Physiological, biochemical and molecular characterisation of hydroxycinnamic acid catabolism by *Dekkera* and *Brettanomyces* yeasts

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Abstract

Dekkera and the closely related *Brettanomyces* are important yeasts in food and beverage production in part due to the metabolism of hydroxycinnamic acids (HCAs). There is a dearth of information concerning the role *Brettanomyces* spp. play in the food or beverage from which they are isolated and although *Dekkera* spp. have been investigated further there are discrepancies and questions yet to be answered. Representatives of both genera were examined to define growth and metabolism of individual HCAs in synthetic media. In addition, growth with combinations of HCAs was investigated for the first time. The results provide a comprehensive overview of HCA metabolism and volatile product formation for these genera. Furthermore, results have been confirmed in a semi-defined wine medium that more closely resembled the physio-chemical parameters found in the typical wine environment.

The enzymes responsible for the metabolism of HCAs were examined in *Dekkera* and *Brettanomyces*. *Dekkera* yeasts are known to enzymatically convert HCAs into vinylphenols (VPs) and ethylphenols (EPs). These products are indicative of *Dekkera* contamination. The first enzyme in the two-step HCA ─ VP ─ EP biochemical pathway is a hydroxycinnamic acid decarboxylase (HCD). This enzyme has been previously characterised from a single *Dekkera* strain. The second enzyme, vinylphenol reductase (VPR) has never been isolated or characterised from any microorganism.

In order to further elucidate the HCA ─ VP ─ EP pathway, cell extracts were prepared from all five *Dekkera* and *Brettanomyces* spp. to evaluate activity against HCAs and VPs. *Brettanomyces* spp. were unable to metabolise HCAs indicating that these yeast do not

have a functional HCD enzyme. Both *Dekkera* spp. have substrate inducible HCD activity. Temperature and pH optima were 40ºC and 5.75-6.00, respectively. The active protein was purified from cell extracts of *D. anomala* CBS 77 and a partial sequence was obtained. 3'RACE PCR was performed and a near complete gene sequence determined. This sequence does not have homology to HCA decarboxylase enzymes previously characterised from yeasts and bacteria and thus may represent a novel enzyme not previously described. Biochemical characterisation of the vinylphenol reductase (VPR) enzyme was also undertaken. VPR activity was found for all 5 *Dekkera* and *Brettanomyces* spp. Activity was greatest at pH 6 and between 40-50ºC and was induced by both VPs and HCAs.

Data obtained during growth experiments indicated that HCAs, and in particular ferulic acid, inhibited the growth of *Dekkera* and *Brettanomyces* spp. On this basis a more detailed study was carried out to determine the concentrations required to prevent growth in various media. In a modified red wine a concentration 0.1 mM ferulic acid inhibited growth and 2 mM prevented cultures of both *D. anomala* and *D. bruxellensis* from becoming established even when re-inoculated into to a fresh HCA-free medium. Scanning electron micrographs revealed that ferulic acid caused physical damage to *Dekkera* cells upon exposure. This work could lead to the development of an alternative method for the control of *Dekkera* in wine or other food products.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference has been made in the text.

Chapters two and three have been published in peer-reviewed scientific journals:
Dekkera and *Brettanomyces* growth and utilisation of hydroxycinnamic acids in synthetic media (2008). Victoria Harris, Vladimir Jiranek, Christopher M. Ford, and Paul R. Grbin. Applied and Microbiology and Biotechnology **78**:997-1006.

Survey of enzymatic activity responsible for phenolic-off-flavour production by *Dekkera* and *Brettanomyces* yeast (2009). Victoria Harris, Vladimir Jiranek, Christopher M. Ford, and Paul R. Grbin. Applied and Microbiology and Biotechnology **81**:1117-1127.

Chapter four has been submitted for review to the Australian Journal of Grape and Wine Research. Investigations into the use of ferulic acid as an antimicrobial against *Dekkera* yeast Victoria Harris, Vladimir Jiranek, Christopher M. Ford, and Paul R. Grbin.

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Finally I'll leave it simple. Thanks mum.

Chapter 1

Review of literature

1.1 Introduction

Dekkera and *Brettanomyces* are closely related yeasts of interest in a number of fields. The key difference between these yeasts is limited to their ability to enter a sexual phase. These organisms are instrumental in the production of some Belgian beers (Spaepen *et al*., 1978; Martens *et al.,* 1997) and both have been isolated from traditional fermented foods and beverages (Kolfschten and Yarrow 1970; Cabranes *et al.,* 1990; Liu *et al.,* 1996; Joshi and Sandhu 2000; Cosentino *et al.,* 2001; Gadaga *et al.,* 2001; Fadda *et al.,* 2001; Coton *et al.,* 2006). *Dekkera* yeasts have also been proposed for use in the industrial production of acetic acid (Freer 2002; Uscanga *et al.,* 2007) and have the potential to produce natural food flavourings such as vinylguaiacol (Rosazza *et al.,* 1995). Recent interest in *Dekkera* has come about in relation to the biofuel industry (Abbott and Ingledew 2005; de Souza Liberal *et al.,* 2007; Passoth *et al.,* 2007; Basílio *et al*., 2008) in its use as a tool in genetic studies (Woolfit *et al.,* 2007). *Dekkera* is of particular interest within the wine industry for its detrimental production of organoleptic effects resulting in wine that is out of specification and financial losses. This yeast is considered a spoilage organism predominantly of red wines (van der Walt and van Kerken 1959; Chatonnet *et al.,* 1992) as white wines are more resilient (Chatonnet *et al*., 1993; Barata *et al.,* 2006). Thus one important aspect of winemaking is the elimination and control of spoilage microorganisms and therefore research into *Dekkera* and *Brettanomyces* yeasts is significant.

1.1.1 Taxonomy and Identification of *Dekkera* and *Brettanomyces* yeast

Brettanomyces was first isolated in 1904 from British beer (Claussen 1904) and all subsequent isolates were associated with this genus. The initial classification of *Brettanomyces* yeast was based on several criteria, distinct ogival shape, acetic acid

production from glucose under aerobic conditions, slow growth in malt medium and a characteristic odour with no ascospore production (Custers 1940). In 1960, the first ascosporogenous strains of *Brettanomyces* were described (van der Walt and van Kerken 1960); consequently a new telomorphic genus, *Dekkera* was proposed (van der Walt 1964).

Establishment of molecular technologies in the 1990's prompted further reclassification of *Dekkera* and *Brettanomyces* genera. Initially mitochondrial DNA maps were developed (Hoeben and Clark-Walker 1986), but subsequently more sensitive methods have been employed to identify and differentiate species and strains (Smith *et al.,* 1990; Cai *et al.,* 1996; Esteve-Zarzoso *et al.,* 1999; Egli and Henick-Kling 2001; Miot-Sertier and Lonvaud-Funel 2007). Currently *Dekkera* and *Brettanomyces* yeast are defined as two distinct genera and 5 species (Barnett *et al*., 2000) this taxonomy will be adhered to throughout this thesis although the names *Brettanomyces* and *Dekkera* remain interchangeably in contemporary literature. Availability of new technologies has resulted in a number of methods that detect and enumerate *Dekkera* directly from the source (Phister and Mills 2003; Barszczewski and Robak 2004; Cocolin *et al.,* 2004; Delaherche *et al.,* 2004). This ability to quickly confirm contamination facilitates appropriate prevention or containment, thus limiting potential financial losses.

Application of molecular methodology has highlighted a significant propensity for genetic diversity between *Dekkera* and *Brettanomyces* spp. and strains. There is up to a four fold variation in mitochondrial size between the species (Hoeben and Clark-Walker 1986) and karyotypes range from 20 Mb to over 30 Mb in size (Woolfit *et al.,* 2007). These striking differences go some way to explaining the observed physiological and

metabolic diversity between species and strains. Currently, genomic sequencing of *D. bruxellensis* CBS 2499 is underway (Woolfit *et al.,* 2007) with around 40% completed.

1.1.2 Isolation of *Dekkera* and *Brettanomyces* from foods and beverages

Dekkera and *Brettanomyces* are isolated from foods and beverages where in some cases they have been linked to production of undesirable flavour and aroma compounds. Research dedicated to *Brettanomyces* is limited as they appear to be less capable of causing unwanted organoleptic modifications (Dias *et al*., 2003b). *Brettanomyces* spp. have been identified from Bantu beer, olives, cocoa bean fermentation, bottled beer, carbonated lemonade, lemon drink, tonic water and soda water (van der Walt 1964; Kolfschten and Yarrow 1970; Smith and van Grinsven 1984; Ravelomanana *et al.,* 1985). Presently, the contribution of *Brettanomyces* to these products is unknown.

