has been shown previously and holds promise for the future screening of *B. bruxellensis* strains. However, this requires the establishment of a substantial spectral database for these specific microorganisms on which predictive modelling could be evaluated.

Genetic investigations

Despite the economic importance of *B. bruxellensis* and the many research initiatives that have been performed on this yeast over the years, this species remains understudied on genetic level and its exact physiological behaviour in wine is yet to be determined. Surprisingly little genetic investigations have been undertaken on *Brettanomyces* species apart from the ribosomal genes (rRNA) that are typically targeted for the development of molecular identification procedures and phylogenetic studies. Woolfit *et al.* (2007) has initiated a large genome sequencing project on *D. bruxellensis* and has revealed the presence of several novel genes and its likely phylogenetic relationship with other hemiascomycete yeasts. These data provide a valuable genetic resource to the evolution and physiological capabilities of this yeast species. In particular, more information about the encoding genes and the enzymes involved in the formation of volatile phenols needs to be ascertained. Discovery of the complete cinnamate decarboxylase and vinyl phenol reductase gene sequences of *B. bruxellensis* will not only be a profound fundamental breakthrough, but can also potentially serve as targets for the development of alternative rapid detection systems. Moreover, the purified enzymes can be studied with the aim of developing additives for the winemaking process that can ultimately prevent volatile phenol production, should it be required. Although we were only partially successful in this study to gain information relating to the cinnamate decarboxylase gene involved in the volatile phenol production pathway of *B. bruxellensis*, we were able to establish a useful platform for further continuation.

7.3 FINAL CONCLUSION

The role of microorganisms and the importance of wine microbiology is an area that remains neglected. The susceptibility of wine to spoilage by microorganisms such as *Brettanomyces* yeasts only seems to be considered by winemakers as a problem once it is realised that the wine quality is affected. For this reason, wine scientists should emphasise more on a specific awareness about the significance of microbiology and its impact on wine quality in future directions of collaborative research with industry.

The data that was gathered and the knowledge that was gained in this dissertation made an original contribution to the current understanding of the contentious yeast species *Brettanomyces/Dekkera* during the winemaking process. The results show how this species is common during winemaking and that the continued development of more sensitive methods alongside fundamental investigations is required for its frequent monitoring in the winemaking environment.

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CHAPTER 8

Addendums

8. ADDENDUMS

A: WINELAND ARTICLE 1 (WINELAND 198: 65-67)

Wynboer A Technical Guide for Wine Producers

Brettanomyces/Dekkera during winemaking - Part 1

What the winemaker should know

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Introduction

Winemaking appears to be a relatively simple process; the conversion of grape sugar into ethanol by wine yeasts. However, the microbiology of wine production is much more complex. Wine quality is an intrinsic compilation of unique characteristics, not only from the wine yeast (*Saccharomyces cerevisiae*), but also from the bacteria and wild yeasts present from the onset of fermentation. These yeasts include the genera *Candida*, *Debaryomyces*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Pichia*, *Toruloaspora*, *Zygosaccharomyces* and *Brettanomyces*. However, from the onset of alcoholic fermentation the influence of the wild yeasts diminishes quickly as the increase of ethanol concentrations drastically influences their growth (Loureiro and Malfeito-Ferreira, 2003).

One of the yeasts that can withstand the toxicity of ethanol levels and which has become the latest concern for most winemakers as a result of phenolic off-flavours, is *Brettanomyces/Dekkera* (Licker et al., 1999; Loureiro and Malfeito-Ferreira, 2003).

The name *Dekkera*, which is often mentioned together with *Brettanomyces* in literature, refers to the interchangeable name for Brettanomyces (Barnett et al., 1990; Heresztyn, 1986). *Dekkera* is the sporogenous form (ascospore-forming) or sexual teleomorph of *Brettanomyces*.

Wines typically associated with a "Bretty character" is commonly recognised by aromatic defects ranging from medicinal smells (elastoplast, band-aid) to farmyard-like odours (horse sweat, wet animal, wet leather) and even spicy clove-like aromas (Licker et al., 1999). However, the true significance of Brett during winemaking is vague and therefore the "Brett problem" has spawned an interest of international magnitude.

