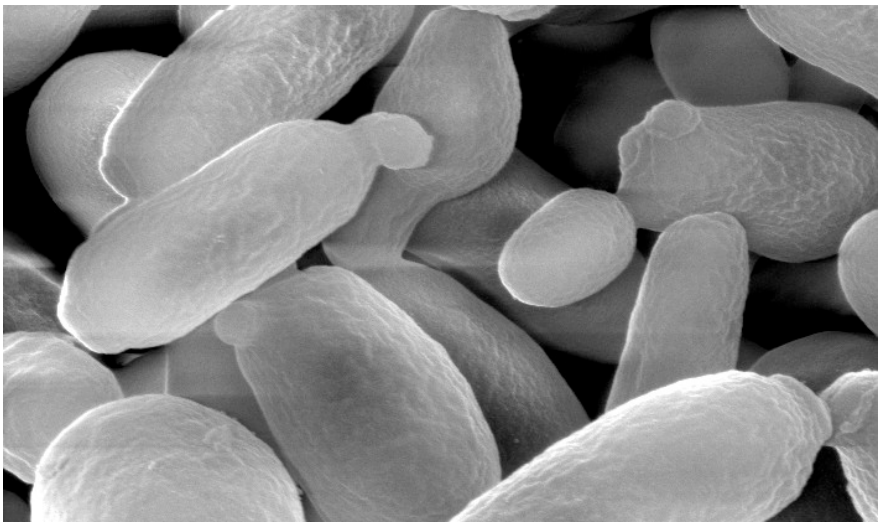
THE UNIVERSITY OF ADELAIDE
AUSTRALIA

Australian Government
Grape and Wine Research and Development Corporation

Growth and physiology of *Brettanomyces/Dekkera* yeast and the production of flavour impact compounds in wine



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **UA03/01**

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Research Organisation: **UNIVERSITY OF ADELAIDE**

Date: **30 MAY, 2008**

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1. Abstract

Volatile phenols are produced by *Dekkera* yeasts and are critical in wine spoilage. *Dekkera* and *Brettanomyces* yeast were cultured in a ranges of media supplemented with hydroxycinnamic acids (HCAs) to investigate their influence on growth and the formation of volatile phenols. The growth rate and substrate utilisation rates of *Dekkera* species differed depending on strain and the acid precursor present. Yeast growth was slowed and inhibited by the presence of ferulic acid.

The enzyme phenolic acid decarboxylase (PAD) converts HCAs to vinyl phenols, which are then converted to ethylphenols via the postulated vinylphenol reductase (VPR). Both steps of the pathway were characterised using cell extracts from of *Dekkera* and *Brettanomyces*. A partial protein sequence for PAD was determined from *D. anomala* and may indicate the presence of a novel enzyme in this genus. The growth of *Dekkera* yeast species was inhibited by ferulic acid and this effect was enhanced in the presence of ethanol. This work introduces a novel mechanism by which *Dekkera* yeast may be controlled in the wine enviroment by naturally occuring grape extracts.

2. Executive Summary

- *Dekkera* and *Brettanomyces* yeast are important spoilage organisms in a number of food and beverage products.
- Isolates of both genera were cultured in defined medium, supplemented with hydroxycinnamic acids (HCAs) and vinylphenols to investigate their influence on growth and the formation of ethyl phenol derivatives.
- The growth rate of *Brettanomyces* species in the presence of acids was reduced and no significant conversion to vinyl or ethyl derivatives was observed. The growth rate and substrate utilisation rates of *Dekkera anomala* and *Dekkera bruxellensis* yeast differed depending on strain and the acid precursor present.
- Growth of *D. bruxellensis* was slowed by the presence of ferulic acid with the addition of 1 mM ferulic acid completely inhibiting growth in chemically defined media.
- The microbial synthesis of volatile phenols is thought to occur in two steps via the enzyme phenolic acid decarboxylase (PAD) converting HCAs to vinyl derivatives, which are the substrates of a second enzyme, postulated to be a vinylphenol reductase (VPR) whose activity results in the formation of ethylphenols.
- Both steps of the pathway were investigated, using cell extracts from a number of *Dekkera* and *Brettanomyces* species.
- *Dekkera* species catabolise ferulic, caffeic and *p*-coumaric acids and possess inducible enzymes with similar pH and temperature optima.
- *Brettanomyces* does not decarboxylate HCAs but does breakdown vinylphenols.
- A partial protein sequence for PAD was determined from *Dekkera anomala* and may indicate the presence of a novel enzyme in this genus.
- The effect of ferulic acid additions on *Dekkera* growth was investigated under various wine-related conditions and in combination with other hydroxycinnamic acids, namely *p*-coumaric and/or caffeic acids.
- The growth of *Dekkera* yeast species was inhibited by ferulic acid and this effect was enhanced in the presence of ethanol.
- In wine the concentration of ferulic acid required to inhibit or kill *Dekkera* is significantly reduced.

- This work introduces a novel mechanism by which *Dekkera* yeast may be controlled in the wine enviroment by naturally occuring grape extracts.

3. Background

Foods and beverages including dairy products (Cosentino et al. 2001), olive products (Coton et al. 2006) soft drinks (Kolfschten and Yarrow 1970) and fermented beverages (van der Walt 1964; Lachance 1995; Teoh et al. 2004;) can be influenced by the growth of *Dekkera* and *Brettanomyces,* however, these yeast are commonly reported as spoilage organisms (Stratford et al. 2000; Silva et al. 2004). Based on molecular similarities (Smith et al. 1990; Cai et al. 1996), these yeasts belong to related but distinct genera, however, the terms *Brettanomyces* and *Dekkera* are commonly used interchangeably. In this report the taxonomy adhered to is that of Barnett et al. (2000). There are 5 recognised species of these yeasts; three *Brettanomyces* species; *nanus, custersianus* and *naardenensis* and two species of *Dekkera*; *anomala* and *bruxellensis*.

D. bruxellensis is present on grapes (Renouf et al. 2007), insects (Sandhu, 1985), workers and equipment within the winery environment (Peynaud and Domercq 1956, Licker et al. 1998) and is commonly associated with wine contamination. *D. bruxellensis* spoilage attributes in wine include sediments or haze (van der Walt and van Kerken 1958) and the formation of mousy off-flavour (Grbin and Henschke 2000, Grbin et al. 2007; Snowdon et al. 2006), acetic acid and ethyl-acetate (Scheffers 1961; Freer et al. 2003), volatile fatty acids (Spaepen and Verachtert 1982; Fugelsang et al. 1993) and the production of volatile phenols (Chatonnet et al. 1992).

Volatile phenols are produced by a number of microorganisms during the catabolism of HCAs, primarily *p*-coumaric and ferulic acids (Chatonnet et al. 1992; Edlin et al. 1995) and to a lesser extent, caffeic (Edlin et al. 1995) and sinapic acids (Heresztyn 1986; Edlin et al. 1995). HCAs are present as tartaric ester conjugates in the grape cell wall (Lynd et al. 2002) and are released into grape juice during crushing. The phenolic compositions of juice and wines is complex and the concentration of compounds present range from trace to 200 mg/l (Reguant et al. 2000). The concentrations of individual compounds are dependent on the grape variety and vinification processes (Reguant et al. 2000). *Dekkera* species produce both vinyl and ethyl phenol derivatives from the phenolic precursors (Chatonnet et al. 1992). These products are considered to be among the most unpleasant spoilage characteristics produced by *Dekkera* yeast.

Little research has focused on *Brettanomyces* species as they do not appear to play a role in wine spoilage and no *Brettanomyces* species have been found to produce less than 1 mg/l of 4-ethylphenol during growth with *p*-coumaric acid (Dias et al. 2003a). There has been no research conducted to ascertain whether *Brettanomyces* produces the vinyl-derivative or if they can catabolise other HCAs besides *p*-coumaric acid or act directly on vinylphenols to form ethylphenols. If *Brettanomyces* can produce vinylphenols this may have a spoilage impact.

The conversion of HCA precursors is speculated to occur in a two step pathway (Chatonnet et al. 1992; Edlin et al. 1995). Initially a decarboxylase enzyme yields the 4-vinyl derivative. The second reaction is a reduction, to form the 4-ethyl derivative (Chatonnet et al. 1992; Donaghy et al. 1999). The vinylphenol reductase (VPR)

enzyme putatively responsible for this step has never been isolated. The initial production of vinylphenols is not exclusive to *Dekkera*. Yeasts such as *Saccharomyces cerevisiae* (Chatonnet et al. 1993; Huang et al. 1993) and *Pichia guilliermondii* (Barata et al. 2006) as well as bacteria including, *Lactobacillus plantarum, Bacillus subtilis* and *Escherichia coli* (Cavin et al. 1997b; Barthelmebs et al. 2000a; van Beek and Priest 2000), are also capable of decarboxylating HCAs. *D. bruxellensis* can additionally convert free 4-vinylphenols into 4-ethylphenols (Dias et al. 2003b) and therefore is able to utilise 4-vinylphenol produced by other wine organisms. It is unclear whether this ability extends to other vinyl-derivatives. *Pichia guilliermondii* produce high levels of ethylphenols in synthetic medium (Dias et al. 2003a; Dias et al. 2003b; Martorell et al. 2006) but are unable to do so in wine (Barata et al. 2006). However, *P. guilliermondii* may convert free HCAs present in grape juice before fermentation. It has not been established if this impacts on final wine sensory characteristics.

Dekkera are therefore the only yeast associated with the winery environment that have been shown to produce significant concentrations of ethyl-compounds in wine (Chatonnet et al. 1995; Barata et al. 2006). Limited research has been conducted to determine the variability displayed by *Dekkera* and *Brettanomyces* species in relation to HCA metabolism and the formation of volatile phenols. The majority of previous studies have focused on the variability of a single species, *D. bruxellensis* and its ability to produce ethylphenols in wine (Chatonnet et al. 1995; Barata et al. 2006). Heresztyn (1986) investigated a single strain of both *Dekkera* species in a chemically defined medium and during a white wine fermentation, demonstrating that *D. anomala*, as well as *D. bruxellensis*, are capable of metabolising *p*-coumaric and

ferulic acids into 4-ethyl derivatives. Dias et al. (2003b) confirmed these results and determined that *Brettanomyces* species do not have the capacity to directly form 4-ethylphenol from *p*-coumaric acid.

A greater understanding of the growth and physiology of *Dekkera* and *Brettanomyces* in relation to wine has a direct benefit to the industry. Through this the ability to control and manipulate the growth of this yeast in wine to avoid undesirable flavour changes will be developed.

4. Project aims & performance targets

The original aims of the project were:

1. Investigate the growth parameters/nutritional requirements related to the production of volatile phenols by *Brettanomyces*/*Dekkera* in a model wine system.
2. Investigate the growth parameters/nutritional requirements related to the production of isovaleric acid by *Brettanomyces*/*Dekkera* in a model wine system.
3. Determine growth limits/minimum requirements for development of these compounds in sensorially significant concentrations.
4. Investigate and characterise biochemical and molecular basis of enzyme activity (intra- and extra-cellular) of selected *Brettanomyces*/*Dekkera* strains in relation to development of the flavour impact compounds and its relationship to growth in a wine model system.
5. Confirm results obtained above in small-scale winemaking trials including microbiological, chemical and sensory analysis.
6. Develop strategies/industry practice to control growth and/or physiology of *Brettanomyces*/*Dekkera* in wine.

The outputs and performance targets are detailed below (Table 4.1). This table reflects the original outputs and the variations approved through the life of the project. As detailed in 2005/2006 significant technical difficulties where faced in the molecular genetic approach that has was undertaken in an attempt to elucicate the volatile phenol biochemical pathway. Therefore the outputs were focused on volatile phenols and the control of *Dekkera* in wine

Table 4.1 Outputs and performance targets as detailed in original applications and variations approved in 2004/2005 and 2006/2007.

Outputs	**Performance Targets**
1. A Review of relevant literature	Completion of literature review and research proposal (PhD student)
2. Information on the physiological basis for growth of *Dekkera*/*Brettanomyces*	Identification of the key growth parameters, nutrition al requirements and limits for the growth of *Dekkera*/*Brettanomyces* in a model wine system
3. Information on the physiological basis for volatile phenol	Identification of the key growth parameters, nutritional requirements and limits for the formation of volatile phenols by *Dekkera*/*Brettanomyces* in a model wine system
4. Biochemical basis of volatile phenol production determined	Identification of the intra and extracellular enzyme activity relative to volatile phenol production
5. Information on the molecular basis of volatile phenol production	Analysis of the enzymology and molecular genetics of volatile phenol biosynthesis that provides a better understanding of the stimuli for the production of these off-flavour compound and hence identify targets to limit or manipulate this formation
6. Finalisation of biochemical and molecular investigations	Completion of the characterisation of the biosynthesis of volatile phenols
7. Information on yeast growth and off-flavour development in small-scale winemaking trials	Validation of results of laboratory research under industry relevant conditions
8. Development of strategies to minimise influence of *Dekkera*/*Brettanomyces*	Completion of analysis of results from laboratory and small-scale winemaking trials outlining impact biochemical and physiological characterisation of growth of this yeast in wine
9. Communication of results	Results presented at one international conference, one industry journal and at least one paper submitted to scientific journal. Results presented at the AWITC. Submission of final report
10. PhD Thesis	Completion of PhD thesis

5. Growth and utilisation of hydroxycinnamic acids in synthetic media

Outputs and performance targets achieved

Output 1: A Review of relevant literature

Output 2: Information on physiological basis for growth of Dekkera/Brettanomyces

Output 3: Information on the physiological basis for volatile phenol

Output 9: Communication of results, scientific paper and conference presentations (Appendix 1)

The aim of this study was to examine all *Dekkera* and *Brettanomyces* species for their ability to produce volatile phenols by investigating the utilisation of four known precursors. *D. bruxellensis* is known to convert 4-vinylphenol into 4-ethylphenol (Dias et al. 2003b), but it is has not been established whether this is also a characteristic of *Brettanomyces* species. Furthermore, no known studies have addressed whether *Dekkera* or *Brettanomyces* utilise the vinylphenol derivatives of ferulic, caffeic or sinapic acids. This study also investigated the metabolism of 4-vinylphenol and 4-vinylguaiacol and ethylphenol derivatives by all species. Further a wine medium based on the fermentation of chemically defined grape juice was developed to allow investigation of *Dekkera* growth under strictly defined wine-like conditions.

5.1 Materials and methods

5.1.1 Yeast strains

Dekkera and *Brettanomyces* yeast were obtained from the Centraalbureau Voor Schimmelcultures (CBS) and The Australian Wine Research Institute (AWRI). *Saccharomyces cerevisiae* strain Maurivin PDM was also used in the production of WM (Table 5.1). Yeasts were maintained on YPD [1% (w/v) yeast extract, 2% (w/v) peptone (Amyl Medium), 2% (w/v) glucose (Univar) and 2% (w/v) bacteriological agar (Amyl Media)] stored at 4^{o}C.

Table 5.1 Yeast strains used during this investigation

Species	Strain
Saccharomyces cerevisae	Maurivin™ PDM wine yeast
Dekkera anomala	CBS 77
Dekkera bruxellensis	CBS 2336; AWRI 1499
Brettanomyces naardenensis	CBS 6042
Brettanomyces nanus	CBS 1945
Brettanomyces custersianus	CBS 4805

5.1.2 *Dekkera* and *Brettanomyces* growth in Chemically Defined Media (CDM)

A single loop of *Dekkera* yeast was inoculated from fresh YPD plates into 25 ml of YPD in 250 ml Erlenmyer flasks and grown with shaking (180 RPM). Starters were incubated at 30ºC until a cell density of 10^{8}cells/ml was attained. Fermentations were undertaken with 50 ml of CDM modified from Heresztyn (1986): [50 g/l glucose, 2 g/l sodium sulfate, 250 mg/l magnesium sulfate, 2 g/l potassium phosphate, 200 mg/l citric acid, 310 mg/l calcium chloride anhydrous, 2 mg/l zinc sulfate, 10 mg/l iron sulfate, 25 mg/l *myo*-inositol, 1 mg/l pyridoxine.HCL, 10 mg/l nicotinic acid, 1 mg/l thiamine.HCl and 30 μg/l biotin (Sigma-Aldrich)] and incubated aerobically in 250

ml Erlenmeyer flasks. Flasks were inoculated with 5 x 10^6 cells/ml and incubated at 30ºC. Previous investigations indicated that 30ºC was unsuitable for growth of *B. nanus* and *B. naardenensis* (data not shown) and it was determined that 25ºC was optimal.

Dekkera and *Brettanomyces* strains were grown in CDM supplemented with 2 mM of one of the hydroxycinnamic acids: *p*-coumaric, ferulic, caffeic and sinapic (Fluka). Determination of total acid utilisation was preformed with the addition of 1 mM to avoid growth inhibition by ferulic acid. Further experiments with *Dekkera* were completed in the presence of a combination of *p*-coumaric, ferulic and caffeic acids. The total concentration of acids was 2 mM and all acids were added at an equal concentration. Cultures were grown in CDM supplemented with 0.5 mM of 4-vinylphenol, 4-vinylguaiacol and 4-ethylphenol (Alfa Aesar). All experiments unless otherwise stated were carried out in duplicate and the analysis on each sample was performed in duplicate.