More research has been conducted on *Dekkera* spp. The rationale is the number of undesirable characteristics formed during industrial production of wines, beers, some dairy products and fuel ethanol production. *D. anomala* has been isolated from soft drinks, concentrates, fruit juices, cider, wine, tequila, stout, tea-beers, feta cheese and milk (Peynaud and Domercq 1956; van der Walt 1964; Smith and van Grinsven 1984; Lachance *et al.,* 1995; Stratford *et al.,* 2000; Cosentino *et al.,* 2001; Fadda *et al.,* 2001; Spanamberg *et al.,* 2004; Coton *et al.,* 2006). Soft drinks are generally resistant to microbial growth due to carbonation and high acidity, however *D. anomala* contamination can lead to the formation of sediments or floating particles with sour or acetic flavours (Stratford *et al.,* 2000). The isolation of *D. anomala* (*B. anomalus*) from agave, used in the manufacture of tequila, has also been related to the production of acetic aromas, it is not clear what role *Dekkera* spp. plays during fermentation although

they are found in high numbers (Lachance *et al.,* 1995). It is not thought that *D. anomala* is significant for spoilage of wines as it is rarely isolated from wine (Stender *et al.,* 2001; Phister and Mills 2003).

In contrast, *D. bruxellensis* is isolated from wine and associated environments such as, grapes, musts, barrels, winery equipment, and insects and can causes spoilage (Peynaud and Domercq 1956; Sandhu and Waraich 1985; Renouf and Lonvaud-Funel 2007; Renouf *et al*., 2007). *Dekkera bruxellensis* has also been isolated from contaminated Bioethanol fermentations, where it decreases the ethanol yield (Basílio *et al.*, 2008). Conversely, these yeasts may be instrumental in production of Irish ciders (Morrissey *et al.,* 2004), Belgian wheat beers, German Weizen and Rauch beers (Spaepen *et al*., 1978; Martens *et al.,* 1997; Vanbeneden *et al.,* 2008). In these and other speciality beers, phenolic flavours are essential characters. *Dekkera bruxellensis* has also been isolated from sherry, Kombucha, bantu beer, ginger ale, olives, milk, cheese and fermented milk (van der Walt 1964; Ibeas *et al.,* 1996; Kotzekidou 1997; Fadda *et al.,* 2001; Gadaga *et al.,* 2001; Teoh *et al*., 2004; Spanamberg *et al.,* 2004), although again the role they play has not been established.

Dekkera bruxellensis contamination in wine is a global phenomenon. However, in Europe some barrel aged red wines have *Dekkera* characteristics which, are incorporated as signature stylings by winemakers and not automatically considered undesirable (Fugelsang *et al.,* 1993; Kunkee and Bisson 1993). Other wines such as Gewürztraminer and Beaujolais wines can have smoky and clove-like aromas typical of vinylphenol compounds (Etièvant 1981; Guth 1997). In new-world wine regions, such as Australia, the trend is to minimize and eliminate *Dekkera* and its characteristics in

wine.

Dekkera are isolated from all stages of winemaking (Martorell *et al.,* 2006) and have been described as the yeast best adapted to the winery environment (Renouf and Lonvaud-Funel 2008). *Dekkera* are tolerant of high ethanol, low sugar, limited nutrient concentrations and minimal oxygen conditions (Medawar *et al.,* 2003; Silva *et al.,* 2004; Renouf and Lonvaud-Funel 2008) thereby allowing its survival through fermentation. *Dekkera* yeast can grow in red wine in the presence of relatively high doses of sulfur dioxide (Barata *et al*., 2008). Finally, unlike other potential spoilage yeast such as *Pichia guilliermondii*, proximity to large populations of *Saccharomyces* does not kill *Dekkera* (Barata *et al.,* 2006), in fact by end of fermentation, *Dekkera* can prevail (Dias *et al.,* 2003a; Renouf *et al.,* 2006). Despite being ever-present during fermentation, spoilage usually occurs when ferments become stuck, malolactic fermentation is delayed or during aging in oak barrels (Renouf *et al.,* 2005; 2006).

1.2 Wine spoilage characteristics associated with *Dekkera bruxellensis*

1.2.1 Production of acetic acid

Undesirable compounds produced by *Dekkera* yeast include acetic acid and the associated ester, ethyl acetate. Both increase wine volatile acidity and have aromas reminiscent of nail varnish remover (Scheffers 1961; Freer 2002). These compounds are perceived as a fault at concentrations around 150 mg/l while *Dekkera* can produce up to 5 g/l from an initial 35 g/l of ethanol (Freer 2002). During wine making, *Dekkera* can be stimulated by the presence of oxygen to produce high quantities of acetic acid (Rozès *et al.,* 1992; Ciani and Ferraro 1997; Ciani *et al*., 2003).

1.2.2 Formation of compounds causing mousy-off-flavour

Nitrogen containing heterocyclic compounds, 2-ethyltetrahydropyridine, 2-acetyltetra-hydropyridine and 2-acetylpyroline produced by *Dekkera* in a strain-specific manner, cause mousy-off flavours in wine (Strauss and Heresztyn 1984; Heresztyn 1986a; Grbin *et al.,* 2007). This fault is not detectable by aroma at typical wine pH but is manifested as a strongly objectionable off-flavour during or after gustation (Grbin and Henschke 2000). The causative N-heterocycles are characteristically found in combination in affected wines although possible synergism between the compounds has not been investigated (Snowdon *et al.,* 2006). The elucidation of the complete biosynthetic pathway required for production is also incomplete (Snowdon *et al.,* 2006), however, essential precursors include amino acids D-lysine, L-lysine or L-ornithine, responsible for ring formation (Snowdon *et al.,* 2006; Grbin *et al.,* 2007), and ethanol or acetaldehyde (Heresztyn 1986a; Grbin *et al.,* 2007). The production of mousy-off-flavour compounds by *Dekkera* and *Brettanomyces* spp. is strain specific (Grbin *et al.,* 1996).

1.2.3 Production of volatile fatty acids

Dekkera produces a variety of short and medium chain (C_3-C_{14}) volatile fatty acids, which contribute to the aroma profile of wine (Rozès *et al.,* 1992; Malfeito-Ferreira *et al.,* 1997; Licker *et al.,* 1998). A prominent volatile fatty acid produced by *Dekkera* yeasts and observed in wine and other fermented beverages is isovaleric acid (IVA; 3-methylbutanoic acid) (Licker *et al.,* 1998). IVA can be produced by the breakdown of L-leucine (Dickinson *et al.,* 2000) an amino acid present in both grapes and wine (Henschke and Jiranek. 1993; Licker *et al.,* 1998). IVA is detrimental to wines, smelling sweaty, putrid or like rancid cheese (Kotseridis and Baumes 2000) at relatively low

concentrations (33.4 μg/l) in wine (Ferreira *et al.,* 2000). Related fatty acids, 2-methylbutanoic acid and isobutyric acid are also produced by *Dekkera* yeasts (Malfeito-Ferreira *et al.,* 1997), and are detected as sweaty, rancid or putrid aromas (Spaepen and Verachtert 1982; Rozès *et al.,* 1992; Fugelsang *et al.,* 1993).

Formation of fatty acids in fermented beverages by *Dekkera* has not been substantially examined (Henschke and Jiranek. 1993; Licker *et al.,* 1998) although their production in lambic beers has been documented (Gilliland 1961; Scheffers 1961; van Oevelen *et al.,* 1976; Spaepen *et al.,* 1978). Since *Saccharomyces* yeast synthesise volatile fatty acid compounds from the branched chain amino acid substrates leucine, isoleucine and valine (Dickinson *et al.,* 1997; 1998; 2000), it is probable that similar metabolic pathways are utilised by *Dekkera*. Branched chain amino acid catabolism is related to a number of cellular functions and therefore the variety of catabolic options makes it difficult to predict conditions that exacerbate fatty acid synthesis by *Dekkera*. Quantities of fatty acids will also depend on initial concentrations of substrates in grape juice (Henschke and Jiranek. 1993; Licker *et al.,* 1998) and will be affected by other microbes in the ferment such as *S. cerevisae* (Henschke and Jiranek. 1993).