Brett in my wine?

What are we talking about? From a microbiologist's viewpoint, are there actual Brett cells? Or are we talking about a flavour characteristic? The latter being more common amongst winemakers and consumers. If we talk about actual cells, then there's a very specific problem. If we are talking about an already formed off-flavour, the question

remains; was it produced by Brett? Research is ongoing in an attempt to answer this question.

Origin and occurrence of Brett

What's in a name? It was shown in 1904 that a slower secondary fermentation followed the primary fermentation in the production of an English stock beer (Claussen, 1904). This fermentation was induced by a non-spore forming non-*Saccharomyces* yeast. This yeast or ''British brewing fungus'' was designated Brettanomyces, seeing that ''Brettano'' means British brewer and ''myces'' means fungus (Licker et al., 1999). The flavours that were produced by *Brettanomyces* during the secondary fermentation were very characteristic of the British beer of that time.

With regards to wine, *Brettanomyces* have been isolated from wineries all around the world and it is therefore unlikely that winemaking regions exist that are free of potential *Brettanomyces* growth (Arvik and Henick-Kling, 2002). There is evidence that Brett comes from the grapes and survives in the vineyard but difficulties are experienced with the detection and isolation of Brett from grapes (personal communication). However, preliminary results indicate very low Brett numbers which are not to be seen as reason for concern, but rather as caution, as with all the other wine microbes coming from the grapes (unpublished data). The detection levels are very low and this is typical of organisms renowned for having a VBNC (viable but non-culturable) state (Millet and Lonvaud-Funel, 2000). It is also interesting to note that Bretts have been isolated from fruit flies (*Drosophila*) and bees, and this may additionally serve as a source of contamination (Henick-Kling, 1998). It appears however, as if these yeasts are more established in wineries (Chatonnet et al., 1995; Fugelsang, 1997) and this can be ascribed to more suitable niches that exist within a winery as a result of ineffective cleaning of the crushing equipment, transfer lines and the barrels (wooden cooperage). Chatonnet et al. (1995) states that used barrels are the most notorious source of Brett contamination.

Origin of Brett flavour

The substrates from which "Bretty aromas" are produced are called hydroxycinnamic acids (also generally described as phenolic acids). Phenolic acids are abundant in plants (e.g. grapes) and form part of the structure of plant cell walls (Barthelmebs et al., 2000a). The action of enzymes (hemicellulases) from fungi and bacteria can however release these weak acids. Phenolic acids serve as natural toxins for the plant to inhibit the growth of unwanted microbes (Barthelmebs et al., 2000b). Some microbes can however degrade these acids, specifically the hydroxycinnamic acids p-coumaric (PCA), ferulic (FA) and caffeic acid (CA), as a method of survival. However, the consequence of this action is the production of phenolic off-flavours. The organisms involved include wine bacteria (lactic acid bacteria), wild yeasts (e.g. Brett) and the wine yeast S. cerevisiae. Some contribute indirectly to the so-called "Bretty flavour" (production of precursors, vinyl derivatives) and others more directly (production of ethyl derivatives). Nevertheless, the significance of the flavours produced by these yeasts is argued both ways. Some consider the slightest hint of Brett character as an indication of spoilage. Others see the contribution of these contentious yeasts as an integral part of a red wine's complexity.
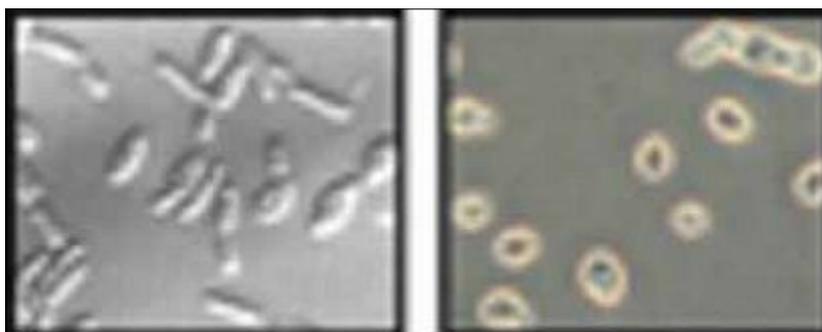