5.1.3 *Dekkera* growth in Wine Media (WM)

Chemically Defined Grape Juice Medium (CDGJM) modified from Henschke and Jiranek (1993) was fermented by *S. cerevisiae* strain PDM, a commonly used commercial winemaking strain. The medium fermented with PDM was used as a base for the WM in further experiments in order to more closely reflect wine-like conditions. Before use the fermented medium was clarified by centrifugation at 15,000 x *g* for 1 hour and sterile filtered through a nitrocellulose filter (0.22 μm, Millipore). Ethanol concentration was determined in an Anton Paar Alcolyser-Wine, as per manufacturer's instructions. The medium was then adjusted to 10% ethanol

(v/v). The pH was adjusted to 3.8 with 10M NaOH. Residual sugar concentrations were determined by HPLC. A 15 μl sample was injected onto a Waters high performance carbohydrate column (WAT044355; 4.6 mm x 250 mm) and a 75% acetronitrile mobile phase was pumped through the column at a flow rate of 1.4 ml/min for 12 minutes. Peak detection was achieved via a refractive index detector, with sugars quantified with reference to standards of known concentration. The total glucose content of the WM was then adjusted to 5 g/l. To ensure vitamins were not deficient, supplementation was applied [25 mg/l *myo*-inositol, 1 mg/l pyridoxine.HCL, 10 mg/l nicotinic acid, 1 mg/ml thiamine.HCL and 30 μg/l biotin]. Additions of 100 mg/l of *p*-coumaric and ferulic acids were also made to WM.

An adaptation period was required prior to growth of *Dekkera* yeasts in WM. As previously described a YPD starter culture was prepared, 5 x 10^6 cells/ml were then inoculated into adaptation medium (25 ml of CDM with 5% v/v ethanol) and cultures grown at 30ºC with shaking at 180 RPM. Adaptation cultures reached a density of 1 x 10^8 cells/ml before being used to inoculate WM, in triplicate, at a rate of 5 x 10^6 cell/ml.

5.1.4 Sampling and analysis

Cultures were sampled at regular intervals during their growth. Growth rates were highly strain dependant and hence samples were taken at intervals of between 6 to 48 hours, depending on the yeast strain. A 1 ml aliquot of culture was removed at each time point, centrifuged and the supernatant stored at -30ºC prior to further analysis. Cell growth was estimated spectrophotometrically at 600 nm (Helios α, Thermospectronic). Glucose concentrations were estimated during the fermentation

from the refractive index of the supernatant and using Clinitest® Tablets (Ames), with end point sugar concentrations being quantified by enzymatic assay (D-glucose oxidase/peroxidase; Megazyme).

5.1.5 Determination of hydroxycinnamic acid utilisation during fermentation by HPLC

Fermentation samples were centrifuged for 1 minute at 16,000 x and 200 µL of supernatant was sterile filtered with a 1 ml syringe filter (0.45 µm, Alltech) prior to analysis. An Agilent 1100 series HPLC fitted with an auto-sampler and DAD detector was used to analyse samples (5 µL) injected onto a Phenomenex Synergi™ 4µ Hydro-rp 80A column (2 mm x 150 mm). Peaks were separated over a 40 minute gradient; 0-100% buffer B, where buffer A was 0.2% v/v phosphoric acid and buffer B was 80% acetonitrile with 20% buffer A, at a flow rate of 0.2 ml/min. Before loading subsequent samples the column was flushed for 5 minute with 100% buffer B and re-equilibrated over 5 minutes with 100% buffer A. Elution was monitored by absorbance at 310, 254 and 280nm. Standard curves were prepared for *p*-coumaric, ferulic, caffeic and sinapic acids (Fluka) and 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol (Alfa Aesar) in the range of 0.01–2.5 mM.

5.2 Results

5.2.1 Growth of strains in defined medium with hydroxycinnamic additions

Yeasts were grown in the presence of 2 mM of *p*-coumaric, ferulic, caffeic and sinapic acids individually or in combination and growth measurements undertaken. The growth of *D. anomala* strain CBS 77 was not affected by *p*-coumaric, caffeic and

sinapic additions, however, growth in the presence of ferulic acid demonstrated an extended lag phase of around 72 hours compared to the control. Despite this lag the final cell density was comparable to that of the control (Fig. 5.1). *D. bruxellensis* strains were inhibited by ferulic acid at 2 mM. Thus *D. bruxellensis* AWRI 1499 did not grow and CBS 2336 exhibited an extended lag phase of 120 hours. Growth in the presence of other acids was consistent with the unsupplemented control. All *Brettanomyces* species were inhibited by the presence of HCAs (Fig. 5.1). Sugar utilisation (data not shown) and culture densities were lower relative to the control. *Brettanomyces* species were unable to grow in the presence of ferulic acid at 2 mM, while sinapic acid inhibited *Brettanomyces* yeast such that *B. nanus* and *B. naardenensis* only reached a cell density 25% and 50% of that of the control, respectively. By comparison, the growth of *B. custersianus* was not greatly influenced by sinapic acid, but rather was most affected by the addition of *p*-coumaric and caffeic acids, which each lead to a 25% decrease in optical density (Fig. 5.1).

5.2.2 Utilisation of hydroxycinnamic acids

The uptake of HCAs was determined by HPLC analysis. Although *Brettanomyces* species were able to grow in the presence of precursors their ability to utilise HCAs (Fig. 5.2) or produce vinyl or ethyl compounds derived from these substrates was low. The exception was sinapic acid, the concentration of which decreased during the fermentation, though the expected production of volatile phenol derivative was not detected by HPLC. *B. custersianus* CBS 4805 utilised around 80% of the sinapic acid, which is higher than the other species. All *Dekkera* strains were able to metabolise *p*-coumaric and ferulic acids. *D. anomala* also utilised over 90% of caffeic acid in the growth medium while only 25% of caffeic acid was used by *D. bruxellensis* strains

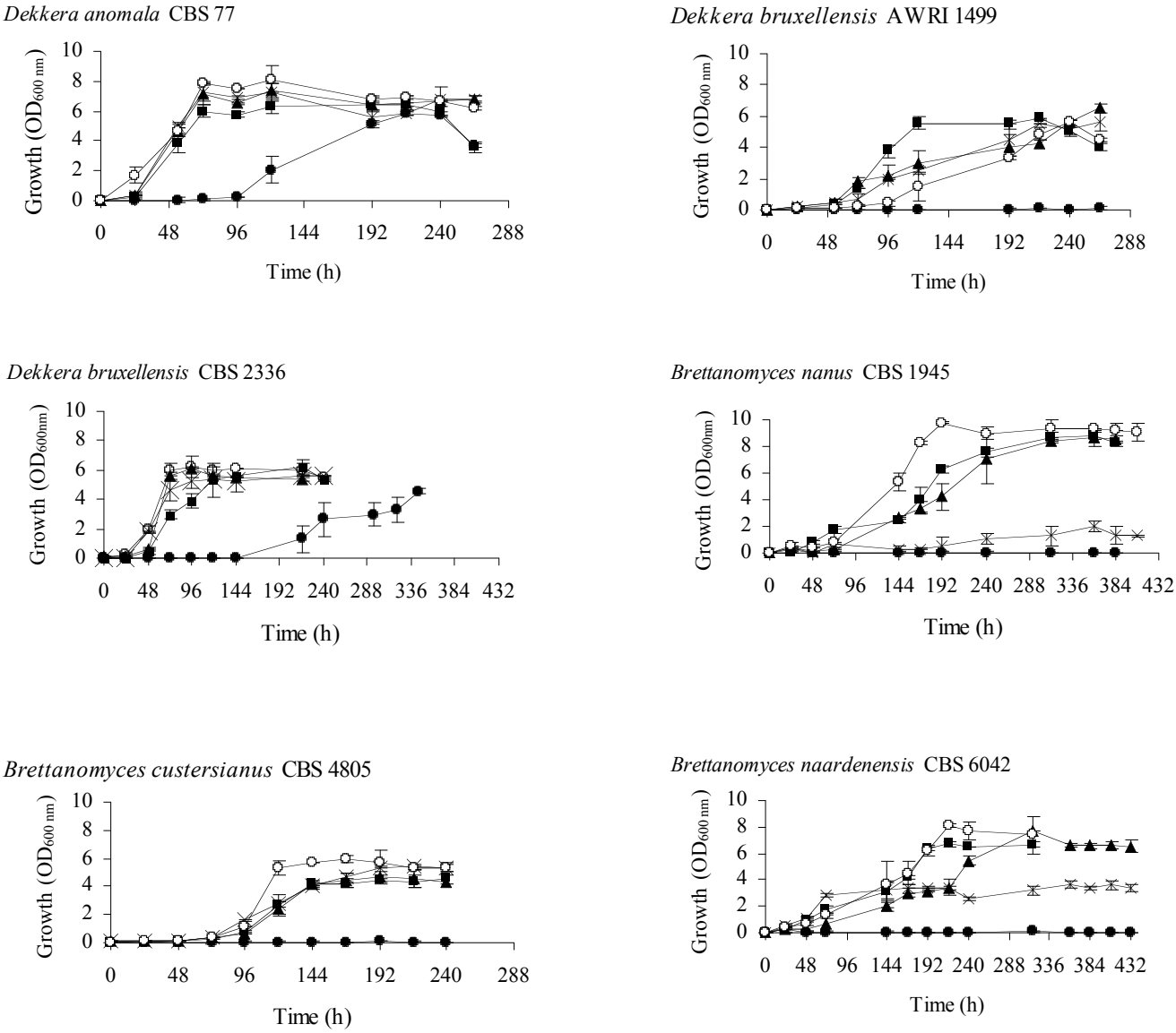


Fig. 5.1 *Dekkera* and *Brettanomyces* growth in the presence of 2 mM HCAs; *p*-coumaric (■), ferulic (●) caffeic (▲) and sinapic acid (×) and control (○) estimated by optical density ($OD_{600\ nm}$). Yeast strains CBS 77, AWRI 1499 and CBS 4805 were monitored over 288 hours while CBS 2336, CBS 1945 and CBS 6042 were monitored over 432 hours

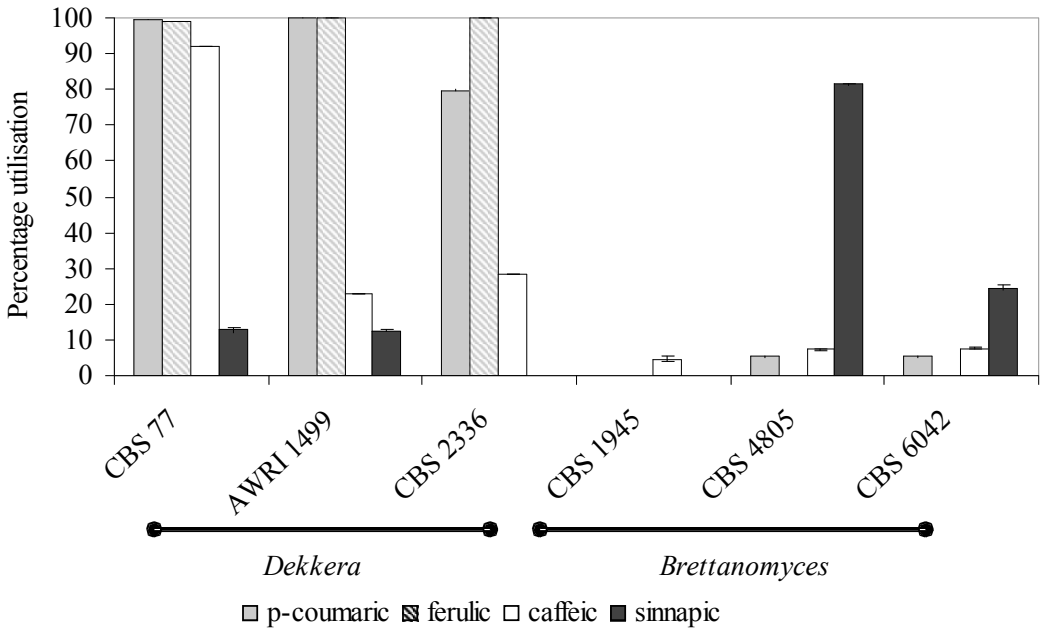


Fig. 5.2 The utilisation of HCAs by *Dekkera* and *Brettanomyces* yeast reported as the percentage of total supplied substrate (1 mM) utilised at the end point of fermentation. Fermentations were preformed in triplicate and HPLC analysis in duplicate. Standard deviations are included and were below 1%

CBS 2336 and AWRI 1499 (Fig. 5.3). *Brettanomyces* species utilised a maximum of 5% of the caffeic acid from the medium.

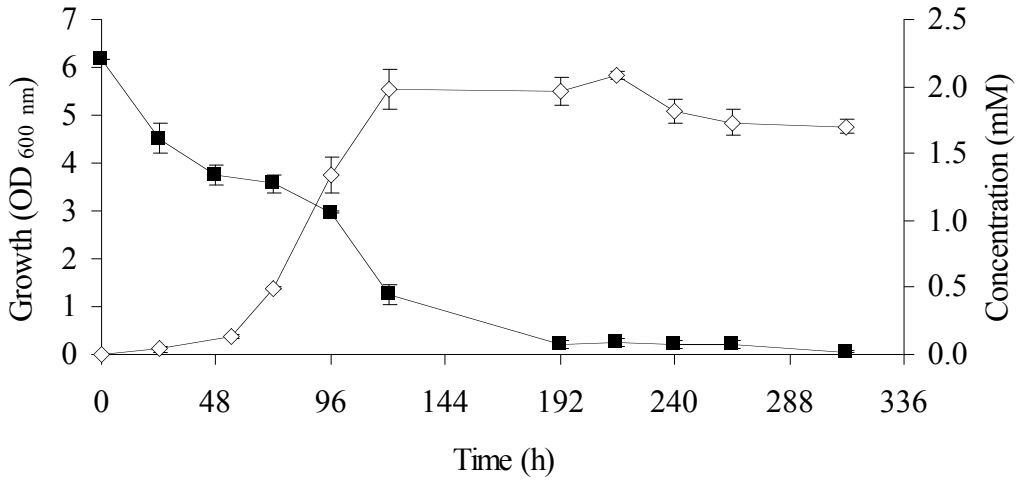
The time course of the utilisation of HCAs is illustrated in Fig. 5.3A using *p*-coumaric uptake by *D. anomala* CBS 77 as an example. The overall pattern remained the same for all utilised HCAs and yeast strains. The initial uptake of acids coincided with the beginning of the log phase of growth and *p*-coumaric concentration rapidly decreased. The product, 4-vinylphenol, was detected immediately after the breakdown of *p*-coumaric, where after its concentration increased to a maximum before the stationary phase of growth was reached. After the peak in 4-vinylphenol concentration, 4-ethylphenol was detected and continued to be released into the medium after the culture entered the stationary phase. In all cases the vinylphenol derivative was not stoichiometrically converted into ethylphenol and both products were present at the end point of fermentation.

The exception to this scheme of product formation was ferulic acid, which was inhibitory to the growth of *Dekkera* and *Brettanomyces* strains. *Dekkera* yeasts that are able to grow in the presence of ferulic acid utilised the majority of the acid (data not shown) before entering log phase presumably detoxifying the medium. The products accumulated in a similar manner to that shown in Fig. 5.3B.

5.2.3 Combinations of hydroxycinnamic aids

Dekkera yeasts were grown in various combinations of HCAs. The total concentration of acids was 2 mM and each compound was supplemented at the same concentration. Both *Dekkera* species were inhibited by the addition of acids in combination. No

A



B

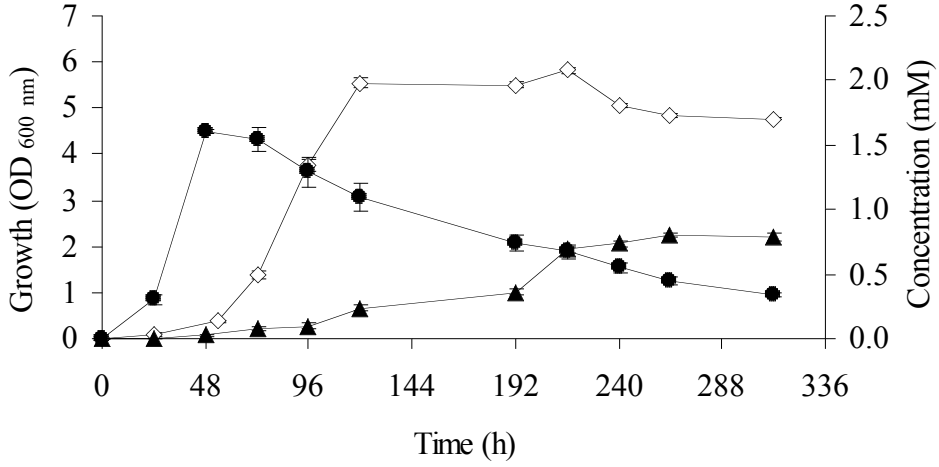


Fig. 5.3 (A) *Dekkera anomala* (CBS 77) growth (◊) as measured by optical density ($OD_{600\ nm}$) and utilisation of 2 mM *p*-coumaric acid (■) during fermentation. (B) Production of 4-vinylphenol (●) and 4-ethylphenol (▲) from *p*-coumaric acid during culture growth (◊)

growth was observed in cultures with caffeic acid supplements (data not shown). Cultures grown with ferulic and *p*-coumaric acids underwent an extended lag phase of 144-168 hours. The observed inhibition of ferulic acid on *D. anomala* CBS 77 in combination with *p*-coumaric acid was greater than the degree inhibition caused by ferulic alone (Fig. 5.4A). The final culture density ($OD_{600\ nm}$) was 75% of that of the control, but was comparable to the end point density of cultures with single HCA additions.