1.2.4 Production of volatile phenol compounds

Volatile phenol compounds of major concern to the wine industry are vinylphenols (VP; hydroxystyrenes) and ethylphenols (EP; hydroxybenzenes). Volatile phenols are derivatives of naturally occurring hydroxycinnamic acids (HCAs) (Chatonnet *et al.,* 1992). They have low threshold limits of perception in wines and are detectable as medicinal, horsy, barnyard, burnt beans, stable-like or clove-like aromas (Chatonnet *et al.,* 1992; Fugelsang 1993; Licker *et al.,* 1998). Despite this, VPs, at low concentrations

have inoffensive and even desirable characteristics e.g. spicy, smoky and sweet (Maga 1978; Heresztyn 1986b; van Beek and Priest 2000) which can be considered to enhance a wine as aroma nuances (Fugelsang *et al.,* 1993; Kunkee and Bisson 1993). Most undesirable aromas are attributed to EP compounds, they are detectable at levels as low as 25 µg/l (Chatonnet *et al.,* 1992) and act in synergy, lowering to threshold of perception by over 50% (Chatonnet *et al.,* 1992; Hesford *et al.,* 2004) and in wines EPs are commonly reported in concentrations many times in excess of this.

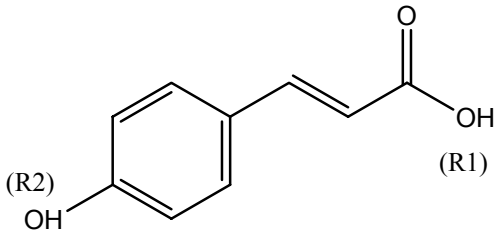
Several wine yeast and bacteria catabolise HCAs to form VPs which can be metabolised further by some microbes (Huang *et al.,* 1994; Chatonnet *et al.,* 1995; Cavin *et al.,* 1997a ;b; Barthelmebs *et al.,* 2000b; Barata *et al.,* 2006). In particular lactic acid bacteria are known to produce EPs in high quantities in synthetic medium (Dias *et al.,* 2003a). However, in the wine environment, only *Dekkera* yeasts produce significant concentrations of EPs (Chatonnet *et al.,* 1995; 1997; Barata *et al.,* 2006). *Dekkera* also metabolise free 4-vinylphenol into 4-ethylphenol (Dias *et al*., 2003b). Hence, VPs produced by other wine organisms during fermentation are potential substrates for *Dekkera,* thus increasing the potential for spoilage and financial losses. This aspect of *Dekkera* spoilage is particularly insidious and will be explored further during this study.

1.3 Hydroxycinnnamic content of grapes, juice and wine

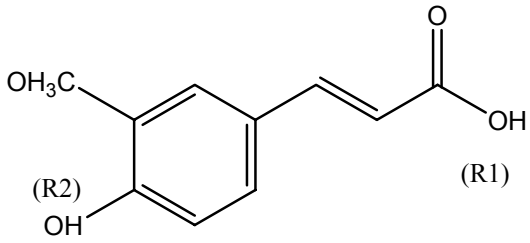
Phenolic compounds are important in foods and beverages. They are naturally derived from plants or through microbial action and consist of a phenyl ring backbone with a variety of substitutive possibilities (Conde *et al.,* 2007). Simple phenolics include the soluble, non-flavonoids *p*-coumaric, ferulic, caffeic and sinapic acids (Figure 1), known collectively as HCAs. These acids are derived from the amino acid phenylalanine,

which is acted upon by phenylammonia lyase to form cinnamic acid, which is then transformed into the other simple phenolic compounds (Conde *et al.,* 2007). HCAs are often found in combination with tartaric acid esters to form tartrates or conjugated with a glucose moiety to form a HCA-glucoside (Figure 1) (Fleuriet and Macheix 2003). A number of chemical and enzymatic reactions occur during grape ripening, juice extraction and wine making that determine the final ratio of bound and free HCAs. (Macheix *et al.,* 1990; Dugelay *et al.,* 1993; Fleuriet and Macheix 2003; Pérez-Magariño and González-San Jose, 2005). HCAs are universally distributed in plants, where they are found in the leaves, roots, fruit and seeds (Wall *et al.,* 1961; Van Sumere *et al.,* 1971; Maga 1978; Ong and Nagel 1978; Nagel *et al.,* 1979; Montealegre *et al*., 2006). In ripe fruit HCA compounds collectively total between 16 - 430 mg/l (Conde *et al.,* 2007). In grapes, HCAs are found in the stem, outer seed coat, skin, pulp and juice where they primarily consist of caffeic, ferulic and *p*-coumaric acids (Fernández de Simón *et al.,* 1992; Thorngate and Singleton 1994; Souquet *et al.,* 1996; Souquet *et al.,* 2000; Montealegre *et al*., 2006). However, in grapes the majority of HCAs are present in their bound forms (Ong and Nagel 1978; Fleuriet and Macheix 2003).

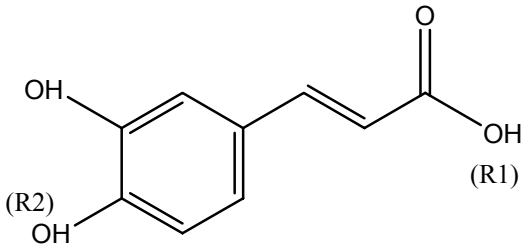
The concentrations and proportions of both bound and free HCAs in grape juice, are dependent on numerous viticultural factors including grape variety, berry size, ripeness, nitrogen availability, sunlight, and geographical region (Nagel *et al.,* 1979; Crippen and Morrison 1986; Keller and Hrazdina 1998; Peña-Neira *et al.,* 2000; Bautista-Ortín *et al.,* 2007). During the first stage, low molecular weight phenolics increase to a maximum around verasion, at this time compounds concentrations decrease. The second stage is characterised by a rapid accumulation of phenolic compounds which peak



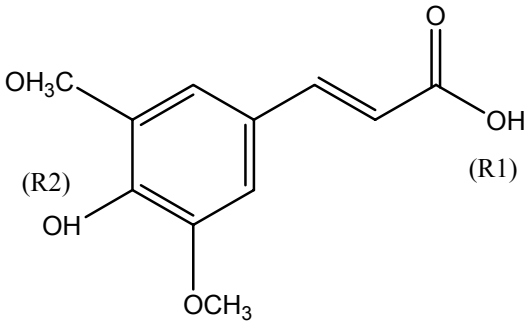
p-Coumaric acid



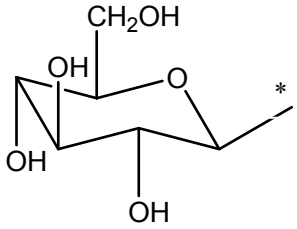
Ferulic acid



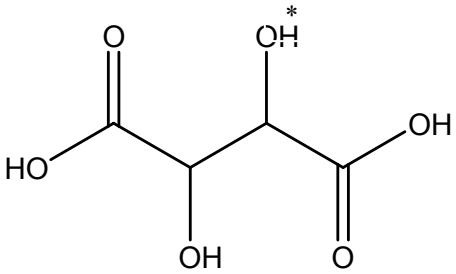
Caffeic acid



Sinapic acid



Glucose moiety



Tartaric acid

Figure 1. Chemical structures of simple hydroxycinnamic acids, glucose and tartaric acid. The R1 and R2 positions on HCAs denote where glucose moieties may be bound to form glucosides and the R1 position where tartaric acid may esterify the compounds. * Binding site.

around one week later than the usual harvest date (Singleton 1966; Fernández de Simón *et al.,* 1992; Pérez-Magariño and González-San Jose, 2005). In the subsequent week the phenolic concentration decrease before again increasing to reach a maximum, one week later (Figure 2; Singleton 1966; Fernández de Simón *et al.,* 1992; Pérez-Magariño and González-San Jose, 2005). Therefore, the date of harvest significantly affects the phenolic content of the resultant juice and wine. Variations in HCA concentrations can be a result of conditions which influence vine and berry development, such as nitrogen availability and exposure to sunlight (Crippen and Morrison 1986; Keller and Hrazdina 1998).

Concentrations of HCAs in grape juice are further dependant on their extraction from grape components and/or liberation from the bound forms. Consequently, the manner in which grapes are harvested; crushed and the time before the juice is separated from the solid material (grape skins and seeds) will determine the final HCA content (Oszmianski *et al.,* 1986; Macheix *et al.,* 1990; Auw *et al.,* 1996). Winery practices such as thermo-vinification, the addition of enzyme preparations, maceration time, yeast strain used, microoxygenation treatment and particularly fermentation temperature moderate the concentration of free HCAs in wines (Ramosa *et al.,* 1999; Netzel *et al.,* 2003; Sacchi *et al.,* 2005; Sartini *et al.,* 2007).