**Figure 1. *Brettanomyces/Dekkera* under a light microscope (1000x**

microscopic).
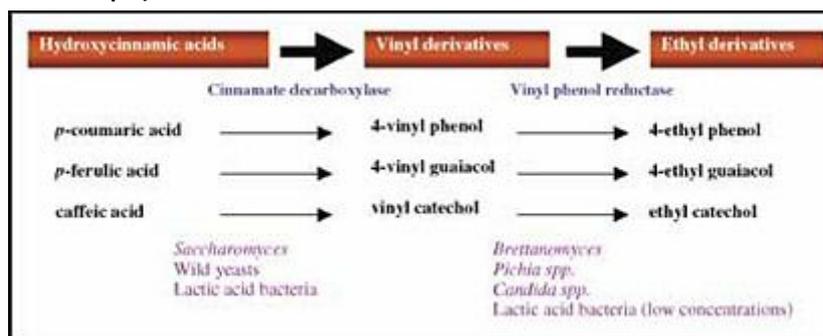
Figure 2. Production of volatile phenols by wine micro-organisms.

Morphology and physiology

Contrary to other wine microbes, Bretts are very unique in the fact that a colony usually exhibits mixed morphology (see Fig. 1), ranging from frequently ogival (pointed arch) cells; spherical; ellipsoidal; cylindrical to elongated (Kurtzmann and Fell, 1998). This makes it difficult to do an identification based on simple microscopy as the shapes represent a large diversity of yeasts. The cells vary in size and range from (1.5-3.5) to (2.0-19.0) µm. This is why the isolation and identification of yeasts always need verification by molecular methods. Low Brett numbers in wine cannot be detected by traditional plating methods and as part of quality control systems; it should be considered monitoring the level of microbial contamination and proliferation by the use of viability techniques (methylene blue staining, epifluorescence) (Du Toit et al., 2005).

With regards to their taxonomy, *Brettanomyces/Dekkera* have seen numerous reclassifications in the last few years and there are currently five recognised species: *D. bruxellensis, D. anomala, B. custersianus, B. naardenensis* and *B. nanus* (Egli and Henick-Kling, 2001). From the research that has been done, *D. bruxellensis* have thus far been associated with wine, and more recently *D. anomala* have also been added after the molecular techniques improved for the identification of isolates (Cocolin et al., 2004). Amongst these two species of Brett, there are many strains that exist and it is still unclear if all strains are bad (some may be harmless). This is being investigated as part of my research and other international research groups.

Factors you should know (Metabolism)

Like most yeasts, *Brettanomyces* can utilise a large number of substrates for their sugar requirement. Any available amounts of glucose, fructose, arabinose and trehalose contributing to the residual sugar of the wine will greatly suit their growth or carbon source needs along with alcohols already present such as ethanol and glycerol. Wine yeasts (Saccharomyces) often do not metabolise the last bit of sugar because of the gradual rise in ethanol levels and consequently small amounts of residual sugar are remaining (<2.0 g/l) that can be utilised by some spoilage wild yeasts (e.g. *Brettanomyces, Schizosaccharomyces*) (Loureiro and Malfeito-Ferreira, 2003). In addition, instances of stuck/sluggish fermentation may create sufficient time for a Brett bloom and any residual sugar would be beneficial to Brett for metabolising ethanol as a carbon source. Brett however, do not need sugar, but it gives them energy to survive. With regards to nitrogen sources, care should be taken with the addition of diammonium phosphate (DAP). Analysis for yeast assimilable nitrogen (YAP) should be performed so that the winemaker can avoid unnecessary additions. Excessive usage in an attempt to improve slow fermentations could lead to increased levels of nitrogen. This could further encourage the growth of Bretts and other spoilage microbes during later stages, such as post MLF (Godden et al., 2004).