When *D. anomala* was grown in the presence of *p*-coumaric and ferulic acid the *p*-coumaric acid was completely removed from the medium before the culture entered the log phase of growth (Fig. 5.4B). Although ferulic acid was initially removed at a rate similar to that of *p*-coumaric acid, this rate decreased after *p*-coumaric utilisation was complete, such that ferulic acid was not completely depleted from the medium. HPLC analysis of the supernatants of cultures inhibited by other combinations of HCAs did not show any uptake of acids from the medium (data not shown).

5.2.4 Utilisation of 4-vinylphenol and 4-vinylguaiacol

Chemically Defined Medium was supplemented with 1 mM of 4-vinylphenol and 4-vinylguaiacol and their uptake by *Dekkera* and *Brettanomyces* species examined by HPLC analysis (Fig. 5.5). *D. anomala* and *D. bruxellensis* utilised between 90–100% of both 4-vinylphenol and 4-vinylguaiacol into corresponding derivatives (data not shown). The growth of *Dekkera* species was also investigated in the presence of 4-vinylphenol and exhibited an inhibitory effect on culture growth. These cultures demonstrated noticeably longer lag phases of around 24–48 hours, depending on the strain (data not shown). *Brettanomyces* species, demonstrated an ability to utilise 4-

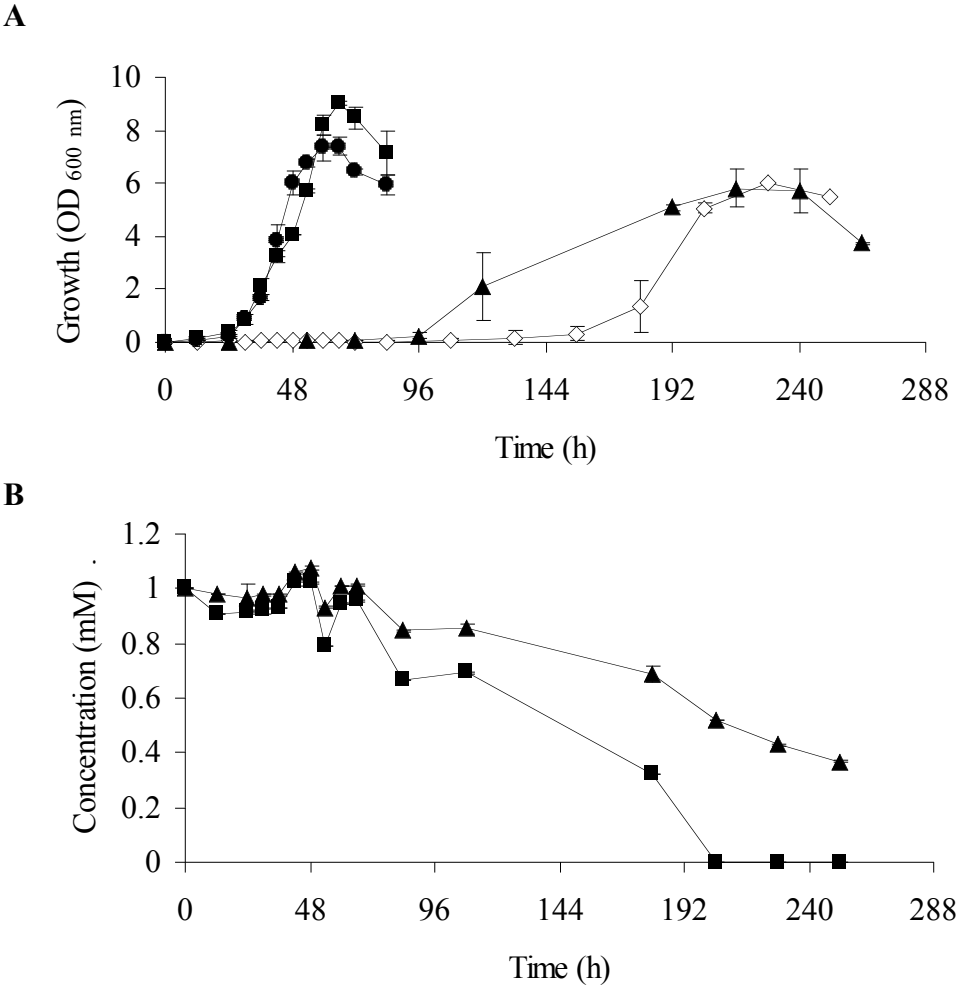


Fig. 5.4 (A) Comparison of *Dekkera anomala* CBS 77 culture growth, estimated by optical density ($OD_{600\ nm}$), in the presence or absence (●) of 2 mM *p*-coumaric (■) ferulic acid (▲) or *p*-coumaric and ferulic acids in combination (◊) each at a concentration of 1 mM. (B) The utilisation of *p*-coumaric (■) and ferulic acids (▲) when combined was determined by HPLC analysis

vinylphenol. However, limited uptake of 4-vinylguaiacol was observed and no breakdown products detected. *B. custersianus* CBS 4805 removed around 60% of 4-vinylphenol from the medium and the remaining two *Brettanomyces* species removed between 20-30% of the supplied 4-vinylphenol (Fig. 5.5). The corresponding 4-ethylphenol product was not detected by HPLC.

5.2.5 Utilisation of 4-ethylphenol

All yeasts were inoculated into CDM with 0.5 mM of 4-ethylphenol. Only limited uptake of 4-ethylphenol was observed (Fig. 5.5) and culture growth was affected. The inhibitory effect of 4-ethylphenol was greater than that of 4-vinylphenol, increasing the lag phase by 48-72 hours (data not shown). 4-Ethylphenol was not metabolised during growth.

5.2.6 Growth in Wine Medium

The aim of the use of WM (a pre-fermented define medium) was to develop a culture system in the laboratory that resembled wine like conditions whilst still allowing conditions important for *Dekkera* growth and volatile phenol production to be established. *D. bruxellensis* AWRI 1499, initially isolated from wine, as well as CBS 2336 and *D. anomala* were used to confirm results obtained from CDM in WM supplemented with *p*-coumaric and ferulic acids.

Additions of *p*-coumaric and ferulic acids to 2 mM inhibited growth of all strains in WM (data not shown). As a result, lower additions of 0.61 mM (100 mg/l) were made according to HCA concentrations previously reported in wine (Baranowski and Nagel 1981; Soleas et al. 1997; Gutierrez et al. 2005). Yet despite this reduction, growth of

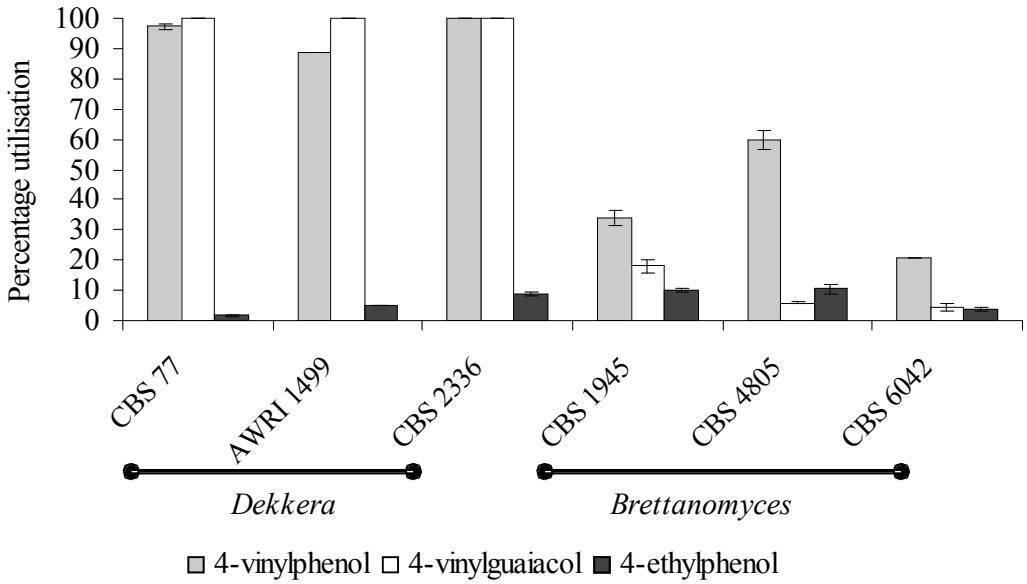


Fig. 5.5 Comparison of the utilisation of 4-vinylphenol, 4-vinylguaiacol and 4-ethylphenol by *Dekkera* and *Brettanomyces* yeasts during growth in CDM

D. anomala CBS 77 and *D. bruxellensis* CBS 2336 was still inhibited by *p*-coumaric and ferulic acids (data not shown). *D. bruxellensis* AWRI 1499 was not inhibited by *p*-coumaric and ferulic acids and utilised the majority of these compounds from the medium (Fig. 5.6). Vinylphenols were detected shortly after HCA removal from the medium, which were in turn converted into ethylphenols (data not shown) as observed for CDM supplemented with HCAs.

5.3 Discussion

The metabolism of HCAs by *Dekkera* and *Brettanomyces* has significance for the production of food and beverages in various industries. Several species of *Dekkera* and *Brettanomyces* yeasts were examined to define differences in their metabolism of HCAs in CDM. These results were confirmed in a model wine fermentation system. Apart from species variation in growth rates their ability to metabolise HCAs into vinyl and ethyl derivatives also differed.

Ferulic acid concentrations of 2 mM prevented the growth of all *Brettanomyces* yeast and some *Dekkera* yeast investigated. Inhibition of *Brettanomyces* species by other HCAs was also evident, with *B. nanus* being most sensitive (Fig. 5.1). While these findings concur with other studies that have reported an inhibitory affect of some HCAs on microbial growth, this appears to be the first report of such an effect on *Dekkera* and *Brettanomyces* species. Although the concentrations of HCAs used in this study are higher than those found naturally in grape juice (Reguant et al. 2000) they are lower than levels investigated to control other microorganisms (Stead et al. 1995).

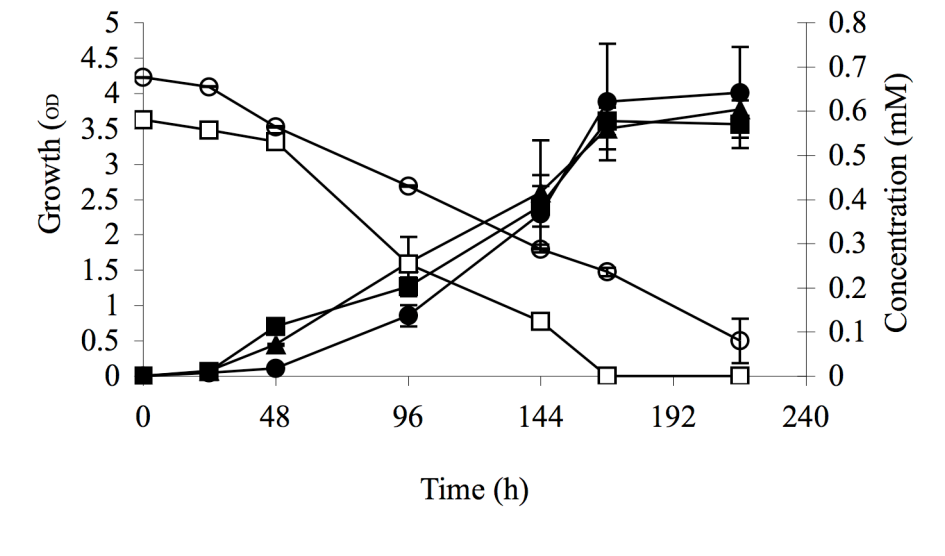


Fig. 5.6 Growth of *Dekkera bruxellensis* AWRI 1499 in wine medium unsupplemented (▲) and supplemented with 0.6 mM (100 mg/l) *p*-coumaric (■) and ferulic (●) acids determined by optical density ($OD_{600\ nm}$). Uptake of *p*-coumaric (□) and ferulic (○) acids from the medium was determined by HPLC analysis

Strain variation was also seen with regard to the metabolism of HCAs (Fig. 5.2). Previous studies (Dias et al. 2003b) have reported *Brettanomyces* species to be unable to produce 4-ethylphenol, hence it has been assumed that *Brettanomyces* do not have the enzymes required to metabolise HCAs. However, in examining a range of HCAs in this study, it has been possible to show that some *Brettanomyces* species in fact are able to decarboxylate some HCAs, but different phenolic acids to that of *Dekkera* species. *B. custersianus* CBS 4805 and *B. naardenensis* CBS 6042 isolates removed 80% and 25% of the initial sinapic acid concentration from medium with the formation of a yet unidentified product. Further work is required to identify and characterise this novel activity.

This investigation is the first to examine growth and acid utilisation by *Dekkera* species in media supplemented with combinations of HCAs. Such combinations are deemed more closely aligned with grape juice and other beverages where multiple HCAs are always present (Soleas et al. 1997). A total concentration of 2 mM, as used in the single addition studies, was applied here. In combinations that included caffeic acid no growth was observed. Furthermore the combination of ferulic and *p*-coumaric acid inhibited growth of strains to a greater extent than that of single acids. These results suggest that in an industrial fermentation the presence of more than one HCA may create a more inhibitory condition than previously considered. The extent and nature of the yeast-HCA interaction is not yet wholly comprehended. In addition to HCA growth inhibition there is evidence that suggests 4-vinylphenol and 4-vinylguaiacol also impede growth. This effect has not been previously described with regards to *Dekkera* or *Brettanomyces* species but antifungal activity of vinylphenols has been described (Ayer et al. 1995; Hammond et al. 1999).

The utilisation of vinyl derivatives by *Brettanomyces* species was also examined for the first time in this research. It has been assumed that since Chatonnet et al. (1995) found *Brettanomyces* to be incapable of metabolising *p*-coumaric acid that these organisms would also lack the vinylphenol reductase enzyme. However since our findings indicate that *Brettanomyces* species can at least utilise sinapic acid, the metabolism of intermediates was also examined. While *B. custersianus* and *B. nanus* removed between one third and one half of the 4-vinylphenol from the medium, no associated 4-ethylphenol production was detected. This may indicate that these isolates metabolise 4-vinylphenol by another previously undetermined pathway. Many species of yeast and bacteria are capable of converting *p*-coumaric and ferulic acid via 4-vinylphenol into vanillin and vanillic acid (Rosazza et al. 1995; Brunati et al. 2004; Ghosh et al. 2006). Therefore it is possible that HCAs are metabolised by *Brettanomyces* via a similar pathway.

The production of 4-vinylphenol and 4-ethylphenol from *p*-coumaric acid by *Dekkera* species agreed with the scheme reported by Edlin et al. (1995) and Heresztyn (1986). However, in contrast to Edlin et al. (1995) a 1:1:1 ratio of HCA to vinylphenol and ethylphenol was not observed. This could be due to *Dekkera* yeasts absorbing the HCA into their cell walls as shown by Salameh et al. (2007). This absorbtion could decrease the concentration of HCA available to undergo decarboxylation and subsequent reduction.

The study reported here confirmed the direct metabolism of 4-vinylphenol and 4-vinylguaiacol by *Dekkera* species. The importance of this observation is that many

yeast and bacteria associated with alcoholic fermentation can convert HCAs into vinyl derivatives (Degrassi et al. 1995; Rosazza et al. 1995; McMurrough et al. 1996; Donaghy et al. 1999; Karmakar et al. 2000). Accordingly any 4-vinylphenol or 4-vinylguaiacol, which has accumulated in wine at the end of primary fermentation through the action of other microbes could be acted on by *Dekkera* to produce the highly undesirable 4-ethylphenol and 4-ethylguiacol.

A semi-defined WM was developed in order to confirm the validity of results from CDM in an environment more analogous to that which occurs in industry. Thus CDGJM was pre-fermented to a model 'wine' using the commercial *S. cerevisiae* Maurivin PDM. The addition of 2 mM of acids to this wine was found to be inhibitory to all *Dekkera* and *Brettanomyces*. Even where the concentrations of HCAs were reduced to 100 mg/l, inhibition of some *Dekkera* and *Brettanomyces* strains was still observed. It therefore appears that inhibition by HCAs becomes more acute in wine-like environments, perhaps due to added presence of ethanol. Since the amounts of HCAs used in this experiment were more in line with total amounts found naturally in wines (Baranowski and Nagel 1981; Soleas et al. 1997; Kennedy et al. 2006), these findings may be indicative of a novel method of controlling unwanted *Dekkera* growth. The investigation of this inhibitory effect will be described in Section 7.