A number of oenological factors also alter the availability of HCAs. Malolactic fermentation and aging on lees can increase the concentration of HCAs, as a result of tartaric ester breakdown (Liu 2002; Hernández *et al.,* 2006; Cabrita *et al.*, 2008). Wine

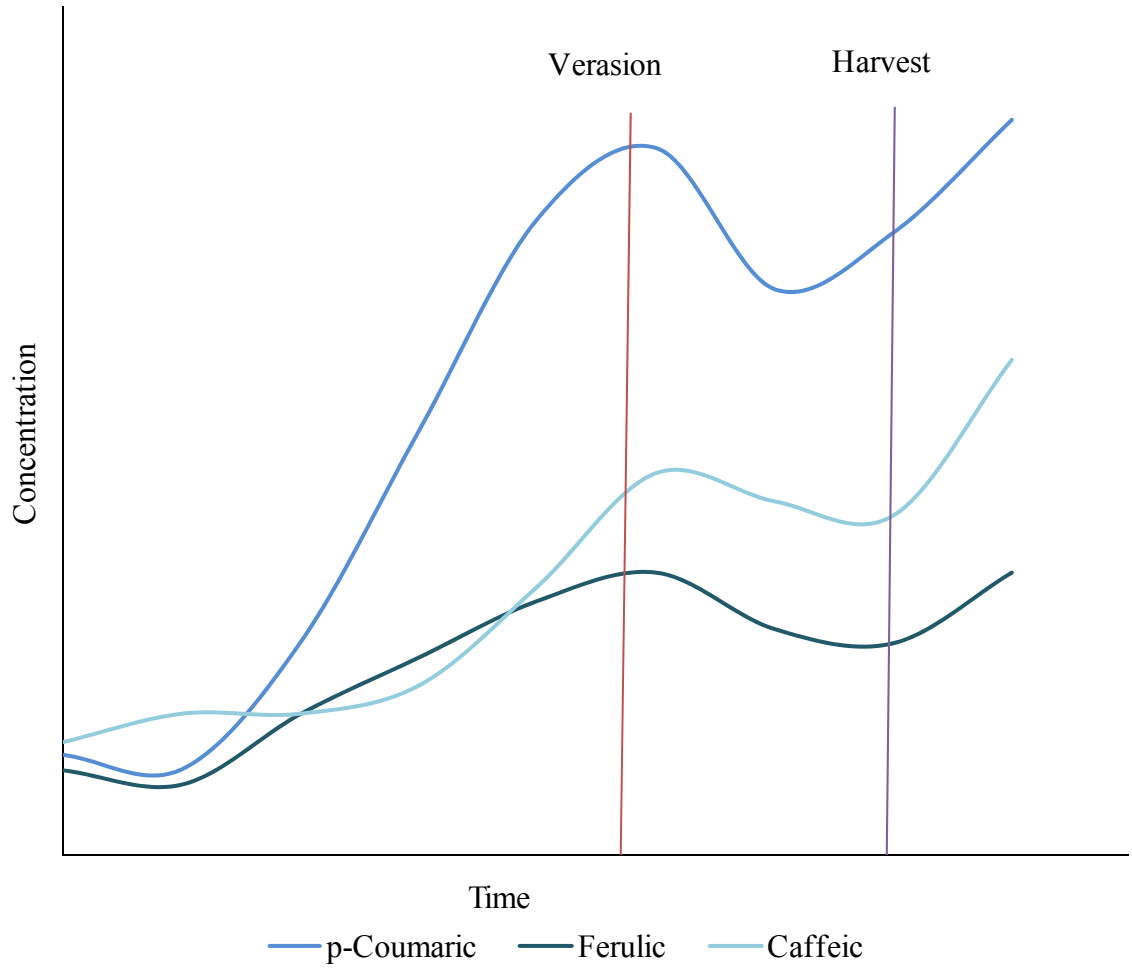


Figure 2. Schematic representation of hydroxycinnamic acid concentrations during grape maturation (Singleton 1966; Fernández de Simón *et al.,* 1992; Peréz- Magariños and González-San Jose, 2004).

yeast and spoilage organisms such as *Dekkera* catabolise HCAs further altering the phenolic profile of the finished wine (Chatonnet *et al.,* 1992; Silva *et al.,* 2005). Lactic acid bacteria also modulate available HCAs as they play an active role in HCA release (Hernández *et al.,* 2006) and can decarboxylate HCAs into VPs (Cavin *et al.,* 1993). Aging also increases the HCA concentrations, with the use of oak resulting in the greatest increase as phenolics such as sinapic acid that is extracted from the wood (Ibern-Gomez *et al.,* 2001; Matejícek *et al.,* 2005; Hernández *et al.,* 2006; Recamales *et al.,* 2006).

HCA-tartrate esters, glycosolated HCAs, free HCAs and products of various reactions that occur under wine conditions affect the appearance, flavour and aroma of both red and white wine (Budić-Leto and Lovrić 2002). HCA-tartrate, contribute to the bitterness of wines (Nagel and Wulf 1979) while higher increased free HCAs correlate to increase in hue and decrease in colour intensity (Budić-Leto and Lovrić 2002). In white wine production oxidation of HCAs causes browning of wine and is therefore an important factor (Cheynier *et al.,* 1990). HCAs are converted non-enzymatically into vinylphenol-anthocyanin adducts which have importance to colour intensity (Schwarz *et al.,* 2003). Finally, yeast and bacteria present in wine either in the natural fauna or inoculated directly act on HCAs converting them into volatile products which impact on wine aroma and flavour (Chatonnet *et al.,* 1992; Cavin *et al.,* 1993).

It is problematic to compare reported values of HCAs in finished wines due to the range of factors that influence their concentrations. However, caffeic acid is typically predominant in both red and white wines occurring at between 4.5–30 mg/l and 3.5–31 mg/l, respectively (Soleas *et al.,* 1997; Kallithraka *et al.,* 2006; Avar *et al.,* 2007).

Ferulic acid concentrations have been reported to range from undetectable up to 11 mg/l (Soleas *et al.,* 1997; Kallithraka *et al.,* 2006; Avar *et al.,* 2007), while *p*-coumaric acid in red wine varies from 3–9.5 mg/l and 1–8 mg/l in white (Soleas *et al.,* 1997; Kallithraka *et al.,* 2006; Avar *et al.,* 2007).

1.4 Biochemical transformations of hydroxycinnamic acids

Organisms that transform HCAs are of relevance during the production of alcoholic beverages where the resultant production of VPs is often deleterious. Understanding the enzymatic breakdown of HCAs is integral to developing preventative strategies against microbial spoilage in beer and wine and may benefit other industries. For example, 4-vinylguaiacol is 25-30 times more valuable than ferulic acid (Mathew and Abraham 2006). 4-Vinylguaiacol also may be further converted by biological or chemical means into acetovanillone and polystyrenes. Thus, the bioconversion is a high value added process (Mathew and Abraham 2006). In this context isolating and characterising the ferulic acid decarboxylating enzyme from *Dekkera* may result in enhancement of the biocatalytic conversion of ferulic acid to manufacture 4-vinylguaiacol for use in a variety of industries.

A number of bacteria and yeast have evolved mechanisms whereby they remove or modify HCAs from the growth medium. The majority of yeast and bacteria catabolise ferulic, *p*-coumaric and caffeic acids (Table 1) with few reported to act on sinapic acid (Hasidoko *et al*., 2001).

Table 1. Summary of bacterial and yeast enzymes that decarboxylate hydroxycinnamic acids pc - *p* -coumaric acid, fer - ferulic acid, caf- caffeic acid, sin - sinapic acid, nd- not determined

Organism	Protein	Protein size (kDa) SDS gel	Native	pH	Temp (ºC)	utilisation from strongest to weakest	Expression	References
Bacillus pumilus	ferulic acid decarboxylase	19 - 23	42 - 45	5.5	37 to 40	fer - pc- caf	induced	Degrassi *et al* .,1994; Zago *et al* ., 1995; Prim *et al* ., 2003
Bacillus subtilis	phenolic acid decarboxylase	23	45	5	40 to 45	fer - pc- caf	induced	Cavin *et al* ., 1998
Lactobacillus plantarum	ferulic acid decarboxylase and *p* - coumaric acid decarboxylase	23.5	nd	5.5 to 6	30	pc- caf - fer	induced	Cavin *et al* ., 1997a; b: Barthelmebs *et al* ., 2000a; 2001b; Gury *et al* ., 2004
Pseudomonas fluorescens	ferulic acid decarboxylase	20.4	40.4	7.3	27 to 30	fer - pc	constitutive	Huang *et al* ., 1994
Klebsiella oxytoca	phenylacrylic acid decarboxylase	21.5	nd	nd	nd	fer- pc -sin- caf	induced	Hashidoko *et al* ., 2001; Uchiyana *et al* ., 2008
Aerobacter aerogenes	4-hydroxycinnamic decarboxylase		nd	5.7	nd	fer - pc- caf	constitutive	Finkle 1962; Parry 1975
Saccharomyces cerevisae	phenylacrylic acid decarboxylase	26.7	nd	6	30	fer - pc	constitutive	Goodey and Tubb 1982; Claussen *et al* ., 1994; Donaghy *et al* ., 1999
Candida lambica	ferulic acid decarboxylase	25.8	nd	4.5 to 6.5	35	fer	nd	Donaghy *et al* ., 1999
Dekkera anomala	phenylacrylic acid decarboxylase	21	39.8	6	40	caf- pc - fer	constitutive	Edlin *et al* ., 1998

acid (Heresztyn 1986b; Hashidoko *et al.,* 2001). In the case of *Dekkera* spp. the conversion of *p*-coumaric acid has been examined during culture growth (Chatonnet *et al*., 1992; Dias *et al*., 2003b; Edlin *et al*., 1995). HCAs are converted during exponential growth of *Dekkera* cultures (Figure 3).