Spoilage products

The growth of *Brettanomyces/Dekkera* in wine has been associated with various forms of spoilage including cloudiness, pellicle formation (less common) and a range of off-flavours (Chatonnet et al., 1995; Henick-Kling, 1998). Depending on the conditions and

precursors available, these yeasts can produce acetic acid, contributing to wine's volatile acidity (Arvik and Henick-Kling, 2002; Guadalupe Aguilar Uscanga et al., 2003); tetrahydropyridines that are reminiscent of mouse-urine (mousiness) (Heresztyn, 1986); isovaleric acid, known for a rancid cheesy aroma and volatile phenols (Edlin et al., 1995; Dias et al., 2003; Coghe et al., 2004). The latter is determined by a few factors, including the substrate and enzyme activity. E.g. if the substrate is p-coumaric acid, it will be converted by an enzyme to form the vinyl derivative, 4-vinyl phenol, which will be further reduced by another enzyme to form the ethyl form or 4-ethyl phenol (See Fig. 2). It is the ethyl derivatives that are typically associated with "Bretty character", as the vinyl derivatives (precursor of ethyl derivatives) can be produced by many other wine microbes as already mentioned (Cavin et al., 1993). Excluding the influence of grape variety, the enzymatic activity or capability of the various organisms indicated differs, depending on the wine conditions, and therefore the production levels, precursors and concentrations of these volatile phenols will also vary in different wines.

As mentioned earlier, there are currently many aromatic descriptions associated with Brett. In many cases a large quantity is falsely attributed to the Brett character, with new ones often developing. The common descriptions include elastoplast, medicinal, sweaty, spicy, smoky, mouldy cheese, vomit, barnyard, horsey etc. (Table 1). There are many others as well, but it is important to note that these aromas associated with the volatile phenols (vinyl- and ethyl-derivatives) produced in wine are perceived differently by people. It all depends on the concentrations and ratios in which these compounds exist and co-exist in wine (see Fig. 2). For example, in a light-bodied red wine with little oak influence, the sensory perception threshold of 4-ethyl phenol may be as low as c. 350 µg/l, compared with 1000 µg/l in a full-bodied red wine with intense fruit and considerable oak influence.

Important: What many should realise is that the precursors of the ethyl derivatives, namely the vinyl derivatives (e.g. 4-vinyl phenol and 4-vinyl guaiacol) can also be produced by the wine yeast, by other non-*Saccharomyces* yeasts (other than Brett) and by lactic acid bacteria (LAB) (van Wyk and Rogers, 2000; Cerdán et al., 2001; Cavin et al., 1997). Therefore, all these organisms contribute to the phenolic off-flavours and form part of the so-called "Bretty character" during some stage. Focusing only on the ethyl derivatives, there have been a few questions: (1) Is it really only Brett that can produce these in wine? (2) Are all strains of Brett equally bad? In my opinion one of the most important issues, (3) What are the concentrations of the substrates PCA and FA (free form) in the grape juice as these will have the most influence on the potential formation of volatile off-flavours? (4) Why aren't the substrates measured? (5) Shouldn't it become a standard measurement of grape juice? How do these levels differ between cultivars? These questions are some of the aspects investigated and will be answered in time.

Table 1. Typical "Bretty" aromas.

Compound	Character
4-EP (ethyl phenol)	Band-aid, elastoplast
4-EG (ethyl guaiacol)	Smoky, spicy, cloves
4-EC (ethyl catechol)	Sweaty, horsey
Isovaleric acid	Rancid, cheesy, vomit
Combination of all the above	Horsey, barnyard, mouldy

Concluding remarks

The microbiology of wine is very complex and this makes it often difficult to pin-point the exact problem and its origin in a habitat such as wine. Nonetheless, large-scale investigations on controlling wine spoilage in attempt to improve wine quality have been undergone for many years with great results and improvements originating. In order to build on this it is very important that the winemaking community, winemakers, researchers, and consumers communicate and keep each other informed.

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8. ADDENDUMS

B: WINELAND ARTICLE 2 (WINELAND 199: 66-67)

Wynboer A Technical Guide for Wine Producers

Brettanomyces/Dekkera during winemaking - Part 2

What the winemaker could do?