5.4 Conclusions

In conclusion this work provides a comprehensive overview of the catabolism of a number of HCAs and the production of vinylphenols and ethylphenols during growth in a defined and a wine-like. The metabolism of acid combinations by *Dekkera* yeasts

was also examined for the first time. The results indicate that the presence of HCAs in combination decreases the growth of yeast and metabolism of HCAs.

6. Survey of enzymatic activity responsible for phenolic-off-flavour production

Outputs and performance targets achieved

Output 4: Biochemical basis of volatile phenol production determined

Output 5: Information on the molecular basis of volatile phenol production

Output 6: Finalisation of biochemical and molecular investigations

Output 9: Communication of results, scientific paper and conference presentations (Appendix 1)

Dekkera yeasts decarboxylate HCAs into vinylphenol derivatives (Chatonnet et al 1992; Edlin et al 1995) via a phenolic acid decarboxylase (PAD) enzyme. Further catabolism into ethylphenols is hypothesised to occur enzymatically via the activity of a vinylphenol reductase (VPR) enzyme (Chatonnet et al 1992). *Dekkera* yeasts can also act directly on vinylphenol compounds released by other yeast or bacteria during wine fermentation to produce 4-ethyl derivatives (Section 5.2, Dias et al 2003b; Harris et al 2008). The ability of *Dekkera* to utilise free vinylphenols from the medium may increase the potential spoilage risk to wines infected by *Dekkera*. It has been indicated that the presence of *Dekkera* even without growth can effect ethylphenol production and therefore wine spoilage (Salameh et al 2008).

The PAD enzyme and homologues have previously been characterised from yeast and bacteria (Barthelmebs et al 2000a; Cavin et al 1997b; Clausen et al 1994; Edlin et al

1998). In *S. cerevisiae* there is a single copy of the *PAD1* gene (Clausen et al 1994). The over expression of this *PAD1* leads to an increased resistance to phenolic acids (Goodey and Tubb 1982). HCA decarboxylases have been identified from bacterial species, including *Pseudomonas fluorescens* (Huang et al 1994), *Lactobacillus plantarum* (Cavin et al 1997a), *Bacillus subtilis* and *pumilus* (Cavin et al 1998; Degrassi et al 1995; Zago et al 1995), *Pediococcus pentosaceus* (Barthelmebs et al 2000b) and *Klebsiella oxytoca* (Hashidoko et al 2001). Known PAD homologues are homodimers with a molecular weight of around 41-45 kDa (Cavin et al 1998; Degrassi et al 1995; Huang et al 1994; Rodríguez et al 2007) and have been related to a stress response caused by exposure to HCAs (Barthelmebs et al 2000a). The amino acid sequences of these enzymes share around 64-84% similarity (Barthelmebs et al 2000a; Cavin et al 1998), but they do not have any sequence similarity to the PAD protein of *S. cerevisiae* (Cavin et al 1998).

An enzyme with decarboxylating activity towards HCAs has been partially characterised from *B. anomalus;* now classified as *D. anomala* (Edlin et al 1998). The enzyme is constitutively expressed and its activity is induced in the presence of HCA substrates. The protein is a homodimer of approximately 38 kDa (Edlin et al 1998) and has specific activity towards caffeic, *p*-coumaric and ferulic acids with optimal pH and temperature, for *p*-coumaric decarboxylation, of 6 and 40°C, respectively (Edlin et al 1998).

N-terminal sequencing of protein purified from *D. anomala* revealed no similarity to other yeast or bacterial PAD enzymes, but instead suggested that the enzyme shares homology to sequence at the N-terminus of the YDR032 open reading frame (ORF)

of *S. cerevisiae* (Edlin 1997). This ORF was subsequently identified as *PST2* although their molecular and biological functions are not yet known (*Saccharomyces* Genome Database). The *S. cerevisiae PST2* ORF was cloned and the protein expressed in *E. coli,* however, the protein expressed had no detectable PAD activity (Edlin 1997).

The metabolism of HCAs and subsequent reduction by *Dekkera* and *Brettanomyces* yeast is important due to the role they play in wine spoilage. Therefore it was desirable to further characterise the enzymes involved in phenolic off-flavour production. Secondly, HCA are considered to be antimicrobial agents (Baranowski et al 1980) and the action of PAD detoxifies these compounds as a stress response (Barthelmebs et al 2000a). This function is not well understood and may have implications in the future control of *Dekkera* and *Brettanomyces* yeasts.

6.1 Methods

6.1.1 Yeast strains

Yeasts used in this study are described in 5.1.1.

6.1.2 Cell extracts

Starter cultures (YPD broth, 50 ml) were grown from single colonies and used to inoculate 500 ml YPD to 5 x 10^6 cells/ml. Cultures were grown with or without the addition of substrates and to a density of approximately 10^8 cells/ml. A 20 ml aliquot of culture was removed for dry cell weight determination. The remaining culture was centrifuged (10,000 x *g* for 30 min). The cell pellet washed with 20 ml of 20 mM bis-Tris (pH 6) and resuspended in the same buffer and homogenised with Zirconia/Silica beads (10 min MSK Braun Homogeniser). Beads and cellular debris were removed by

centrifugation (10 min at 4500 x *g*) before clarification of the supernatant by ultra-centrifugation (30 min at 100,000 x *g* and 4ºC). The total protein concentration of extracts was determined using the modified Lowry method as per manufacturer's instructions (Pierce Biotechnology).

6.1.3 Dry cell weights

Dry cell weights of cultures were determined in duplicate, using 10 ml of culture, filtered through a pre-weighed glass-fibre membrane (Millipore) and washed with 25 ml sterile MQ water. The filter was microwaved (650W) on a medium power setting for 10 min and held in a desiccator chamber overnight. Filters were re-weighed and the dry weight calculated in g/l.

6.1.4 Enzyme activity assays

All assays were performed in triplicate with 50 µl of cell extract added to 450 µl of 20 mM bis-Tris buffer pH 6 with either 2 mM HCA or 0.5 mM vinylphenol and 0.1 mM $NADH^+$ in a 1.5 ml microcentrifuge tube. The standard assays were incubated at 40ºC for 10 min and the reaction was stopped by heating at 80ºC for 10 min. Protein precipitate was removed by centrifugation (20,800 x *g*, 1 min). The supernatant was analysed by HPLC for a decrease in substrate concentration and/or the detection of product formation. A blank of 450 µl of buffer with 50 µl boiled extract was included for all assays. Activity was reported as units of activity per mmol per second.

6.1.5 Purification of PAD enzyme

Purification was achieved using a modification of the protocol described by Edlin et al (1998). Clarified cell extract (50 ml) was loaded onto an anion exchange column 50

mm x 10 mm (Q-sepharose resin, GE Healthcare), with a 12 ml column volume (CV). The column was equilibrated before loading with 10 x CV and washed after sample loading with 3 x CV of buffer A (20 mM bis-Tris buffer pH 6, 0.01 mM DTT, 20% glycerol) at 1 ml/min. A gradient of 0 to 50% buffer B was applied over 12 column volumes at the same flow rate. Buffer B consisted of buffer A, plus 1M NaCl. Fractions of 5 ml were collected using an automated fraction collector (BioRad) and protein elution was recorded by monitoring absorption at 280nm. The protein concentration in each fraction was assayed using the Modified Lowry method. Representative fractions were analysed by SDS PAGE electrophoresis (Fling and Gregerson 1986). Fractions were assayed for activity (see above) and the active fractions pooled and concentrated using an Amicon stirred cell microconcentrator fitted with YM30 membrane.

Concentrated fractions were prepared for hydrophobic interaction chromatography by the addition of ammonium sulfate to a final concentration of 1.7 M and loaded onto a Phenyl-Sepharose HP column, (GE Healthcare) of the same dimensions used for anion exchange. The column was equilibrated before sample loading with 10 x CV of buffer C (20 mM Bis-Tris pH 6, 1.7 M $(NH_4)_2SO_4$) and washed after sample loading with 3 x CV at a flow rate of 1 ml/min. A gradient of 1.7–0 M $(NH_4)_2SO_4$ was applied to the column over 10 CV and 5 ml fractions collected. Fractions were analysed and concentrated as described above. Active fractions from hydrophobic interaction chromatography were combined and injected onto a second Q-sepharose column and proteins eluted using the same conditions for anion exchange chromatography as described above.

Sequencing was achieved using a combination of ESI and MALDI MS/MS and results were analysed by MS-BLAST (Papayannopoulos 1995).

6.1.6 PAD enzyme stability

The stability of PAD activity was determined over time under different storage conditions. Extracts were stored at -80, -30, 4, 24, 40 and 80ºC in 500 µl aliquots. The activity was measured in triplicate at 0, 1, 2, 7, 14 and 28 days as described above.

6.1.7 Survey of optimal conditions of enzyme activity involved in volatile phenol production

PAD activity was measured over a range of temperatures using the assay described previously, incubated at 30, 38, 39, 40, 41, 42, 45 and 50ºC. In further assays, the effect of pH was examined across the range pH 5 to 7, using 20 mM acetic acid/acetate buffer for pH 5.0 and 5.5, 20 mM Bis-Tris buffer for pH 6 and 6.25 and 20 mM Tris-HCl buffer between pH 6.5 and 7. Assays for pH-dependence were performed at 40ºC. The pH range was clustered around pH 6 as this had previously been identified as the optimum pH of PAD enzyme activity from one strain of *D. anomala* (Edlin et al 1998).

Characterisation of the optimal temperature of VPR activity was achieved over a temperature range of 20-60ºC at 10ºC increments. A pH range of between 3–9 was investigated using the following buffers prepared at a 20 mM final concentration with 0.5 mM 4-vinylphenol: pH 3 and 4 acetate buffer, pH 5 and 6 bis-Tris buffer and pH 7-9 with Tris-HCl.

6.1.8 Substrate specificity of PAD/VPR enzyme activity from *Dekkera* and *Brettanomyces* cell extracts

The specificity of PAD activity was determined using *p*-coumaric, ferulic, caffeic, sinapic, sorbic and gallic acids, as well as vanillin at a concentration of 2 mM. Reductase activity was determined for 0.5 mM 4-vinylphenol and 4-vinylguaiacol.

6.1.9 HPLC analysis of enzyme activity

HPLC analysis of assays was carried out in duplicate using the method described in 5.1.5.

6.1.10 3'RACE PCR

RNA was isolated from *D. anomala* CBS 77 using the RNeasy mini prep (Qiagen) as per manufactures instructions. cDNA was made from 2 µg of RNA, using superscript 3 reverse transcriptase (Invitrogen) as per manufacturers instructions. 2nd strand synthesis was preformed with PFU DNA polymerase (Promega) using 2 µl of first strand reaction and 1 µl, 10 mM nucleotide mix (Roche). Oligo DT (Invitrogen) was used to prime the reverse reaction. The sense primer (AAGGTCG CTATTATYATCTAC) was designed from the back-translated N-terminal amino acid sequence (Edlin et al 1997). Reactions were preformed in an Eppendorf Mastercycler Gradient thermocyler for 35 cycles, denaturing at 95ºC for 30 secs, annealing 54ºC, 30 secs, extension 72ºC, 30 secs with a final 5 minute extension. Reactions were run on a 1% TAE gel with ethidium bromide. Resultant bands were excised and purified using the Wizard SV gel and PCR clean up system (Promega). 20 ng of product was sequenced by the Australian Genome Research Facility (Brisbane) purified DNA sequencing service.

6.2 Results

6.2.1 Storage of extract

The PAD activity in cell extracts from *D. anomala* and *D. bruxellensis* was examined for stability under different storage conditions (Table 6.1) to determine the optimum conditions for future protein investigations. The activity of fresh cell extract was designated to be 100%. Activity was analysed over a period of up to 28 days from extract preparation with storage at between -80ºC to 80ºC. In general the lower the storage temperature the more enzyme activity was retained in cell extracts while the longer the extract was stored the more activity was lost. When held at 40ºC and above, the activity of extracts from all strains was reduced to less than 40% after 1 day and lost completely after 1 week or more. Storage at room temperature (25ºC) or with refrigeration (4ºC) for 1 day resulted in greater than 60% of the original activity being retained. Freezing of samples proved to be the optimal method of storage such that. When frozen at -80ºC all extracts retained between 71 and 86% of their original activity after 1 month.

Table 6.1. Effect of storage on PAD activity over time preformed and analysed in duplicate, standard deviation for all values are below 5%

Storage (°C)	% activity remaining after								
	1 day			1 week			1 month		
	CBS 77	AWRI 1499	CBS 2336	CBS 77	AWRI 1499	CBS 2336	CBS 77	AWRI 1499	CBS 2336
-80	100	100	100	93	88	82	86	78	71
-30	83	91	100	77	77	87	64	73	63
4	65	60	86	30	42	53	24	40	55
25	63	61	76	27	13	0	0	0	0
40	10	33	36	0	0	0	0	0	0
80	0	0	0	0	0	0	0	0	0

The VPR activity was highly unstable and while activity could be measured in freshly produced cell extract, no activity could be detected after 24 hours, even when the extract was immediately frozen at -80ºC. All further experiments were therefore carried out using fresh cell extracts to maintain the integrity of the enzyme.

6.2.2 Substrate specificity and induction of PAD enzyme activity

Dekkera and *Brettanomyces* cultures grown with no addition of substrates acted as experimental controls. Neither induced nor uninduced *Dekkera* or *Brettanomyces* yeast utilised gallic acid, sorbic acid or vanillin (data not shown). Cell extracts prepared from *Dekkera* yeasts had low constitutive activity towards HCA (Fig. 6.1). No activity under any conditions was detected for any cell extracts prepared from *Brettanomyces* species. Growth in the presence of *p*-coumaric or ferulic acids increased the activity of cell extracts from *D. anomala* and *D. bruxellensis* by up to 64%-80%, respectively (Fig. 6.1). The induction effect of ferulic acid was greater for *D. bruxellensis* strains than *D. anomala* and the opposite was determined for *p*-coumaric acid induction.

D. anomala was the only yeast species investigated that was able to utilise sinapic acid (Fig. 6.1) and in fact *D. anomala* utilised all four HCA examined, with the greatest utilisation being found for caffeic, ferulic, *p*-coumaric and then for sinapic acids. The two strains of *D. bruxellensis* varied in their utilisation of substrates, AWRI 1499 converted less than 30% of the *p*-coumaric acid in the assay while CBS 2336 removed over 80% when induced with *p*-coumaric acid, however, AWRI 1499 demonstrated higher utilisation of ferulic acid.

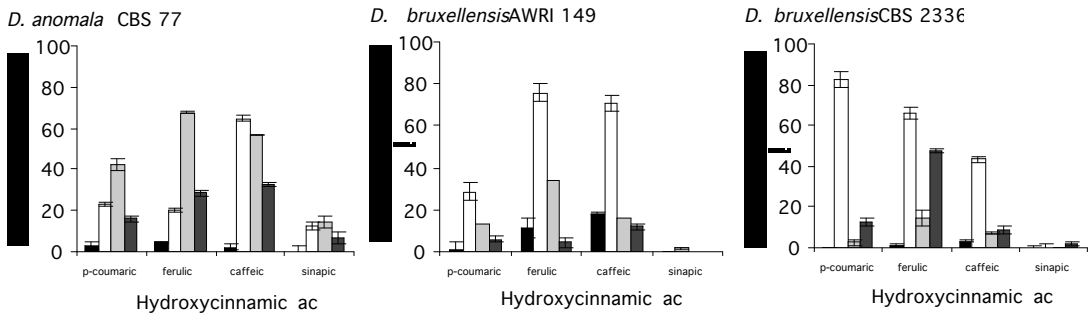


Fig. 6.1 Influence of growth with (white) or without (black) *p*-coumaric, ferulic acid (grey) or 4-vinylphenol (dark grey) on the utilisation of hydroxycinnamic acids by *Dekkera* species where 100% is equivalent to 2 mM initial concentration. Values are the means of duplicate assays measured by HPLC in duplicate ± standard deviation.

6.2.3 Substrate specificity and induction of VPR enzyme activity

Investigations to determine the substrate specificity of the VPR enzyme were undertaken. *Dekkera* and *Brettanomyces* cultures were grown in the presence or absence of vinylphenol compounds. Constitutive activity was present in all extracts, towards at least one vinyl compound (Fig. 6.2). *D. bruxellensis* AWRI 1499 had the highest uninduced activity towards 4-vinylphenol but had only trace activity towards 4-vinylguaiacol. Similarly *B. naardenensis* utilised 20% of the 4-vinylphenol but no 4-vinylguaiacol. *D. anomala* in comparison to *D. bruxellensis,* removed more of the substrate 4-vinylguaiacol than 4-vinylphenol.

Extracts prepared from cultures grown with 4-vinylphenol showed induction of VPR activity in both *Dekkera* species and only one *Brettanomyces* species (Fig. 6.2). The activity of *D. bruxellensis* CBS 2336 towards 4-vinylphenol and 4-vinylguaiacol was increased 3–5 fold by growth with vinylphenol. Activities of VPR in cell extracts from *B. nanus* and *B. naardenensis* were not affected by prior growth with the substrate, while utilisation of both vinyl-derivatives by *B. custersianus* increased 3 fold (Fig. 6.2). Extracts made from cultures induced with HCA did not influence the VPR activity of any yeast screened (data not shown).