In general the HCA ─ VP reaction is a non-oxidative decarboxylation (Chatonnet *et al*., 1993) that occurs in two stages: 1) addition of a nucleophilic group in either the cis- or trans- orientation to form an intermediate, 2) spontaneous decarboxyl-group elimination from the opposite orientation, such that decarboxylation continues with geometry retention (Manitto, 1975; Parry, 1975; Huang, 1994). There is no available information for the mechanism by which *Dekkera* and *Brettanomyces* spp. metabolise HCAs, however, it is probable that *Dekkera* spp. follow this established route as they only metabolise acids with a free hydroxyl position (Edlin, 1998). The theoretical ratio of 1:1:1 of HCA:VP:EP is not observed. Up to 92% of HCAs may be removed from media but the collective total of VPs and EPs produced is less than the starting HCA concentration (Chatonnet, 1992; Dias *et al*., 2003b). This suggests that compounds are lost, either, the HCA-VP-EP pathway is not closed and compounds may enter other biochemical pathways, or through absorption to cellular material (Salameh *et al*., 2008).

HCAs are catabolised into corresponding 4-VPs by a HCA decarboxylase enzyme (HCD). This enzyme is also known as phenolic off-flavour 1 (POF1) (Goodey and Tubb 1982), phenylacrylic acid decarboxylase 1 (*PAD1*) (Clausen *et al.,* 1994) or by the primary substrate i.e. ferulic acid

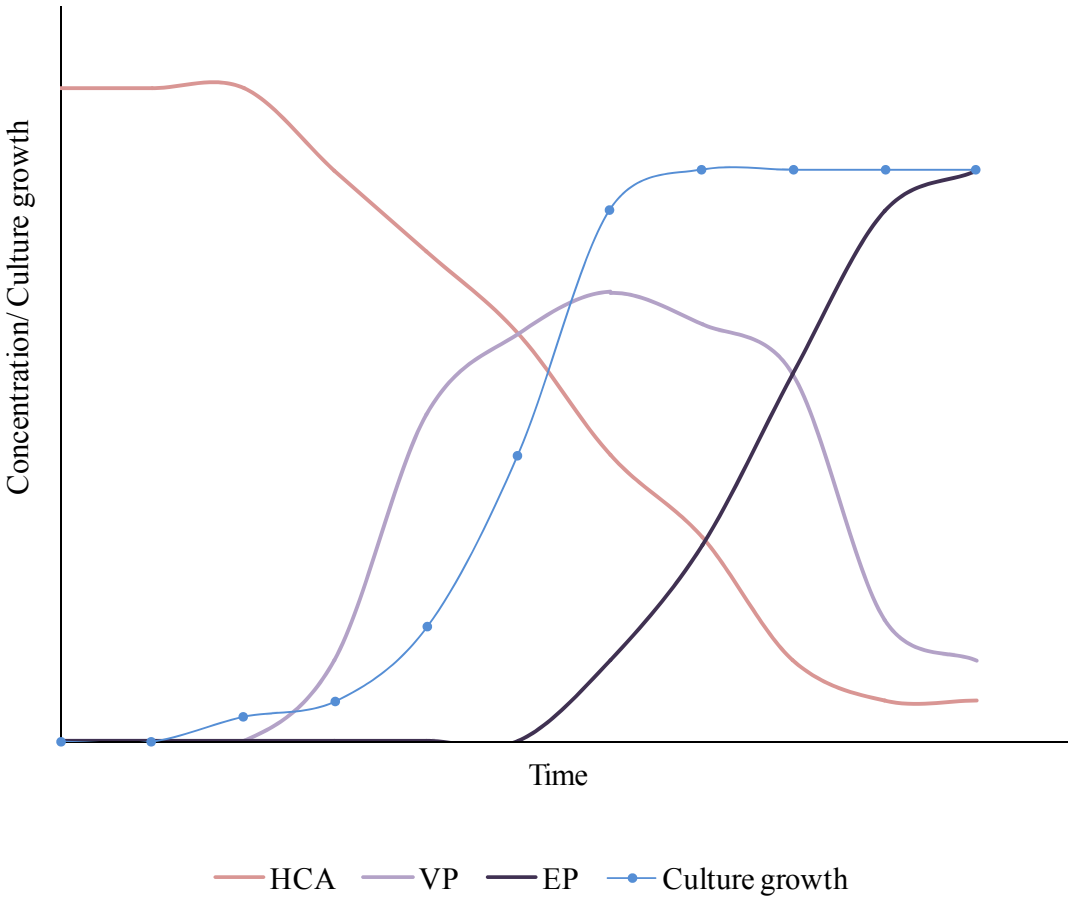


Figure 3. Schematic representation of the HCA ─ VP ─ EP pathway during the culture growth of *Dekkera anomala*. *p*-Coumaric acid is rapidly converted into 4-vinylphenol and these are simultaneously degraded into 4-ethylphenol (Chatonnet *et al.,* 1992; Edlin *et al.,* 1995; Dias *et al.,* 2003b).

or *p*-coumaric acid decarboxylase (FDC or PDC) (Huang *et al.,* 1994; Barthelmebs *et al.,* 2001b). A variety of organisms with HCD activity have been identified, including Gram-negative, Gram-positive bacteria, yeast and fungi (Parry 1975; Goodey and Tubb 1982; Nazareth and Mavinkurve 1986; Huang *et al.,* 1994; Zago *et al.,* 1995; Edlin *et al.,* 1998; Donaghy *et al.,* 1999). Wine organisms that have HCA decarboxylase activity include *S. cerevisiae, Pichia, Rhodotorula, Candida* and *Dekkera* spp. (Goodey and Tubb 1982; Edlin *et al.,* 1998; Donaghy *et al.,* 1999; Shinohara *et al.,* 2000) along with some lactic acid bacteria (Cavin *et al.,* 1997a: b). The majority of these are considered to be wine spoilage organisms, although *S. cerevisiae* and lactic acid bacteria are also fundamental to wine production. Characterised HCD enzymes are all homodimers (Edlin *et al.,* 1998; Prim *et al.,* 2003; Rodríguez *et al.,* 2007) with a total molecular weight of around 40-45 kDa (Huang *et al.,* 1994; Degrassi *et al.,* 1995; Cavin *et al.,* 1998; Edlin *et al.,* 1998; Rodríguez *et al.,* 2007). The average pH optimum of HCD enzymes is pH 6, although *Pseudomonas* has a relatively high optimum pH of 7.3 (Huang *et al.,* 1994), while *Bacillus* species can be as low as 5 (Cavin *et al.,* 1998). Optimum temperatures range from 27–45ºC (Table 1; Huang *et al.,* 1994; Degrassi *et al.,* 1995; Cavin *et al.,* 1998; Edlin *et al.,* 1998; Rodríguez *et al.,* 2007).

One important difference between enzymes from different organisms is whether the enzyme is constitutively expressed. Regardless, microorganisms appear to up-regulate the transcription of HCD enzymes when exposed to HCAs. A second important factor is the presence of more than one enzyme with differing specificity towards one or more HCA. *Lactobacillus plantarum* has two inducible enzymes (pdc1 and pdc2) in competition for HCA substrates, whose induction is dependent on which substrate is present (Barthelmebs *et al.,* 2000a; 2001b).