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Summary

In order to control the levels of volatile phenol production in wine, Brett populations have to be kept low throughout the winemaking process. This way the volatile phenol contribution may well add to the complexity of the wine, rather than detracting from the favourable aroma profile when at higher concentrations. This section (part 2) follows on a previous section entitled "Brettanomyces during winemaking - What the winemaker should know" (Winelands, Feb 2006) and mentions some strategies for controlling Brett and minimising Brett-associated off-flavours.

Introduction

As with numerous other wine spoilage microbes, it is not a question of prevention, but rather a question of control. Bretts (referring to actual cells) cannot be prevented as it is part of the natural wine microbial flora, but it can be controlled along with the consequent "Brettiness" (referring to the actual off-flavour produced by Brett cells). Good winemaking practices would definitely control Brett along with many other wine spoilage microorganisms, but a holistic approach is required as wine parameters such as, grape quality, sulphur dioxide (SO_2), pH, wine temperature, residual nutrients, O_2, barrel condition and oenological practices are interdependent. For more information on Brettanomyces and its role in winemaking see Part 1 - February edition 2006.

So the question is: What can the winemaker do to limit the growth of *Brettanomyces* and the potential volatile phenol off-flavours?

What Encourages Brett? - Prevention or Control

If we know what conditions, parameters and/or environments stimulates the growth of Brettanomyces and its enzymes involved in the production of volatile phenols, we have half of the answer. We can use this knowledge to then prevent or control them. The following has been shown to enhance Brett and the levels of the ethyl-derivatives:

- From the vineyard

The vineyard does not appear to be a serious contamination source. However, as part of a holistic approach to improve quality, damaged grapes may well contain a higher microbial load than healthy grapes and the removal thereof can be a consideration, along with judicial SO$_2$ usage at harvest. Winemaking for lighter style wines requires more consideration as these can exhibit phenolic off-flavour due to lower threshold levels.

- Poor sanitation (maintain good cellar hygiene)
- Longer maceration periods

This can lead to increased amounts of substrates and precursors for Bretty off-flavour (Gerbeaux et al., 2002)

- Red wine is more prone to Brett and phenolic off-flavours

"Brettiness" is discouraged by whites, although they do occur in some. This is largely due to higher levels of SO$_2$, lower pH and fewer pre-cursors.

- pH > 3.6 (pH below 3.6 is better)
- Temperatures between 25-30°C will encourage their growth (Below 18°C will help with control).
- Poor SO$_2$ usage

There are still a few concerns with regards to the prevention of Brett growth in the wine. Although SO$_2$ is applied for its antimicrobial properties, the sensitivity of Brett is strain dependant and recently genetic variability among Brett strains has been reported (Godden et al., 2004). The critical additions are at picking and/or crushing and again after MLF before barrel ageing.

Because SO$_2$ is more effective at lower pH values the potential risk of unwanted microbial populations is much higher with a higher pH as encountered in warmer climate regions (like South Africa). Molecular SO$_2$ < 0.4 ppm will give poor antimicrobial properties (rather maintain above this level). It has been reported that molecular SO$_2$ around 0.8 ppm should be sufficient for inhibiting Brett growth in red wine (Australian Wine Research Institute, 2004 annual report). Additionally, research from the AWRI has suggested that it is not just the concentrations of free or bound levels, but rather the ratios thereof. The advised ratio of 0.4 of free to bound/total SO$_2$ is more ideal to ensure microbial stability (e.g. 30 ppm free to 75 ppm total).

- Alcohol below 13%
- Residual sugar > 0.2 g/l

It is important to realise that wines classified as dry (<5.0 g/l) still have enough sugar to support the growth of Brett. This is why the residual sugar should be minimised and this can be performed by using a very active starter culture to ferment to dryness.

- Additions of amino acids and vitamins, especially biotin and thiamine
- Presence of yeasts lees

Yeast lees is a very rich source of nutrients and will encourage many spoilage microbes if the above mentioned points are not controlled.

- Delayed racking

During this time the levels of free SO$_2$ is very low. It has been shown that delayed racking led to increased levels of 4-ethyl phenol (Chatonnet et al., 1995) and therefore winemakers are advised to add SO$_2$ and clarify the wine as soon as possible after MLF.