6.2.4 Effect of pH and temperature on PAD enzyme activity

Extracts from *Dekkera* species induced with *p*-coumaric acid were assayed for PAD activity at pH values in the range of 5–7. Increments of 0.25 were used to more accurately define the optima and determine species and strain differences. Both strains of *D. bruxellensis* examined had optimal activity at pH 6 (Fig. 6.3A) while *D. anomala* had slightly lower pH optima of 5.75. Assays to ascertain the optimal

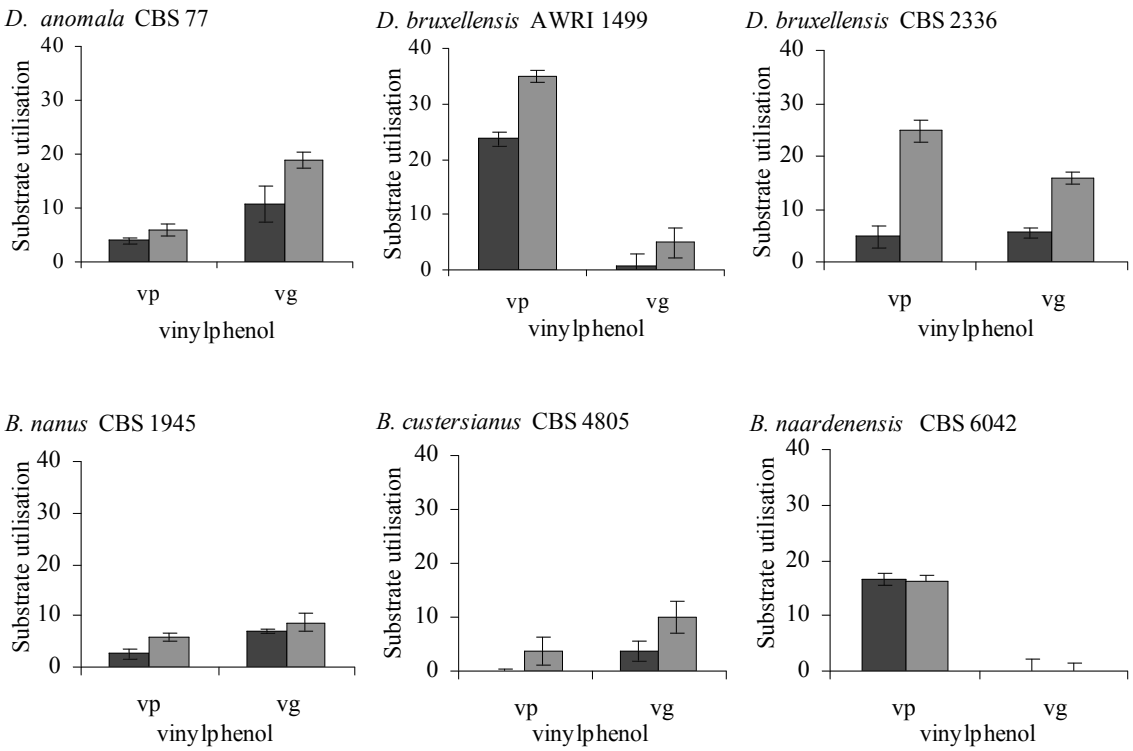


Fig. 6.2 Influence of *Dekkera* and *Brettanomyces* species culture growth with (grey) or without (black) 4-vinylphenol on the utilisation of vinylphenols where 100% is equivalent to 1 mM initial concentration. Values are the means of duplicate assays measured by HPLC in duplicate ± standard deviation.

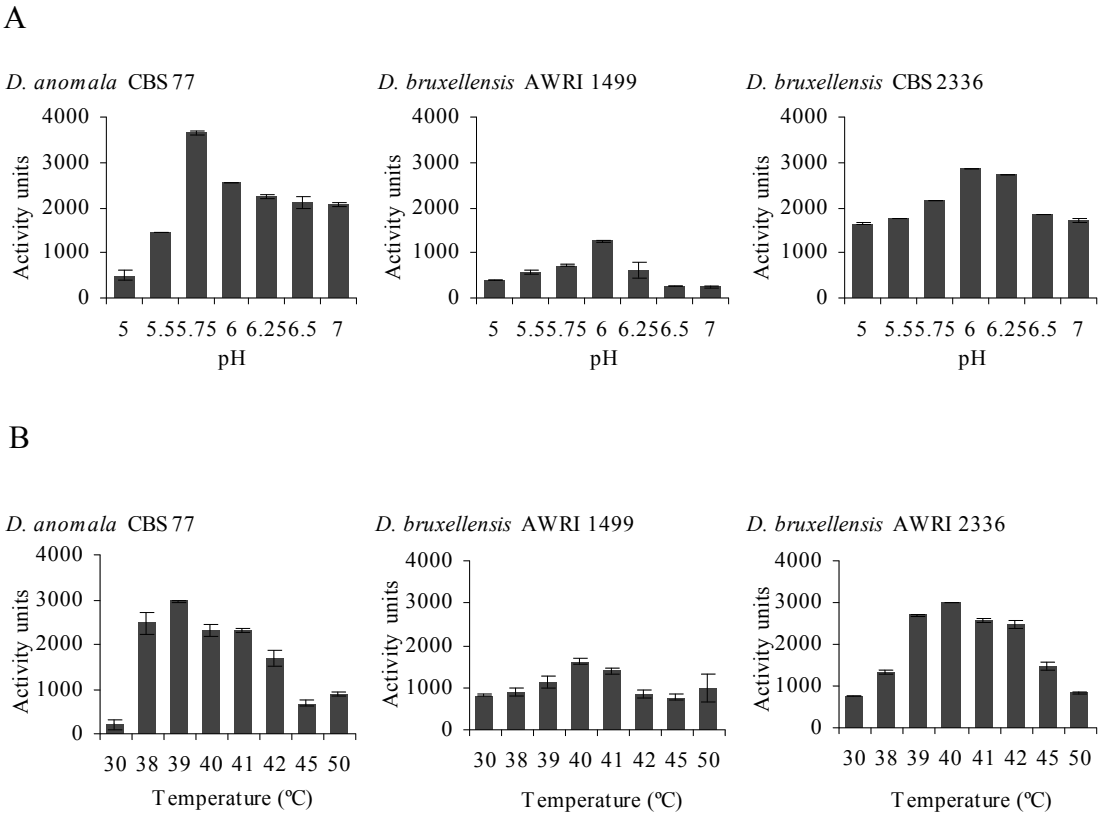


Fig. 6.3 Influence of pH (A) and temperature (B) on phenolic acid decarboxylase activity of *Dekkera* species. Units of activity are defined as pmol of *p*-coumaric acid removed from assay per mg of protein in 1 second. Values are the mean of triplicates ± standard deviation.

temperature for PAD activity were conducted at temperatures ranging from 30–50ºC. The optimal temperature for the *D. bruxellensis* strains was 40ºC and 39ºC for *D. anomala* (Fig. 6.3B).

6.2.5 Effect of pH and temperature on VPR enzyme activity

Assays were conducted with crude cell extracts of *Dekkera* and *Brettanomyces* yeast cultures, grown in the presence of 4-vinylphenol, to determine the optimal pH and temperature for VPR activity. A pH range of 3–9 was used followed by temperatures between 20–70ºC. The optimal pH for activity was pH 7 for all yeast strains except *D. anomala,* which had highest activity at pH 6 (Fig. 6.4).

Temperature assays were preformed at pH 6 and revealed no activity amongst any yeast at the extremes temperatures of 20 and 70ºC but all yeast had an optimum of around 40–50ºC (Fig. 6.5). *Dekkera* species in particular had activity over a large temperature range of 30–60ºC.

6.2.6 PAD Protein purification from *D. anomala*

Protein purification of PAD from *D. anomala* was undertaken using methods modified from Edlin et al (1998). Crude cell extract was made from 5 l of culture grown in YPD media until exponential phase, and the PAD protein purified using FPLC. Fractions were analysed for HCA decarboxylase activity by mixing 50 µl of the fraction with 450 µl *p*-coumaric reaction buffer and analysing for the production of vinylphenols by HPLC (Edlin et al 1998). These active fractions were combined and run on 12% SDS-PAGE gels until the protein band at around 21 kDa clearly resolved.

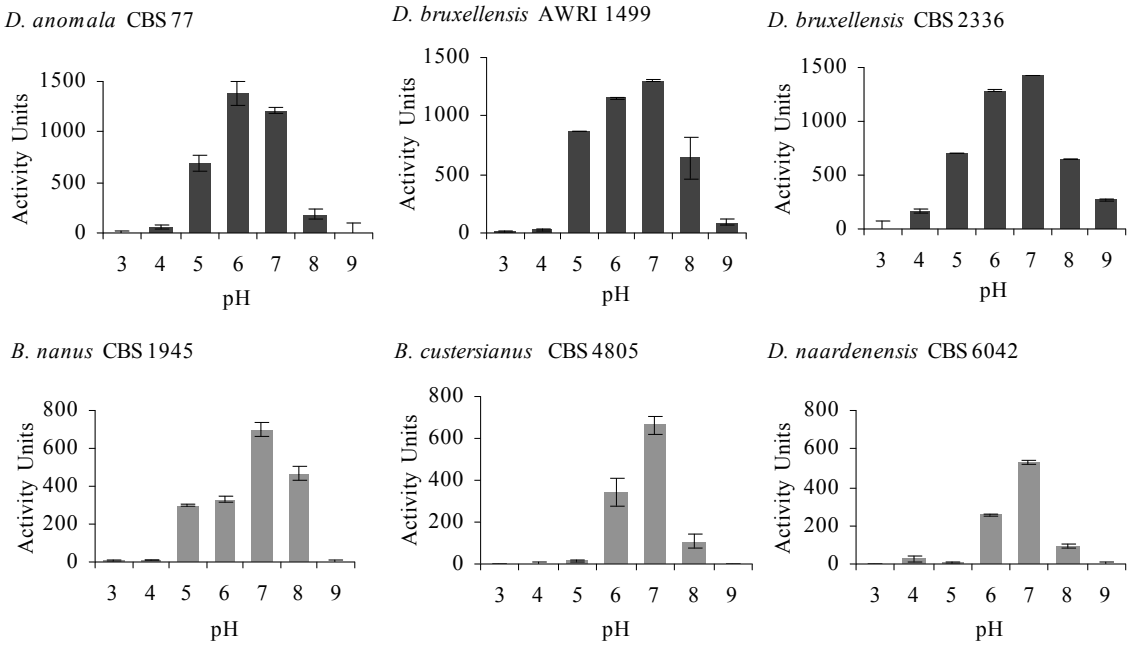


Fig. 6.4 Influence of pH on vinylphenol reductase activity of *Dekkera* species. Units of activity are defined as pmol of 4-vinylphenol removed from assay per mg of protein in 1 second. Values are the mean of duplicate assays analysed in duplicate by HPLC ± standard deviation.

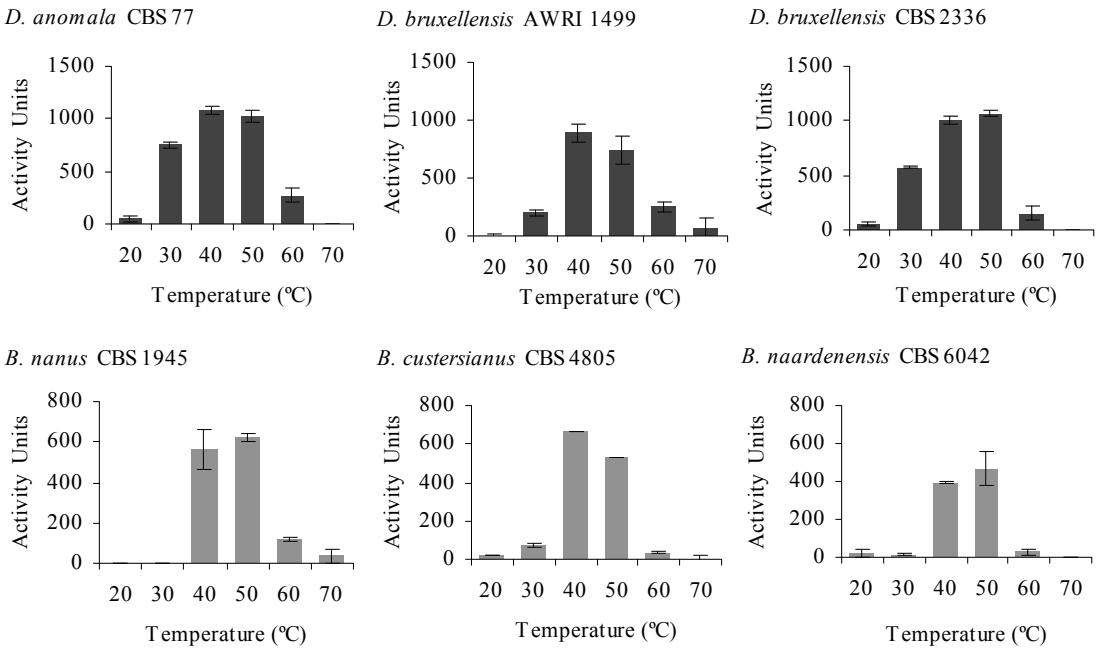


Fig. 6.5 Influence of temperature on vinylphenol reductase activity of *Dekkera* and *Brettanomyces* species. Units of activity are defined as pmol of 4-vinylphenol removed from assay per mg of protein in 1 second. Values are the mean of duplicate assays analysed in duplicate by HPLC ± standard deviation.

This protein band was sequenced at a commercial facility (Adelaide Proteomics Centre). Thus the isolated protein was excised from the SDS gel, destained and digested with trypsin, the resultant peptides were extracted sequentially and sequenced using combined ESI and MALDI MS/MS. A partial amino acid sequence of 22 amino acid residues was obtained, YPLATTETLTAYDGVLFGVPTR (where L can be interchanged for I). This amino acid sequence had a high degree of similarity to the *Candida albicans* protein PST2 and other fungal PST2 entries, especially *S. cerevisiae* (Fig. 6.6). Sequence alignment tools (Bioedit version 7.0.0) were used to align the *Dekkera* sequence to that of PST2 protein sequence of *C. albicans* and *S. cerevisiae* (Fig. 6.6).

The protein fragment obtained here aligned to the PST2 protein, from residues 60–81 of *Candida* and 58–79 of *Saccharomyces*, sharing 50 and 68% homology to *Candida* and *Saccharomyces,* respectively (Fig. 6.6). Additionally, the N-terminal sequence previously obtained from the same strain of *Dekkera* was aligned with the PST2 sequences. This sequence aligns with around 50–56% homology to PST2 further strengthening the assumption that the PAD active protein from *D. anomala* is a PST2-like enzyme. In comparison the isolated protein, compared to the PAD1 gene from *S. cerevisiae* only has around 20% homology.

6.2.6 Sequencing of postulated phenolic acid decarboxylase gene

RNA was isolated from *D. anomala* CBS 77 and cDNA was prepared. A sense primer was designed from the back translated N-terminal sequence of the protein isolated by Edlin (1997) and used in conjunction with oligo dT in the PCR reaction. A fragment of 600 bp was isolated and sequenced.



Fig. 6.6 Alignment of *Dekkera* protein sequence, obtained by tryptic digest during this investigation, to *PST2* sequences from S. *cerevisiae* and *C. albicans*.

Sequence analysis was preformed using NCBI-Blast 2 nucleotide tool, available from the EBI website (EMBL-EBI). Results indicate that the isolated DNA had a high homology to the *PST2* gene of *S. cerevisiae* and *C. albicans*. When translated into an amino acid sequence, the partial protein sequence also aligned (Bioedit version 7.0.0) with the amino acid sequence obtained during this work. The PST2 proteins of a number of yeast species are shown along with the known *D. anomala* protein fragments in Fig. 6.7. From this alignment it is possible to determine where the N-terminal sequence aligned with the translated sequence obtained during this work to obtain a near complete protein sequence.

6.3 Discussion

The metabolism of HCA acids by *Dekkera* can be important in sensory development of wine. It is therefore vital to understand the metabolic processes that are involved in this flavour modification. This investigation expands on previous research conducted by (Edlin et al 1998) in which the PAD enzyme of *D. anomala* was partially purified and characterised. The study presented here includes strains of *D. bruxellensis* and *D. anomala*, which are relevant to wine production, and examined the closely related *Brettanomyces* genera.