HCAs in the growth medium passively diffuse through the cell wall of Gram-positive and Gram-negative bacteria (Gury *et al.,* 2004). Entry of HCAs into the cell induces decarboxylase enzyme activity via a substrate-sensing protein that binds to the *HCD* gene to activate its transcription (Hashidoko *et al.,* 2001). Barthelmebs *et al.* (2000b) has described an alternative regulation of HCD expression via a negative transcriptional, autoregulated operon. In the absence of HCAs, a repressor protein (PadR) inhibits the transcription of the gene encoding the *PAD* (*PADA*). When the hypothesised sensor protein detects HCAs, a conformational change of PadR occurs, allowing the transcription of the *PADA* gene. Constitutive activity, observed for both *Saccharomyces* and *Dekkera* spp. indicates that transcription is not as tightly regulated in these organisms (Barthelmebs *et al.,* 2000b). However, the increase in HCD activity when the organism is exposed to a substrate rich environment suggests that a substrate sensing mechanism is involved.

HCA decarboxylase enzymes require substrates with a free para-hydroxyl group on the E-cinnamic skeleton (Hashidoko *et al.,* 2001). *p*-Coumaric, ferulic, caffeic and sinapic acids all have these structural characteristics. Most HCD enzymes have lower substrate affinity for caffeic and sinapic acids, as these compounds have an additional 3-methoxy group as well as para-hydroxyl groups essential for decarboxylation (Finkle *et al.,* 1962). This methoxy-group may interfere with enzyme recognition and binding to produce the observed decrease in affinity (Finkle *et al.,* 1962).

The HCD protein, from *D. anomala* is proposed to be a homodimer, with activity towards *p*-coumaric acid. The protein was isolated from *D. anomala* and then partially

sequenced (Edlin 1997; Edlin *et al.,* 1998). N-terminal sequencing revealed no similarity to yeast or bacterial HCD enzymes, but instead demonstrated homology to the N-terminus of YDR032 from *S. cerevisiae* (Edlin 1997). This open reading frame was subsequently identified as *PST2* (Edlin 1997), although the molecular and biological functions are not known (*Saccharomyces* Genome Database). It is thought to be unlikely that *PST2* is involved in HCA metabolism.

It is vital to determine the *HCD* sequence of *Dekkera* spp. for further characterisation and comparison. Sequences of genes with HCD activity from bacteria indicate that the C and N terminals have the greatest variability and that these regions are responsible for differences in specificity (Barthelmebs *et al.,* 2001a). However, *HCD* genes of yeast and bacteria share no homology hence no conclusions can be drawn of yeast *HCD* genes from their bacterial counterparts. These large differences between genes that have similar phenotypes suggest that at least two families of *HCD* genes may exist.

A partial genome sequence of a *D. bruxellensis* petite mutant derived from CBS 2499 (Woolfit *et al*., 2007). Assuming CBS 2499 is haploid, around 40% of the genome has been determined and information is available from EMBL (Woolfit *et al.,* 2007). The genomes of *Dekkera* spp. and strains vary greatly in size (Hoeben and Clark-Walker 1986; Woolfit *et al.,* 2007). Yeast strain CBS 2499 was chosen for sequencing as it has a relatively small genome and a petite negative strain could be made (Woolfit *et al.,* 2007). Based on the 40% sequenced it was determined that CBS 2499 has a largely single copy genome of a hypothesised 7,430 genes of which approximately 3,000 have been identified (Woolfit *et al.,* 2007). A large number of these genes are orthologs of *S. cerevisiae* genes or other yeast, although one case of horizontal gene transfer from a

bacterial species was documented (Woolfit *et al.,* 2007). The genome has a high GC content especially at the 3rd codon position, although codon reassignment has not occurred and conventional usage is observed (Woolfit *et al.,* 2007). *Dekkera* strains with genomes up to five times larger than CBS 2499 may have a larger percentage of duplicated regions.

Of the identified genes, many are involved in lipid metabolism and in alternate respiratory pathways representing an increase in genes for survival under harsh conditions (Woolfit *et al.,* 2007). Genes related to HCD activities were not identified. There are two conclusions, firstly, the *HCD* gene was not included in the obtained sequence or secondly, the *HCD* gene from *Dekkera* is poorly conserved. The genes so far identified by the sequencing project are mostly "housekeeping" genes that are relatively conserved between organisms. The *PAD* gene is not a gene essential for survival and hence is not necessarily as conserved and therefore it may not be identified by homologue searches.

An enzyme with HCA decarboxylase activity has been characterised and partially sequenced from *B. anomalus* (now classified as *D. anomala*) (Edlin *et al.,* 1998). This HCD enzyme was constitutively expressed, although its activity was further induced in the presence of substrates. Caffeic, *p*-coumaric and ferulic acids were decarboxylated by cell extracts from *D. anomala*, however, whole cell experiments also showed that sinapic acid can act as a substrate (Heresztyn 1986b). This discrepancy is possibly due to strain specific action of the HCD enzyme. HCD activity was greatest at pH 6 and a temperature of 40°C for *p*-coumaric (Edlin *et al.,* 1998). These values are similar to

HCD enzymes characterised from both yeast and bacteria (Goodey and Tubb 1982; Cavin *et al.,* 1997a: b; Donaghy *et al.,* 1999; Shinohara *et al.,* 2000).

There are few investigations focusing on *Brettanomyces* spp. in relation to vinylphenol formation. Dias *et al.* (2003b) concluded that no *Brettanomyces* spp. formed EPs directly from *p*-coumaric acid. The intermediate VP was not quantified and as such it is not clear whether it is the decarboxylation or reduction reaction or both that do not occur in this yeast. Confirming which enzymes *Brettanomyces* spp. have is essential. It will allow comparisons between the closely related *Brettanomyces* and *Dekkera* spp. to be made and could reveal key parameters required for VP production.

1.5 Biochemical transformations of vinylphenols

Numerous bacteria, fungi and yeast produce VPs. Some exceptional yeast and bacteria are capable of reducing VPs to EPs (Chatonnet *et al.,* 1992; Dias *et al.,* 2003a). This could be in response to the toxic effects of VPs. Microorganisms can produce high concentrations of VPs and although less toxic than HCA compounds, VPs reduce growth and cause cell death (Ayer *et al.,* 1995; Barthelmebs *et al.,* 2001a). In harsh wine environments where organisms are also exposed to high ethanol concentrations, low pH and nutrient concentration this toxicity could be enhanced.

Pichia, *Candida* and *Dekkera* yeasts along with *Lactobacillus* and *Pediococcus* bacteria produce ethylphenols (Cavin *et al.,* 1993; Chatonnet *et al.,* 1992; 1995; Dias *et al.,* 2003a; Barata *et al.,* 2006; Couto *et al.,* 2006; Suárez *et al.,* 2007). In particular, *P. guilliermondii* can produce EPs in similar concentrations to those synthesised by

Dekkera (Dias *et al.,* 2003a: b). Nevertheless, in the wine environment only *Dekkera* produce significant concentrations of EPs (Barata *et al.,* 2006). *Brettanomyces* spp. do not produce EPs from HCA precursors (Dias *et al.,* 2003a). It is unknown whether this is related to a lack of decarboxylase activity or an absence of the vinylphenol reductase (VPR) enzyme.

Both *Dekkera* spp. rapidly metabolise VPs (Heresztyn 1986b; Edlin *et al.,* 1998) into EPs in concentrations many times in excess of the detection threshold in wine or beer (Chatonnet *et al.,* 1992; Vanbeneden *et al.,* 2008). Once released into wine, EPs can only be removed by sophisticated and expensive techniques such as reverse osmosis, which can also remove colour and other essential flavour and aroma compounds (Ugarte *et al.,* 2005). EPs are therefore potent spoilage compounds that decrease beverage quality and at worst leave the product unpalatable and unsaleable.

Dekkera synthesise EPs in a two-step pathway from HCAs or they can act directly on the VP intermediates (Dias *et al*., 2003b). *Dekkera* can therefore convert vinylphenols released into the growth medium by organisms such as *S. cerevisiae*. All HCAs present may be metabolised by *Dekkera* and up to 92% will be converted into EPs (Chatonnet *et al.,* 1992; Dias *et al.,* 2003a). Metabolism of HCAs into VPs is the rate limiting stage in this sequential pathway (Vanbeneden *et al.,* 2008). Conversion of VPs to EPs by *Dekkera* spp. occurs shortly after VPs are first detected in growth medium (Figure 3) (Chatonnet *et al.,* 1992; Edlin *et al.,* 1995; Dias *et al.,* 2003b). Production of EPs continues until almost all VPs are converted. This corresponds to the stationary phase of growth (Chatonnet *et al.,* 1992; Edlin *et al.,* 1995; Dias *et al.,* 2003b). Wine conditions such as low pH, high ethanol concentrations, reduced oxygenation and limited carbon

sources moderate the production of EPs in wines by *Dekkera* (Dias *et al.,* 2003b; Romano *et al.,* 2008).