- Oxidative conditions (Keep containers topped)

The small available amounts of oxygen will support Brett better during conditions of higher ethanol concentrations (Du Toit et al., 2005). In addition, excessive amounts of oxygen will enhance the growth and consequent acetic acid production by *Brettanomyces.*

- Malolactic fermentation

Thus far, it appears as if wine is at its highest risk to Brett spoilage from the onset of MLF and ageing. As MLF is performed when the levels of free available SO$_2$ are very low, the

antimicrobial potential of the wine is also low. The increasing pH (resulting from MLF) and presence of nutrients (resulting from yeast autolysis) can create an environment in which many microbes can proliferate. During early stages of spontaneous MLF, the lactic acid bacterium (LAB) populations are low and Brett populations have more access to substrates and nutrients. Chatonnet et al. (1992) additionally reported that with greater tannic extractions, the LAB exhibit a greater period of latency. Therefore, the use of a strong MLF starter cultures can limit Brett activity. It was observed in certain wines that have high pediococci counts, that they also have high Brett levels or volatile phenol levels (Personal communication, Gafner).

- Cross contamination

Use clean wines to top-up and don't use pre-infected barrels.

- Other means of Brett elimination that has been mentioned as possibilities are:

Filtration (0.45 um)/Cross flow (0.22 um)
A method believed to be effective for removing Brett is sterile filtration (0.45 µm) (Gafner et al.) This however, along with the other methods mentioned has detrimental effects on the organoleptic quality of the wine and should be carefully considered. Sterile filtration/cross-flow may however be a good option when filling up the barrels. It is crucial that the "top-up" wines and even wines destined for blending are tested or decontaminated before use as this may destroy the entire outcome.

Addition of DMDC (Velcorin) Dimethyldicarbonate, O.I.V. approved, cost implications.

Photon hydro ionization (Winterthur©) (not investigated for Brett)

Thermal inactivation (Couto et al., 2005) (not investigated for Brett).

Bretts in Barrels

Bretts are also capable of growing in barrels. These yeasts contain á-glucosidase enzyme activity, which cleaves the disaccharide cellobiose (wood sugar) into glucose molecules thus making it readily available for growth. This conversion could however also be the result of the action of other microbial inhabitants of the barrels. With regards to new barrels, the action of fungal cellobiohydrolases on the cellulose could serve as sites for Brett growth. In older barrels the cellobiose might have been previously metabolised (Fugelsang et al., 1993; AWRI). New barrels contain higher amounts of cellobiose and therefore should have a higher potential for supporting Bretts than used barrels. However, when barrels are produced the toasting and firing process should ensure that the wood is microbially clean. With older barrels, research has shown drastic decreases in the amounts of volatile ethyl derivatives produced (up to 85%) after shaving and refiring (Pollnitz et al., 2000). This is ascribed to the destruction of the microbial load in the first few inner layers of barrels. Other things to mention; new barrels pose greater risk if wine is already Brett infected. New barrels can bind more free SO_2 (15 mg/l) than older barrels over 6 months (AWRI) and this should be taken in considereation.

General misconceptions about Brett
- Brett only comes in on the grapes
- Is in all red wines
- Comes in with new barrels
- Is not spread by infected barrels
- Brett is only found in dirty cellars
- Won't grow in dry wine
- Won't grow in wine with alcohol above 13%
- Brett is a surface film yeast that needs oxygen
- Always makes a lot of acetic acid and ethyl acetate
- Only Brett gives a mousy taint

Concluding remarks

With regards to Brett, well, only small steps have been taken and there are still many things to be researched. Bretts are but a few of the many organisms that exist in the winemaking environment and because it is a living entity, there will be a lot of diversity amongst its species. Not all Bretts should be deemed bad, although others should. Not all wines are bretty, although in some cases bretty can be good! It is the fluctuation in perception, which is influenced by knowledge that shapes the different opinions. Nevertheless, everybody appreciates and enjoys a good wine within their personal taste!

There is no better control for Brett at this stage than ensuring GOOD WINEMAKING PRACTICES!

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