The presence of *p*-coumaric or ferulic acid substrates in growth media augments the activity of *Dekkera* towards HCA, increasing the decarboxylation to vinylphenols and therefore enhancing the probability of spoilage. The PAD enzyme activity from *D. bruxellensis*, the species most commonly found in wine (Curtin et al. 2007), is most reactive to HCAs in growth media. The enzyme activity is greatly increased and this induction could be due to an adaptation to growth in environments rich in the

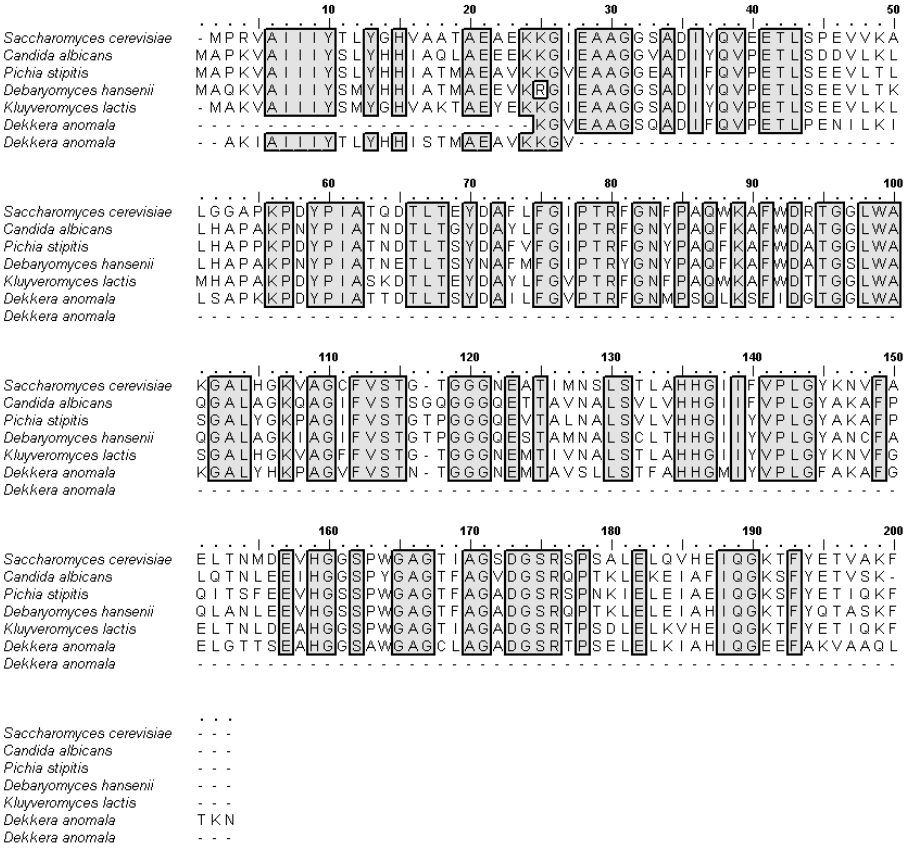


Fig. 6.7 Alignment of protein sequence obtained by 3'RACE from *D. anomala* CBS 77 and the previously sequenced N-terminal region to yeast proteins with high homology (EMBL-EBI BLAST)

antimicrobial HCA compounds (Section 5.1.1; Hammond et al 1999; Harris et al 2008; Stead 1995). *p*-coumaric acid had the greatest effect on enzyme induction in both *D. bruxellensis* and *D. anomala*, which may explain why these yeast are more resistant to *p*-coumaric acid than ferulic acid (Fig. 5.1; Harris et al 2008)

Research described above in Fig. 5.2 suggested that *Brettanomyces* species may be capable of utilising limited quantities of sinapic or caffeic acids. However, the production of vinyl and ethyl derivatives was not observed. The study using crude cell extracts did not confirm the conversion of HCAs even when these activities were induced (Fig. 6.2). Therefore *Brettanomyces* species appear unlikely to have a PAD-type enzyme. However there is the possibility that an alternative enzyme, of different function, can utilise caffeic and/or sinapic acid, but which is not active under the conditions investigated here.

The enzyme activity towards substrates varied depending on species and strain of *Dekkera* yeast. For example *D. anomala* was more active towards caffeic acid, than ferulic and *p*-coumaric acid, confirming results obtained by Edlin et al (1995). By comparison, *D. bruxellensis* strain CBS 2336 had the highest activity for *p*-coumaric acid and AWRI 1499 for ferulic acid. This variation may explain why some wines infected with *Dekkera* accumulate a high concentration of spoilage compounds whilst others do not. This result again highlights the unpredictability of wine spoilage and the complex interactions that occur. The crude extract activity towards substrates does not reflect results found in fermentation studies where *p*-coumaric acid is preferentially utilised (Fig. 5.1). The uptake of HCAs into the cell has not been studied and could give further insights into this phenomenon.

Previous work had determined the pH and temperature optimum of PAD activity for *D. anomala* over a broad range (Edlin et al 1998). By using smaller increments, the pH optimum was found to be slightly lower (pH 5.75) than previously reported (pH 6) for *D. anomala*. The optimal temperature and pH of PAD activity for *D. bruxellensis* was investigated for the first time in the present work and found to be almost identical to that of *D. anomala* at pH 6 and 40ºC.

To our knowledge and despite the production of ethylphenols by *Dekkera* species being well established, this is also the first report of the characterisation of VPR activity *in vivo*. This study has attempted to characterise from cell extracts some key parameters for the activity of the protein. The enzyme appears to be highly unstable with activity lost after only 24 hours under any storage condition, but the utilisation of vinylphenols was detected in fresh extracts. All species of *Dekkera* and *Brettanomyces* were shown to utilise 4-vinylphenol and 4-vinylguaiacol. This agrees with previous fermentation evidence that *Brettanomyces* species can also catabolise vinylphenols (Fig. 5.5) and increases the known number of yeast genera which have VPR activity. The VPR activity from *Dekkera* and *Brettanomyces* appears to be induced in the presence of both HCAs and vinylphenols in the growth medium. 4-Vinylguaiacol is shown to have the greatest effect on VPR activity in *Dekkera* species. Induction of VPR activity by HCA is most likely to be indirect as vinylphenols are released into the medium after HCAs are catabolised by PAD during growth, however, direct induction cannot be ruled out without further investigation.

The isolated and partially sequenced protein from *D. anomala* with HCA decarboxylase activity described here has homology to the PST2 protein of *S.*

cerevisiae and *C. albicans*. In addition, the identification of a partial DNA sequence from *D. anomala* using primers designed to the N-terminus of a phenolic acid decarboxylating protein (Edlin 1997) corresponded to the *PST2* gene. The DNA and protein sequence identified in this work and by Edlin (1997) is unlike any enzymes previously described as having PAD activity. Therefore, the protein isolated from *D. anomala* appears to be a novel enzyme with PAD activity. By combining this new sequence information, gained from tryptic digestion of the protein, the translated sequence obtain by 3' RACE with the N-terminal sequence obtained from the same yeast strain (Edlin 1997) an almost complete protein sequence has been determined (Fig. 6.7). Small differences in the sequences obtained by protein sequencing and 3' RACE indicate that there may be at least 2 isoforms of this gene within the *Dekkera* genome.

Multi-sequence alignment of the PST2 protein of several yeast species and the sequence obtained during this study confirms the novel identity of a protein with PAD activity from *D. anomala*. The isolated protein fragment had a total of 66 and 61% homology to *S. cerevisiae* and *C. albicans* PST2 proteins, which is slightly higher than the predicted 54% from previous protein sequence comparisons (Woolfit et al 2007). The translated protein sequence has a predicted flavodoxin mononucleotide binding site (InterProScan), indicating it is a member of the flavodoxin-like family. Previously identified PAD and PST2 proteins are members of the flavoprotein or flavodoxin families (Grandori and Carey 1994; Rangarajan et al 2004). These proteins are of diverse function, but all are comprised of flavin mononucleotide binding site.

6.4 Conclusions

The results from Section 6 characterised enzyme activities involved in the degradation pathway of naturally occurring HCA into ethylphenols, which are responsible for wine spoilage. The activity of the PAD enzyme from a range of *Dekkera* and *Brettanomyces* species has been discussed including substrate specificity, pH and temperature optima. The VPR enzyme has never previously been characterised and results presented here confirm the presence of such an enzyme, which has previously only been postulated and determined key parameters for its activity.

A partial PAD protein sequence from *D. anomala* has also been described. This sequence does not share homology with previously sequenced PAD genes from other yeast or bacteria but does align with a putative flavodoxin-like protein from *S. cerevisiae* and *C. albicans*. This sequence aligns to the same protein as the N-terminal sequence reported by Edlin (1997). This may indicate the presence of a novel enzyme in *Dekkera* species, which is responsible for HCA decarboxylation. Further protein and molecular characterisation is required to confirm this.

7. Ferulic acid toxicity on *Dekkera* yeast as a possible mechanism of control in wine

Outputs and performances targets achieved

Output 7: Information on yeast growth and off-flavour development in small-scale winemaking trials

Output 8: Development of strategies to minimise influence of Dekkera/Brettanomyces

Output 9: *Communication of results, scientific paper and conference presentations (Appendix 1)*

In investigating the utilisation of HCAs by *Dekkera* and *Brettanomyces* yeasts (Sections 5 and 6), we also observed a significant inhibition of growth for several isolates. Section 7 expands on the previous investigations and examines a broader range of HCA concentrations and synergistic effects arising from combinations of HCAs, pH and ethanol. The objective of this work was to evaluate the potential of ferulic acid as a novel method for control of *Dekkera* in wine

7.1 Materials and methods

7.1.1 Yeast strains

The AWRI and CBS culture collection yeasts used in this study are described in Table 7.1. The wine isolates were obtained from a winery that reported *Dekkera* contamination. Yeast were isolated on YPD agar [1% (w/v) yeast extract, 2% (w/v) peptone (Amyl Media), 2% (w/v) glucose (Univar) and 2% (w/v) bacteriological agar (Amyl Media)] supplemented with 0.01% cycloheximide. To confirm species identity, genomic DNA preparations were made (Adams et al. 1997) and the internal transcribed spacer (ITS) region was amplified by PCR as described by (Guillamón et al. 1998). PCR products were sequenced at the Australian Genome Research Facility (Brisbane). Sequence alignment gave 99 to 100% homology to the *D. bruxellensis* ITS region (EMBL-EBI). All yeasts were routinely maintained on YPD agar stored at 4^{o}C.

Table 1 Yeast strains used, VH strains were isolated from wine as part of this investigation.

Species	Strain
Dekkera anomala	CBS 76, CBS 77, CBS 8139
Dekkera bruxellensis wine isolates	AWRI 1499, CBS 2336 VH01, VH04, VH05

7.1.2 Investigation of the effect of ferulic acid on *Dekkera* growth in a chemically defined medium (CDM) at various pH values and ethanol contents

To prepare starter cultures, a single loop of *Dekkera,* freshly grown on YPD plates was inoculated into 25 mL of YPD broth and grown at 30ºC with agitation (180 RPM) to a density of 10^8 cells/mL. Cells were subcultured to 5 x 10^6 cells/mL into 250 mL Erlenmeyer flasks containing 50 mL of CDM supplemented with 0–2 mM ferulic acid, pH 3.8 (Section 5.1.2). All cultures were prepared in triplicate and flasks were incubated at 30ºC.

The influence of pH and ethanol on the growth of *Dekkera* isolates was examined by preparing cultures as above but with media adjusted to pH values between 3 and 5 or else containing ethanol at concentrations of up to 10% (v/v). All treatments were conducted in triplicate and included ferulic acid at a concentration of 0.5 mM.

7.1.3 Minimum reducing, inhibitory and fatal concentrations in CDM, wine medium and wine (MRC, MIC, MFC)

The minimum concentration at which ferulic acid significantly reduced the growth of the *Dekkera* culture (i.e. the MRC), completely inhibited growth (i.e. the MIC) or as fatal to *Dekkera* cells (i.e. the MFC) in was determined in triplicate using 10 mL of

media supplemented with HCAs. Supplementation rates for CDM were 0–20 mM of ferulic, *p*-coumaric or caffeic acids, for wine medium (Section 5.1.3 WM; Harris et al. 2008) 0–20 mM of ferulic acid was used, while ferulic acid was only used at 0–8 mM in wine. Prior to being used the wine (Shiraz, 12.8% v/v ethanol, < 2 g/L sugars, pH 3.4) was centrifuged for 1 hour at 10,000 x *g* and filter sterilised (0.22 μm). The wine was adjusted to 10 g/L sugar (equimolar glucose and fructose) and pH 3.8, and vitamins (25 mg/ L myo-inositol, 1 mg/L pyridoxine HCL, 10 mg/L nicotinic acid, 1 mg/ L thiamine HCl and 30 mg/L biotin were added).

MRC, MIC and MFC were determined in wine media. Starter cultures were grown YPD with the addition of 5% ethanol for adaptation and then inoculated as above. Yeasts were grown for 7 days with a range of ferulic acid concentrations and cell growth was monitored at OD_{600}. Cultures were treated as above and inoculated into fresh WM with no ferulic acid. Growth was determined after 4 days by OD measurement.

In the case of wine, only ferulic acid was examined and each was inoculated with 5 x 10^6 cells/mL in triplicate and incubated at 30ºC for 2 weeks and the OD_{600} measured, cultures were washed as above and then inoculated into CDM with no additions. Growth was then determined after 3 days incubation at 30ºC. Control cultures (i.e. media lacking HCAs) and treatments in which OD_{600} increased by < 10% of the control were washed and inoculated into fresh media with no HCA additions. Growth was measured after 3 days by OD_{600} measurement. Cultures were incubated at 30ºC

7.1.4 Examination of *Dekkera* by Scanning Electron Microscopy (SEM)

7.1.4.1 Culture growth in presence of ferulic acid

Cultures of *D. bruxellensis* AWRI 1499 and *D. anomala* CBS 77 were grown in the presence of ferulic acid (0, 0.5 and 1–2 mM respectively) for 3 days. Cells were harvested and washed in PBS buffer pH 7.2 (8 g NaCl, 0.2 g KCL, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4), then resuspended into PBS in preparation for SEM imaging.

7.1.4.2 Incubation of cells in presence of fatal concentrations of ferulic acid

Otherwise, cultures of *D. bruxellensis* AWRI 1499 and *D. anomala* CBS 77 were grown in YPD until late-exponential phase then cells washed in PBS buffer. An aliquot of cells was added to PBS with 0, 2 or 20 mM of ferulic acid additions and incubated for 16 hours at 37°C.

7.4.1.3 SEM analysis

Washed cells (1 mL) were filtered through a 0.22 μm membrane. The cells were then fixed to the membrane with fixing solution (4% (v/v) paraformaldehyde/1.25% (w/v) glutaraldehyde/4% (w/v) sucrose in PBS, , pH 7.2) for 20 minutes, then washed in buffer (4% (w/v) sucrose in PBS) for 5 minutes. Cells were post-fixed in 2% OsO_4 in water for 30 mins. Cells were dried in ethanol (v/v) 70%, 90%, 95% and 100% for 10 mins each. A critical point dryer was used to exchange remaining 100% ethanol for liquid CO_2 over 10 cycles. Filters were removed, stuck to a stub, with carbon added for conductivity prior to image analysis.

7.1.5. Sensory evaluation of ferulic acid additions to red wine

Duo trio difference testing was performed to evaluate the aromatic effect of 4 mM ferulic acid addition to red wine (Lawless and Heyman 1999).

7.2 Results

7.2.1 *Dekkera* yeast growth in varying concentrations of ferulic acid

D. anomala and *D. bruxellensis* yeasts were grown in CDM with the presence of varying concentrations of ferulic acid from 0-2 mM (Fig. 7.1). The growth of all strains examined was decreased in the presence of ferulic acid although in general *D. bruxellensis* was more susceptible than *D. anomala*, with the growth of CBS 2336 and AWRI 1499 completely inhibited at a concentration of 2 mM.

D. bruxellensis isolated from the winery environment during this study exhibited longer lag phase, than the culture collection strains, even without the addition of ferulic acid (Fig. 7.1). Further, wine isolates were less susceptible to the higher ferulic acid concentrations compared with the culture collection isolates (Fig. 7.1). The three wine isolates grew poorly in CDM with 2 mM ferulic acid, where as, neither AWRI 1499 or CBS 2336 grew despite originally being isolated from wine.

D. anomala strains CBS 76 and CBS 8139 did not show an increased lag period when grown with concentrations of ferulic acid below 1 mM, however, 2 mM ferulic acid increased the lag phase by 24 hours (Fig. 7.1). *D. anomala* CBS 77 was more sensitive, and had a longer lag phase, compared to the control, when grown with only 0.25 mM acid and the lag phase was four times the control when 2 mM of acid was present.