Substrates for EP production include 4-vinylphenol, 4-vinylguaiacol, 4-vinylcatechol and 4- vinylsyringol (Heresztyn 1986b; Chatonnet *et al.,* 1992; Dias *et al.,* 2003a; b; Hesford *et al.,* 2004). The VPR enzyme is proposed to reduce these substrates into their corresponding 4-ethyl derivatives (Chatonnet *et al.,* 1992). The VPR enzyme has never been isolated from any organism and the gene sequence was not identified during sequencing of *D. bruxellensis* CBS 2499 (Woolfit *et al.,* 2007). Nothing further is currently known about parameters required for VPR activity.

1.6 Antimicrobial action associated with hydroxycinnamic acids

HCAs are mild preservatives with antibacterial and antifungal properties (Baranowski *et al.,* 1980; Stead 1995; García-Ruiz *et al.,* 2008) similar to that exhibited by the weak organic acid sorbate, commonly added to foods as a preservative (Stratford *et al*., 2007). A wide range of microorganisms are sensitive to HCAs but the effects depend on concentration and the acid to which it is exposed (Chesson *et al.,* 1982; Herald and Davidson 1983; Stead 1993; 1995; Campos *et al.,* 2003; Wen *et al.,* 2003; García-Ruiz *et al.,* 2008). A number of wine microorganisms are inhibited by HCAs but at concentration higher than those reported in wine (Stead 1993; 1995; Campos *et al.,* 2003; Reguant *et al.*, 2000).

p-Coumaric and ferulic acids tend to be the most toxic (Baranowski *et al.,* 1980; Chesson *et al.,* 1982; Ravn *et al*., 1989; Stead 1993; 1995). This is probably linked to their polarity and therefore their ability to cross cellular membranes (Baranowski *et al.,*

1980; Campos *et al*., 2003). Gram-positive, Gram-negative bacteria, fungi and yeast can detoxify HCAs into vinylphenols (Goodey and Tubb 1982; Clausen *et al.,* 1994; Degrassi *et al.,* 1995; Cavin *et al.,* 1997a; b; Donaghy *et al.,* 1999). This is beneficial to the survival of microorganisms in environments rich in HCAs, the ability to decarboxylate these phenolics confers a degree of resistance to the antimicrobial properties of HCAs (Clausen *et al.,* 1994; Larsson *et al.,* 2001). Generally, VPs are less toxic than HCAs, but they do inhibit growth of some fungi and bacteria (Ayer *et al.,* 1995; Barthelmebs *et al.,* 2001b) and may be why a few organisms catabolise VPs further.

1.7 Additional benefits of hydroxycinnamic acids

HCAs not only protect food from microbial spoilage, they play important roles in human health. HCAs are protective against human pathogens and viruses such as *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, influenza and AIDS (Edeas *et al.,* 1995; Wen *et al.,* 2003; Ou and Kwok 2004; Papadopoulou *et al.*, 2005; Puupponen-Pimiä *et al.,* 2005). They act as antioxidants, 250 mg/l of ferulic acid scavenges almost 100% of hydroxyl-radicals in a Fenton-type reaction system (Graf 1992; Chen and Ho 1997; Kikuzaki *et al.,* 2002; Ou and Kwok 2004). Ferulic, caffeic and sinapic acids can also scavenge nitrogen dioxide radicals (Zhouen *et al.,* 1998). Antioxidant properties associated with HCAs have been demonstrated for wines where these compounds are in part responsible for health benefits associated with moderate consumption (Tselepis *et al.,* 2005; García-Ruiz *et al.,* 2008).

Ferulic acid in particular has been investigated in a number of medical studies. It is a potential chemopreventive agent against atherosclerosis, due to its anti-inflammatory

properties, as it inhibits thrombosis and lowers cholesterol (Ou and Kwok 2004; Nagasaka *et al.,* 2007). This anti-inflammatory action also decreases the risk of neurodegenerative disease (Srinivasan *et al.,* 2007). HCAs may help prevent some cancer, in particular oral and bowel cancers (Ogiwara *et al.,* 2002; Ou and Kwok 2004; Lee *et al.,* 2005). Further, HCAs decrease lipid peroxidation while enhancing glutathione levels and antioxidant enzymes in relation to diabetes (Sri Balasubashini *et al.,* 2004). Ferulic acid has additionally been demonstrated to increase sperm vitality (Zheng and Zhang 1996) and have anti-aging effects (Srinivasan *et al.,* 2007).

Consumers are becoming more informed and concerned about the use of preservatives in food, hence many industries are investigating alternative or natural antimicrobials. In the wine industry, sulfur dioxide is the most commonly used preservative. Sulfites, however, are known to cause adverse reactions, including anaphylactic shock, asthmatic attacks, nausea, abdominal pain, diarrhea, seizures and death in some susceptible individuals (Yang and Purchase 1985). Concerns over detrimental effects of sulfur dioxide has led to incidences of limits being decreased by regulators and/or a reduced usage of this preservative by winemakers (García-Ruiz *et al.,* 2008). The development of alternate and ideally natural antimicrobials is therefore warranted to combat the potential for increasing susceptibility of wines to microbial spoilage. Given purported health benefits linked to HCAs and their antimicrobial properties against wine-related yeast and bacteria (Stead 1993; 1995; García-Ruiz *et al.,* 2008), HCAs may have a future role in the control of unwanted spoilage organisms.

Dekkera sp. are highly resistant to sulfur dioxide preservatives in wine, thus contaminated wines often require large additions to reduce infection. As well as the

health associated risks, high concentrations of sulfite preservatives affect the organoleptic properties of wine (García-Ruiz *et al.,* 2008). Alternative methods to control *Dekkera* investigated include the addition of weak acids such as sorbic and benzoic acids (Loureiro and Malfeito-Ferreira, 2003; Suárez *et al*., 2007), the use of physical methods such as filtration (Suárez *et al.,* 2007) or reverse osmosis to remove taint (Ugarte *et al.,* 2005).

Phenolic compounds are already under consideration for the control of lactic acid bacteria in wine (García-Ruiz *et al.,* 2008). The inhibition of *Dekkera* by HCAs has not been directly investigated, however, several authors have observed an inhibitory effect of HCA on *Dekkera* however the significance of this has not been discussed (Edlin *et al.,* 1998; Couto *et al.,* 2005). Inhibitory effects were observed in a laboratory medium ideal for *Dekkera* growth. Under harsh wine parameters the concentration required to inhibit microbes could be significantly reduced. In addition there is the potential to extract HCAs from grape derivatives that could be subsequently used for biofortification of wine, following appropriate regulatory processes.

1.8 Research aims

Dekkera produce volatile phenols in excess of the threshold of detection in wine and beer. VPs and EPs are the resultant compounds of a two-step enzymatic pathway that catabolises HCAs present in grape juice and grains (Chatonnet *et al.,* 1992; Vanbeneden *et al.,* 2008). These enzymes have not been satisfactory characterised from *Dekkera*. Thus far, isolation of the VPR enzyme has not been reported. The closely related yeast, *Brettanomyces* spp. have not been characterised with respect to volatile phenol production. These yeasts have less impact on industrial product quality, although they

have been isolated from a range of beverages the role they play is undefined (Stratford *et al.,* 2000). In this thesis, the extent of volatile phenol production by both *Dekkera* and *Brettanomyces* spp. will be elucidated.

The primary aim of this research is to expand the current understanding of *Dekkera* and *Brettanomyces* spp. One priority will be to examine the growth of *Dekkera* spp. and the production of VP and EP compounds, under defined conditions with additions of HCAs. Previous studies have only examined one strain of either *D. anomala* or *D. bruxellensis.* These studies were limited to the conversion of *p*-coumaric acid only. Different HCA concentrations were used therefore making comparisons between studies impossible. Investigations into the influence of four HCAs on growth of all five *Dekkera* and *Brettanomyces* spp. will be undertaken. Comparison of wine and non-wine isolates of *D. bruxellensis* will also be made.

In nature, HCAs occur in combinations. No studies have been undertaken to elucidate the effect of the presence of multiple HCAs. The ability of yeasts to metabolise several acids and the order in which they do so may alter the final wine composition. Determining how *Dekkera* contend with more than one acid is important in order to replicate the grape juice and wine environments and thus determine how *Dekkera* may alter the composition of wine while also examining the effect those combinations may have on the yeast. Comparisons of *Dekkera* in regards to HCA metabolism may determine distinctive strain dependent characteristics relevant to industrial spoilage.