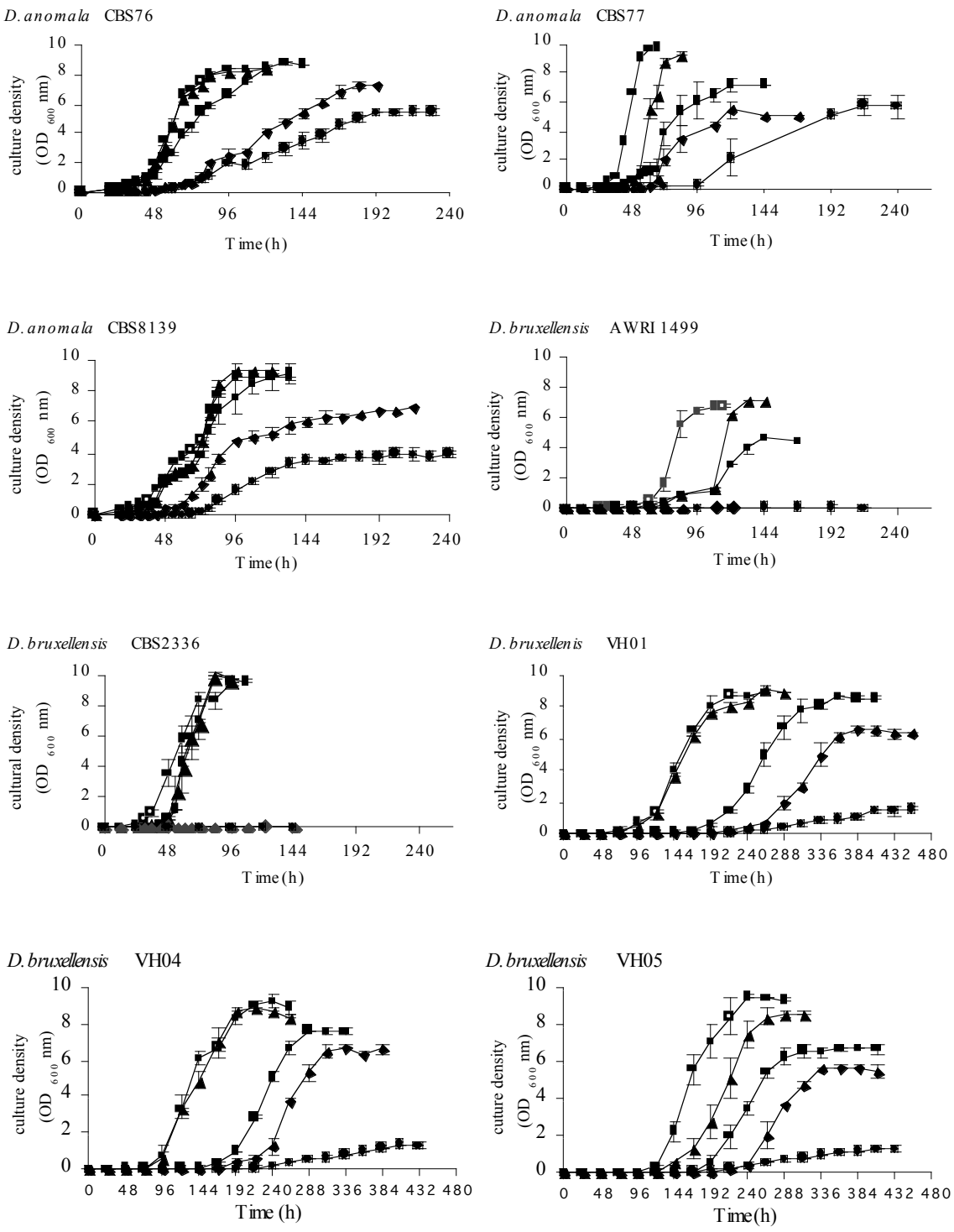


Fig. 1 *Dekkera* growth in the absence (empty square) and presence of ferulic acid; 0.25 mM (triangle) 0.5 mM (filled square) 1 mM (diamond) and 2 mM (circle) estimated by optical density (OD_{600} nm). Yeast strains CBS 76, 77, 8138, 2336 and AWRI 1499 were monitored in duplicate over 240 hours while wine isolates (VH01, 04 and 05) of *D. bruxellensis* were monitored over 480 hours.

In addition to the increased duration of culture growth for both *D. anomala* and *D. bruxellensis*, biomass production (OD_{600}) was reduced with respect to substrate concentration (Fig. 7.1). Concentrations of ferulic acid as low as 0.5 mM were sufficient for some strains (AWRI 1499 and VH05) to decrease their final cell density by almost 25%.

7.2.2 The effect of ethanol and ferulic acid on the growth of *Dekkera*

The combined effect of ferulic acid and ethanol on the growth of *Dekkera* yeast was examined. Fermentations were conducted in CDM containing 0-10% (v/v) ethanol with no ferulic acid addition, showed a reduction in growth rate of *D. bruxellensis* CBS 2336 and *D. anomala* CBS 77 above 3 and 5% (v/v) ethanol respectively (Fig. 7.2). *D. bruxellensis* CBS 2336 was completely inhibited by 7% ethanol and showed limited growth with 5% ethanol. With the addition of 10% ethanol, *D. anomala* growth was delayed for 12 days and the biomass was reduced by approximately 25% from that of the control (Fig. 7.2).

Ethanol in combination with ferulic acid inhibited both species more than ethanol alone (Fig. 7.2). *D. bruxellensis* was more senstive to inhibition by ethanol and ferulic acid combinations than *D. anomala*. Furthermore, the increased ethanol concentration enhanced this synergistic effect. Stationary phase took longer to reach, with a concomitant reduction of culture density than with ethanol, or ferulic acid additions alone (Fig. 7.2).

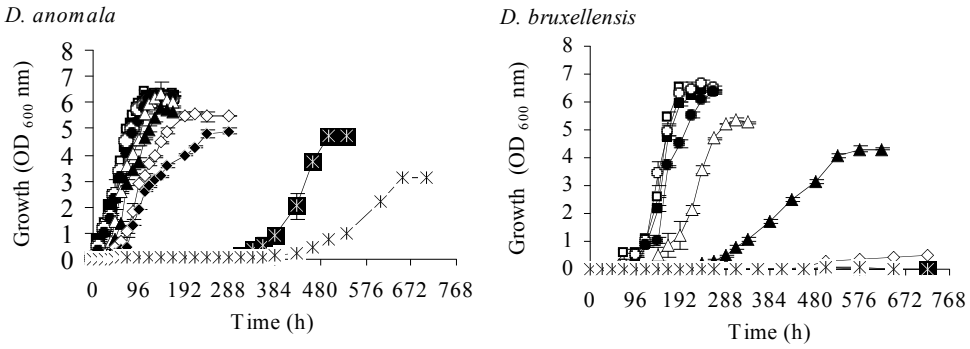


Fig. 2 Growth of *D. anomala* CBS 77 and *D. bruxellensis* CBS 2336 with (solid) or without (empty) 0.5 mM ferulic acid in media with 0% (square), 2% (circle), 5% (triangle), 7% (diamond) 10% (asterisk) ethanol (v/v). Growth was measured by optical density at OD_{600} ± SD

7.2.3 Effect of pH and ferulic acid on the growth of *Dekkera*

Control experiments were conducted to determine the effect of pH on *Dekkera* growth without ferulic additions in CDM. A pH of 3 prevented growth of *D. bruxellensis* (Fig. 7.3). Cultures grown in media at higher pH, of up to 5, had higher growth rates and cell densities than that of the control (pH 3.8). The pH of the media had little effect on the growth of *D. anomala* CBS 77. The addition of 0.5 mM ferulic acid to media at pH 3.8 had a no effect on growth of either species (Fig. 7.3). A slight decrease, of less than 5%, was observed in culture growth at pH 4–5.

7.2.4 Inhibition and fungicidal activity of ferulic acid in different media

The inhibitory and fungicidal properties of ferulic acid on *Dekkera* yeasts were examined in CDM, WM and wine. In CDM *D. bruxellensis* CBS 1499 growth was significantly reduced by 1 mM ferulic acid and 4 mM was fatal However, 8 mM ferulic acid was required to kill CBS 2336 (Table 7.2). *D. anomala* growth was reduced by 2 mM and fungicidal activity was observed at 8 mM.

The MRC, MIC and MFC of ferulic acid on *D. bruxellensis* AWRI 1499 was investigated in a WM. *D. bruxellensis* was significantly inhibited at a concentration of 1 mM and a MFC of 4 mM was determined (Table 7.2). *D. anomala* CBS 77 and *D. bruxellensis* AWRI 1499 were also examined in red wine with additions of ferulic acid concentrations from 0–8 mM. *D. anomala* CBS 77 had a MRC of 0.25 mM and was entirely inhibited by 1 mM while an addition of 4 mM ferulic acid was fatal (Table 7.2). AWRI 1499 was inhibited at a MRC of 0.1 mM, with a ten fold increase of ferulic acid causing death.

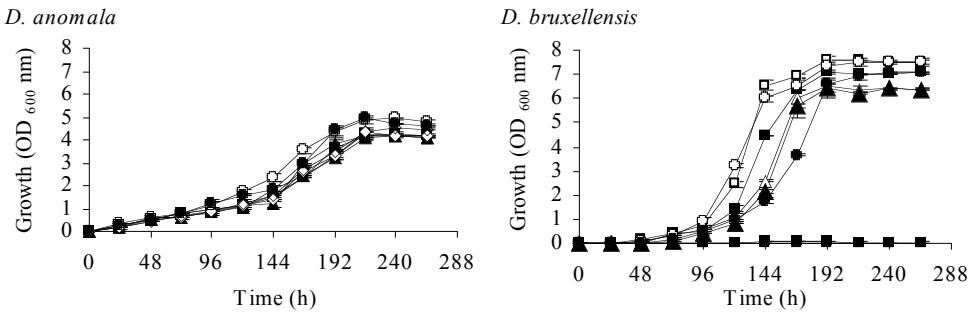


Fig. 3 Growth of *D. anomala* CBS 77 and *D. bruxellensis* CBS 2336 with with (solid) and without (empty) 0.5 mM ferulic acid in CDM at pH 3 (diamond) pH 4 (square) pH 5 (circle) control pH 3.8 (triangle), growth was measured in duplicate by optical density at OD_{600} ± SD.

Table 2. Inhibitory and fatal concentrations of ferulic acid against *Dekkera* yeast in various media and in combination with common hydroxycinnamic acids

Dekkera strain	ferulic acid: CDM			ferulic acid: WM			ferulic acid: wine			*p*-coumaric acid: CDM			caffeic acid: CDM			ferulic and *p*-coumaric acids: CDM			ferulic and caffeic acid: CDM			ferulic, *p*-coumaric and caffeic acids: CDM		
	MRC	MIC	MFC	MRC	MIC	MFC	MRC	MIC	MFC	MRC	MIC	MFC	MRC	MIC	MFC	MRC	MIC	MFC	MRC	MIC	MFC	MRC	MIC	MFC
CBS 77	2	8	8	-	-	-	0.25	1	4	2	10	10	4	20	20	1+1	1+4	1+4	1+1	1+4	1+4	1+1+1	1+1(2)+2(1)	1+1(2)+2(1)
AWRI1499	1	4	4	1	2	4	0.1	0.75	1	1	8	8	2	8	8	1+1	2+2	2+2	1+1	2+2	2+2	1+1+1	1+2+1	1+2+1
CBS 2336	1	4	8																					

Abbreviations: MRC; minimum repressive concentration, as defined as the minimum concentration required to reduce the culture density below that of the control. MIC; minimum inhibitory concentration, as defined as the minimum concentration required to inhibit culture growth completely, this is taken to be the concentration whereby below cultures growth reaches less than 5% of the control. MFC; minimum fatal concentration: as defined as the minimum concentration required to be fatal to cultures. CDM; chemically defined media. WM; wine media.

7.2.5 Determination of the MRC/MIC and MFC of ferulic acid in combination with common wine HCAs

The effect of the presence of multiple HCAs in media was investigated to replicate interactions which may occur in the typical wine environment. The MIC and MFC was determined for single additions of *p*-coumaric and caffeic acids.

Both *Dekkera* species were less sensitive to *p*-coumaric acid and caffeic acids than ferulic acid (Table 7.2). *Dekkera* growth was stimulated by low additions (1–2 mM) of caffeic acid, and *D. anomala* was also stimulated in the presence of 1 mM *p*-coumaric acid (data not shown). *D. bruxellensis* was significantly inhibited by 1–2 mM of caffeic or *p*-coumaric acids and 8 mM was a fatal concentration of both compounds. Where as *D. anomala* culture growth was reduced at 2 mM *p*-coumaric or 4 mM caffeic acid, with a fatal concentration of 10 mM of *p*-coumaric or 20 mM caffeic acid.

The MRC/MIC/MFC for combinations of HCAs was determined. The concentrations of HCAs used were below that of the MRC, *i.e.* caused significant inhibition for each acid. *p*-Coumaric or caffeic acids were combined with ferulic acid in the growth medium, in variable concentrations in a block design. All combinations decreased the total concentration of HCAs required to inhibit and kill *D. anomala* (Table 7.2). Only half the concentration of combined HCAs was required, compared to individual HCAs. *D. bruxellensis* required a total concentration of 2 mM HCA to completly inhibit culture growth with 4 mM HCA fatal. However, only 1 mM of ferulic in combination with *p*-coumaric or caffeic acid was required to achieve this same effect.

Combinations of all three HCAs were used in further MIC/MFC experiments. Concentrations used were based on previous results (Table 7.2). Combinations of the three HCAs lowered the total concentration needed to be fatal to *D. anomala* further than the 2 HCA combinations (Table 7.2). This effect was not observed for *D. bruxellensis*, the total concentration required for cell death remained at 4 mM, although 1 mM of each acid reduced culture growth by 92%.

7.2.6 Sensory evaluation

A red wine spiked at the MFC of 4 mM ferulic acid for *D. bruxellensis* was compared to the same wine with no addition using duo trio sensory difference testing. Results showed no significant difference between samples at $p = 0.05$.

7.2.7 SEM observations of *Dekkera* treated with ferulic acid

Addition of ferulic acid to culture media decreased the growth of *Dekkera* and led to cell death. The action of ferulic acid on the cell surface was investigated using scanning electron microscopy. Experiments were carried out with *D. anomala* CBS 77, the most resistant to ferulic acid and *D. bruxellensis* CBS 1499 the most sensitive.

Electron micrographs of *D. bruxellensis* AWRI 1499 grown in the presence of 0.5 mM and 1 mM ferulic acid both showed morphological changes from that of the control culture. Cells appeared wrinkled and misshapen although budding was still evident. The higher concentration of ferulic acid augmented these effects further (Fig. 7.4A). *D. anomala* grown in CDM (no ferulic acid addition) showed a large variety of cell sizes of 2 μm to greater than 20 μm in length. Cells also formed large masses of highly extended pseudohypha (Fig. 7.4C). Cultures grown with 0.5 mM of ferulic

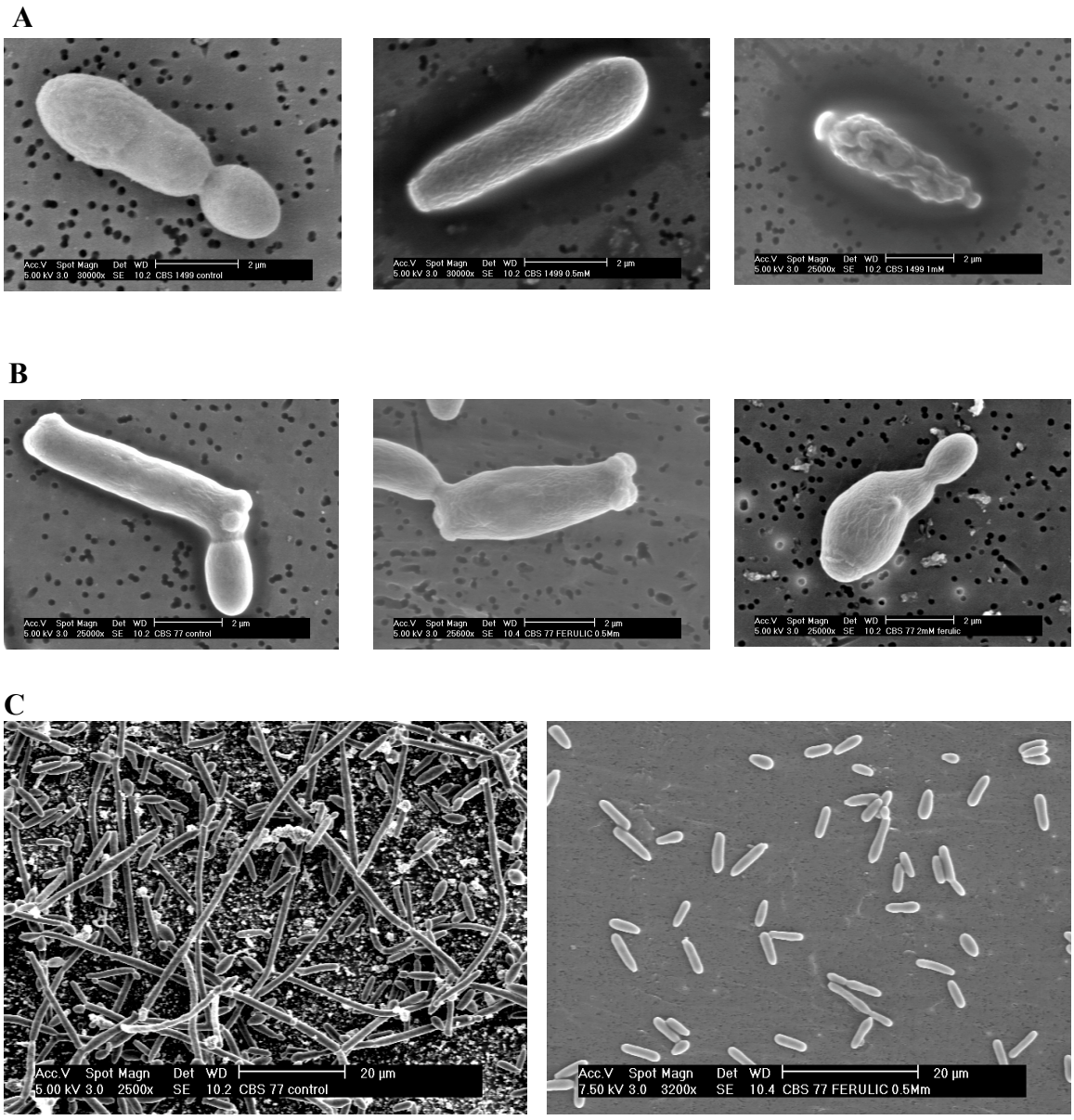


Fig. 7.4. Scanning electron micrographs of *D. bruxellensis* (AWRI 1499) (A) and *D. anomala* (CBS 77) (B and C). Cultures were grown in YPD with additions of, from left to right, 0 mM, 0.5 mM and 1–2 mM ferulic acid. Note the magnification is between 25000 and 30000. Part C shows pseudohyphae in untreated cells compared to cells treated with 0.5 mM ferulic acid.

acid showed no wrinkling of cells (Fig. 7.4B) however at 0.5 mM and above, the majority of cells were reduced in size, with few greater than 10 μm in length and without pseudohyphal growth (Fig. 7.4C). The electron micrographs of *D. anomala* CBS 77 grown with 2 mM ferulic acid indicated morphological changes when compared to the control (Fig. 7.4B).

To observe *Dekkera* under conditions known to be toxic, cultures were grown in YPD with no addition of ferulic acid. These cultures were washed and incubated with 0, 2 and 20 mM of ferulic acid in PBS buffer overnight. Small differences were observed between the *D. anomala* control culture incubated without ferulic acid and the 2 mM incubation (Fig. 7.5) . Cells incubated with 2 mM ferulic acid had some wrinkling of the cell surface and were smaller than the control. Cultures incubated with a fatal concentration of ferulic acid (20 mM) (Fig. 7.5) were highly wrinkled and with a larger proportion of cells that appeared ruptured or damaged. Additionally there were indications that budding was affected, as new buds appeared deformed (Fig. 7.5).

7.3 Discussion:

Dekkera yeasts play a role in the spoilage of red wine and current control strategies are often limited and inadequate. Section 7 reports an investigation of the addition of phenolic grape components as a possible method to prevent *Dekkera* contamination. Results presented here confirm the results decribed in Section 5 that ferulic acid decreases the growth of *Dekkera* yeasts. It also further highlights the differences between *Dekkera* species in resistance ferulic acid. Described in Section 7 was that concentrations as low as 0.25 mM could decrease culture density and increase lag times of a range of species and strains. Concentrations of 2 mM ferulic acid strongly

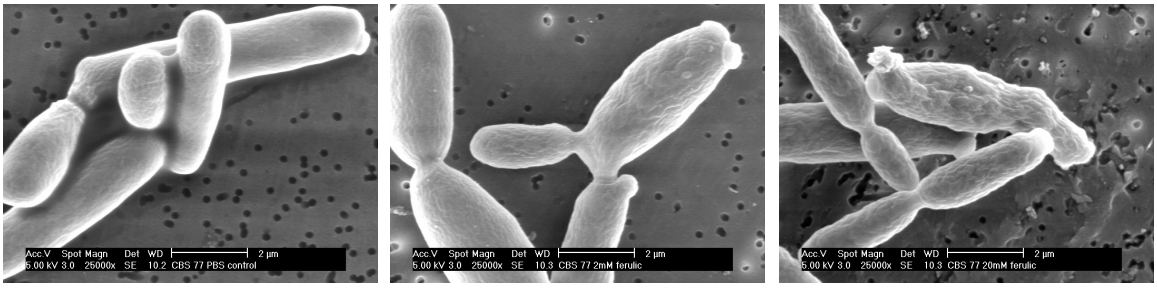


Fig. 5 Scanning electron micrographs of *D. anomala* incubated overnight in PBS buffer with 0, 2 and 20 mM ferulic acid.

inhibited wine isolates of *D. bruxellensis* (VH01, VH02 and VH03) and prevented the growth of the strains AWRI 1499 and CBS 2336 (from culture collections). That the wine isolates grew, may suggest that they were better adapted to growth with ferulic acid, due to their relatively recent environmental exposure.

D. anomala is more resistant to ferulic acid and this supports the results described in Section 5 (Fig 5.1; Harris et al. 2008) that also demonstrated that the inhibition of *D. bruxellensis* strains was greater than that of *D. anomala*. Previous studies examining the effect of HCAs on other microorganisms have found variations in sensitivity to ferulic acid, where the inhibitory effect depends on the organism and HCA under investigation (Campos et al. 2003; Stead 1993; Van Sumere et al. 1971).

Inhibition of *Dekkera* by ferulic acid in situations more closely resembling that of typical winemaking conditions was investigated using a range of ethanol concentrations and pH values. Increasing ethanol concentrations repressed *Dekkera* growth and this effect was augmented by the addition of ferulic acid. This suggests that higher ethanol concentrations require less ferulic acid to have a comparable effect on *Dekkera* growth. The implication of this finding has a direct impact on the application of ferulic acid as an antimicrobial for wine production. Many spoilage organisms cannot grow in high concentrations of ethanol (Kalathenos et al. 1995), however, *D. bruxellensis* can grow in ethanol concentrations higher than 10% (Boulton et al. 1996; Silva et al. 2004) therefore an antimicrobial which acts in synergy with ethanol concentrations would be advantageous in winemaking.

Media pH alone or with the addition of ferulic acid did not have a strong impact on culture growth. The pKa of HCA compounds are between 4.5 and 5 (Price et al. 1979) and therefore a lower pH would result in a greater proportion of the undissociated forms crossing the cell membrane acidifying the intracellular environment (Reguant et al. 2000). However, the role of pH and ferulic acid in relation to microbial control may be more relevant when sulfur dioxide additions are made during the winemaking process. Lower pH could enhance the intracellular acidification of HCAs and the toxic effect of sulfur dioxide further. Investigations combining wine parameters of ethanol concentration, pH and sulfur dioxide with ferulic acid are required to gain insight into these complex interactions. Previous studies have found that lowering pH increases the antibacterial ability of HCAs (Herald and Davidson 1983; Wen et al. 2002). However, the pH examined here are closer to wine pH and are lower than employed in previous studies. If a higher pH range was examined the effects may be more substantial.

Determination of MRC, MIC and MFC values for HCAs against *D. anomala* (CBS 77) and *D. bruxellenesis* (AWRI 1499, CBS 2336) was undertaken in a variety of culture media. In all media *D. bruxellensis* was more sensitive to ferulic acid than *D. anomala.* In CDM *D. anomala* required double the concentration of ferulic acid to inhibit or kill the culture compared to *D. bruxellensis*. Caffeic and *p*-coumaric acids were not as inhibitory towards *Dekkera* yeasts as ferulic acid. Ferulic acid is also a more potent inhibitor of several other wine spoilage yeast including *Pichia* and *Saccharomyces* (Baranowski et al. 1980; Stead 1995). However, this is in contrast to ferulic acid antibacterial activity where *p*-coumaric and caffeic are reported as stronger inhibitors of *Oenococcus oeni* and *Lactobacillus hilgardii* (Campos et al.

2002; Reguant et al. 2000). These results confirm data from *Dekkera* growth experiments reported in Section 5 which showed a decrease in growth rate and final culture density of both species with increasing ferulic acid concentrations. Further confirming previous reports that *D. bruxellensis* is more sensitive than *D. anomala* to ferulic acid (Fig. 5.1; Harris et al. 2008). MIC values are greater than the concentration shown to inhibit AWRI 1499 in CDM growth experiments (Fig. 7.1), however, this variance could be due to differences in growth conditions.

For the first time, inhibition of *Dekkera* yeast by ferulic acid has been examined in various media. The MIC and MFC of ferulic acid in WM for *D. bruxellensis* (AWRI 1499) was determined to be 50% less than in CDM (Table 7.2). An important difference between these two media is the ethanol concentration. Interestingly, *D. bruxellensis* (AWRI 1499) is unable to grow in CDM with 10% ethanol but does grow in WM containing 10% ethanol. This suggests that as yet unidentified components of WM are essential for growth of *Dekkera* in the presence of ethanol.

Ferulic acid inhibition of *Dekkera* yeast has been examined in wine. Inhibitory and fatal concentrations for ferulic acid in a red wine were determined. *D. bruxellensis* AWRI 1499 was inhibited by 0.1 mM of ferulic acid and no growth was observed in cultures with 1 mM. Results for both species are lower than that of CDM and WM with as little as 25% of the inhibitory concentration in CDM required for the same effect in wine. This data illustrates that ferulic acid is more effective at preventing growth of *Dekkera* under wine like conditions and should be investigated further. Any addition of ferulic acid would act in synergy with the background concentration of wine HCAs to enhance toxicity.

A lower total concentration of HCA was required to kill *D. anomala* CBS 77 but not *D. bruxellensis* AWRI 1499 when ferulic acid was combined with either *p*-coumaric or caffeic acids. However, a lower concentration of ferulic acid, together with a second HCA was fatal to *D. bruxellensis* in comparison to ferulic acid alone. This supports the study described in Section 5, where ferulic acid combined with a second HCA had a greater inhibitory effect than ferulic acid alone (Fig 5.4). This support a previous study that found plant derivied phenolic compounds act in synergy to inhibit *Saccharomyces* yeast (Delaquis et al. 2002).

To our knowledge this is the first investigation to reported that cellular morphological changes are caused by the action of ferulic acid on *Dekkera*. The cell morphology of *D. bruxellensis* AWRI 1499 was altered by increasing concentrations of ferulic acid (Fig. 7.4A), further contributing to evidence that this yeast is more sensitive to this HCA. Growth experiments and MIC results indicate that 2 mM of ferulic acid inhibits but is not fatal to *D. anomala* cultures. The electron microscope images reflect these findings; while there are some morphological alterations budding is still occurring (Fig. 7.4B, 7.4C).

To induce alterations in cells a fatal concentration of ferulic acid was added to *D. anomala* in PBS buffer at 2 and 20 mM concentrations. 2 mM causes some modifications to *D. anomala* but at 20 mM cells are obviously altered, they appeared wrinkled and misshapen, further, buds were also deformed (Fig. 7.5). This suggests that modifications to *Dekkera* cells induced by ferulic acid may have effect on both the cell membrane and as a result reproduction.

The mechanisms by which HCAs inhibit cell growth are not fully understood. Ferulic acid has been linked to both specific and non-specific means of growth reduction in microorganisms, these include the inhibition of enzymes (Mason and Wasserman 1987), substrate deprivation (Peres et al. 1997) cellular energy depletion (Baranowski and Nagel 1983) and membrane disruption (Fernandez et al. 1996). The mechanism by which the small phenolic antimicrobial compounds thymol and eugenol act against *S. cerevisiae* have been examined (Bennis et al. 2004). These small phenolic compounds are similar to ferulic acid and also cause modification and damage to the cell membrane possibly related to the cellular proliferation phase (Bennis et al. 2004). Further studies are required to determine why ferulic acid causes cell damage.

7.4 Conclusions

The use of ferulic acid alone or in combination with other HCAs in wine may have a number of benefits, such as better antimicrobial control of wine while using reduced sulfur dioxide additions. There are also speculated possible health benefits associated with ferulic acid (Ogiwara et al 2002). Further, in this investigation no aromatic changes were detected in wine with ferulic acid addition at the fatal antimicrobial concentration. Ferulic acid is not permitted as an additive to wine, however investigation of viticultural and oenological practices could be undertaken to optimize endogenous ferulic acid concentrations. Opportunities may also exist to explore the production of grape extracts rich in HCA for use in winemaking. Investigations are required within an industrial setting to ascertain the effectiveness of using ferulic acid as an antimicrobial and its effect on wine quality.

8. Outcomes/Conclusions

As a result of this investigation the majority of outputs and performance targets have been met (Table 4.1). These include: a review of relevant literature and a detailed analysis of the physiological basis for growth and volatile phenol production by *Dekkera* and *Brettanomyces* under model and wine-like conditions. The biochemical pathway of volatile phenol production has been confirmed and information on the molecular basis of the biosynthesis of these critical aroma compounds has been derived. A comprehensive study of yeast growth and off-flavour development in small-scale winemaking trials has been undertaken, and critically the development of a novel strategy to minimise the influence of *Dekkera* in wine has been proposed. Communication of results has been undertaken throughout the life of the project and fully detailed in Appendix 1. The final output, submission of the PhD thesis is outstanding at the time of writing of this report, however, it is anticipated to occur shortly.

This work has provided a detailed overview of the catabolism of a number of HCAs and the production of vinylphenols and ethylphenols during growth in defined and a wine-like conditions. The metabolism by *Dekkera* yeasts of the combinations of HCAs, a condition more truly reflecting the wine environment, was also examined for the first time. The results indicated that the presence of HCAs combinations decreases yeast growth and metabolism of HCAs.

The characterisation of enzymes directly involved in the degradation pathway of HCAs into ethylphenols has been undertaken. PAD enzyme activity from a range of

Dekkera and *Brettanomyces* species has been analysis and characterized *viz* substrate specificity, pH and temperature optima. VPR enzyme activity has never previously been reported and results presented here confirm the presence of such an enzyme, which has previously only been postulated. Further, key parameters for its activity have been determined.

A partial PAD protein sequence from *D. anomala* has also been described. This sequence does not share homology with previously sequenced PAD genes from other yeast or bacteria but does align with a putative flavodoxin-like protein from *S. cerevisiae* and *C. albicans*. The results from the study reported here indicate the presence of a novel enzyme in *Dekkera* species, which is responsible for HCA decarboxylation. Further protein and molecular characterisation is required to confirm this. No *Dekkera* genetic information related to volatile phenol production has previously been reported.

The use of ferulic acid alone or in combination with other HCAs in wine may have a number of significant advantages. These include better antimicrobial control of wine while using reduced sulfur dioxide concentrations, along with the speculated health benefits associated with ferulic acid. Further, no significant change in aroma was detected in wine with ferulic acid added at the fatal antimicrobial concentration. While ferulic acid is currently not permitted as an additive to wine it has GRAS status. Investigation of viticultural and oenological practices could be undertaken to optimize endogenous ferulic acid concentrations in wine. Opportunities may also exist to explore the production of grape extracts rich in HCA for use in winemaking. Investigations are required within an industrial setting to ascertain the effectiveness of

using ferulic acid as an antimicrobial and its effect on wine quality. The antimicrobial action of ferulic acid and in combination with other HCAs, along other wine parameters such as ethanol, could also offer winemakers greater insight into *Dekkera* spoilage and may explain was some wines become contaminated and others not.

9. Recommendations:

As described above a number of important outcomes have been established from this project. However, several questions remain unanswered. Therefore, further investigation is required.

While a partial protein sequence for PAD has been reported, the complete sequence along with the VPR enzyme has not been determined. As these molecular and biochemical data are not available, a detailed comparison with regards to volatile phenol production between the *Dekkera* and *Brettanomyces* genera could not be undertaken. This type of study would provide further insight into this complex spoilage phenomenon.

The use of ferulic acid as an antimicrobial in wine alone or in combination with other HCAs and ethanol requires further investigation under an industrial scale. There are a number of possible benefits of this approach, including reduced sulfur dioxide additions. Further, there are potential health benefits associated with the use of ferulic acid. A greater understanding of grapevine/berry accumulation of ferulic acid and other HCAs could provide winemaking outcomes directly related to protection from microbial spoilage and healthfullness.

Appendix 1: Communication

Publications

Harris V, Ford CM, Jiranek V & Grbin PR (2008) Ferulic acid as a possible means for control of *Dekkera* in wine. Aust J Grape & Wine Res. in preparation

Harris V, Ford CM, Jiranek V & Grbin PR (2008) Survey of enzyme activity responsible for phenolic-off-flavour production by *Dekkera* and *Brettanomyces* yeast. Appl Microbiol Biotech. in preparation

Harris V, Ford CM, Jiranek V and Grbin PR (2008) *Dekkera* and *Brettanomyces* growth and utilisation of hydroxycinnamic acids in synthetic media. Appl Microbiol Biotech 78:997-1006

Curtin C, Grbin P and Henschke P (2007) Molecular characterisation of the wine spoilage yeast *Dekkera (Brettanomyces) bruxellensis*. Microbiol Aust 28:62-64.

Conference Presentations & Seminars

Harris V, Jiranek V, Ford CM and Grbin, PR (2008) *Dekkera* and *Brettanomyces* utilisation of hydroxycinnamic acids and their ability to synthesis volatile spoilage components during fermentation. *13th Australian Wine Industry Technical Conference*, 28 July–2August, 2007 Adelaide. Blair RJ, Williams PJ & Pretorius IS (eds). (Adelaide: Wine Industry Technical Conference), p 310-311 (ISBN 0 9751054 4 2)

Harris V, Stocker N and Grbin PR (2008) Survey of common laboratory microbiological media for the growth of *Dekkera* and *Brettanomyces* yeast. *13th Australian Wine Industry Technical Conference*, 28 July–2August, 2007 Adelaide. Blair RJ, Williams PJ & Pretorius IS eds. (Adelaide: Wine Industry Technical Conference), p 313 (ISBN 0 9751054 4 2)

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Appendix 2: Intellectual property

It is the author's intention to publish all results from this project in scientific peer reviewed papers.

Appendix 3: References

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