A secondary aim of this research is to characterise the HCD enzyme at the molecular level. There is a dearth of genetic information available for *Dekkera*. No DNA sequence

for the *HCD* gene has previously been determined. It was first assumed that the gene responsible would be similar to the *PAD1* gene of *S. cerevisiae* and molecular characterisation was attempted on this basis but was unsuccessful (Appendix 1). Edlin *et al.* (1998), characterised the activity of a HCD enzyme from *D. anomala.* Further unpublished research, details a short amino acid sequence determined for this HCD protein, which corresponds to the N-terminal region (Edlin 1997). Sequence obtained had no homology to known HCD enzymes. HCD enzymes with similar phenotypes from yeast and bacteria share no homology. It is possible that HCD from *Dekkera* is a member of a third separate group of HCD enzymes. This could explain why molecular characterisation based on *PAD1* sequences was unsuccessful (Appendix 1). Continuing this isolation to obtain more HCD sequence in combination with molecular methods to ascertain DNA sequence is vital for further understanding of this enzyme. Comparative characterisation of HCD enzyme activity from *Dekkera* and *Brettanomyces* spp. will be carried out.

There have been no reports of VPR being characterised from any organism. Therefore, confirmation of the presence and action of VPR enzymes from *Dekkera* and *Brettanomyces* will be investigated. The aim is to carry out a broad biochemical characterisation to determine optimal conditions for VPR activity such as pH and temperature and to investigate the substrate specificity.

The wine industry requires a simple, cheap method of controlling *Dekkera* that has minimal impact on wine quality. It is possible that HCAs have the potential to prevent the problem they are responsible for causing. HCAs are known antimicrobials and concentrations of 2–2.5 mM may inhibit *Dekkera* growth in model medium (Edlin *et*

al., 1995; Couto *et al.,* 2005). It is hypothesised that under wine conditions toxic effects of HCAs may be enhanced and therefore lower concentrations could prevent *Dekkera* growth. Since a minimal viable population is needed to produce volatile phenols, preventing growth could eliminate spoilage. Investigating conditions under which *Dekkera* can no longer grow and whether HCAs inhibit or eliminate *Dekkera* could lead to the development of novel methods to control these yeasts.

Chapter 2

Dekkera and *Brettanomyces* growth and utilisation of hydroxycinnamic acids in synthetic media

Statement of authorship

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Supervised work and helped in the preparation of the manuscript.

Sign: Date:

Vladimir Jiranek (co-supervisor)

Supervised work and helped in the preparation of the manuscript.

Sign: Date:

Paul Grbin (principal supervisor)

Supervised work and helped in the preparation of the manuscript and acted as communicating author.

Sign: Date:

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Dekkera and *Brettanomyces* growth and utilisation of hydroxycinnamic acids in synthetic media

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Abstract *Dekkera* and *Brettanomyces* yeast are important spoilage organisms in a number of food and beverage products. Isolates of both genera were cultured in a defined medium and supplemented with hydroxycinnamic acids 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. This study provides an insight into the spoilage potential of these organisms and possible control strategies involving hydroxycinnamic acids.

Keywords *p*-Coumaric acid · Ferulic acid · Caffeic acid · Sinapic acid · Wine spoilage · Microbial spoilage

Introduction

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 paper, the taxonomy adhered to is that of Barnett et al. (2000). There are five 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 and Waraich 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; 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 phenol production in wine is indicative of *Dekkera* contamination and the presence of concentrations as low as 425 µg/l can impact on wine sensory properties (Chatonnet et al. 1992). At low concentrations, 4-vinylphenol, 4-vinylguaiacol and 4-vinylsyringol, may have inoffensive and even desirable aromatic properties, e.g. ‘spicy’, ‘woody’ and ‘sweet’ (Maga 1978; Heresztyn 1986; van Beek and Priest 2000). When present at concentrations higher than 725 µg/l, their aroma and flavour contributions are deleterious to wine quality, and high

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quantities of 4-vinylphenol is commonly associated with 'medicinal' and 'phenolic' off-aromas (Chatonnet et al. 1993), while 4-ethyl derivatives elicit descriptors such as 'barnyard', 'animal' and 'Bandaid®' (Chatonnet et al. 1992; Licker et al. 1998).

Despite being well-documented spoilage organisms, *Dekkera* yeasts are important in the brewing of the Belgium style lambic and gueze beers where they, in combination with a number of other microorganisms, are responsible for producing characteristic aromas and flavours (Martens et al. 1997). *Brettanomyces nanus*, *Brettanomyces naardenensis* and *Brettanomyces custersianus* have not been isolated from the winery environment, however, *Brettanomyces* yeasts have been isolated from a number of other food and beverage products including soft drinks, beer and traditional fermentation products, such as bantu beer and Kombucha, where they play a role in spoilage or contribute to the sensory properties (van der Walt 1961; Kolfschten and Yarrow 1970; Cabranes et al. 1990; Stratford et al. 2000; Teoh et al. 2004). Finally, *Dekkera* and *Brettanomyces* have also been isolated during the production of some olive, milk and cheese products (Fadda et al. 2001; Carrasco et al. 2006) where the role they play is less clear.

Volatile phenols are produced by a number of micro-organisms during the catabolism of hydroxycinnamic acids (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 are complex, and the concentration of compounds presents 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 play no role in wine spoilage and no *Brettanomyces* species have been found to produce more 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 an effect on the spoilage of other products.

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. 1997; Barthelmebs et al. 2000; 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. *P. guilliermondii* produce high levels of ethyl-phenols 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.

The aims of this study are to examine all *Dekkera* and *Brettanomyces* species for their production of 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 will determine the uptake of 4-vinylphenol and 4-vinylguaiacol by all species. Confirmation that ethylphenol derivatives are not further metabolised by *Dekkera*/*Brettanomyces* species will also be investigated.

Materials and methods

Yeast strains

Dekkera and *Brettanomyces* yeast (Table 1) were obtained from the Centraalbureau Voor Schimmelcultures (CBS) and The Australian Wine Research Institute (AWRI). *S. cerevisiae* strain Maurivin PDM was also used in the production of wine media (WM). Yeast 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°C.

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 Erlenmeyer flasks and grown with shaking (180 rpm). Starters were incubated at 30°C 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 sulphate, 250 mg/l magnesium sulphate, 2 g/l potassium phosphate, 200 mg/l citric acid, 310 mg/l calcium chloride anhydrous, 2 mg/l zinc sulphate, 10 mg/l iron sulphate, 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)], pH 3.8, and incubated aerobically in 250-ml Erlenmeyer flasks. Flasks were inoculated with 5×10^6 cells/ml and incubated at 30°C. Previous investigations indicated that 30°C was unsuitable for growth of *B. nanus* and *B. naardenensis* (data not shown), and it was determined that 25°C 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.

Table 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

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 wine-making strain. The medium fermented with PDM was used as a base for the WM in further experiments to more closely reflect wine-like conditions. Before use, the fermented medium was clarified by centrifugation at 15,000×*g* for 1 h 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 10 M NaOH. Residual sugar concentrations were determined by high-performance liquid chromatography (HPLC). A 15-μl sample was injected onto a Waters high performance carbohydrate column (WAT044355; 4.6 mm×250 mm), and a 75% acetronitrile mobile phase was pumped through the column at a flow rate of 1.4 ml/min for 12 min. 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 that 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 before growth of *Dekkera* yeasts in WM. As previously described, a YPD starter culture was prepared, 5×10^6 cells/ml were then inoculated into adaptation medium (25 ml of CDM with 5% *v*/*v* ethanol) and cultures grown at 30°C with shaking at 180 rpm. Adaptation cultures reached a density of 1×10^8 cells/ml before being used to inoculate WM, in triplicate, at a rate of 5×10^6 cell/ml.

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 and 48 h, depending on the yeast strain. A 1-ml aliquot of culture was removed at each time point, centrifuged and the supernatant stored at −30°C before further analysis. Cell growth was estimated spectrophotometrically at 600 nm (Heλios α, Thermospectronic). Glucose concentrations

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

Determination of hydroxycinnamic acid utilisation during fermentation by HPLC

Fermentation samples were centrifuged for 1 min at 16,000×*g*, and 200 μl of supernatant was sterile filtered with a 1-ml syringe filter (0.45 μm, Alltech) before 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×150 mm). Peaks were separated over a 40-min 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 min with 100% buffer B and re-equilibrated over 5 min with 100% buffer A. Elution was monitored by absorbance at 310, 254 and 280 nm. 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.

Results

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 h compared to the control. Despite this lag, the final cell density was comparable to that of the control (Fig. 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 h. Growth in the presence of other acids was consistent with the unsupplemented control. All *Brettanomyces* species were inhibited by the presence of HCAs (Fig. 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. 1).

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. 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, although 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 CBS 2336 and AWRI 1499 (Fig. 2). *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. 3, 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* yeast 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. 3b.

Combinations of hydroxycinnamic acids

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 concentra-

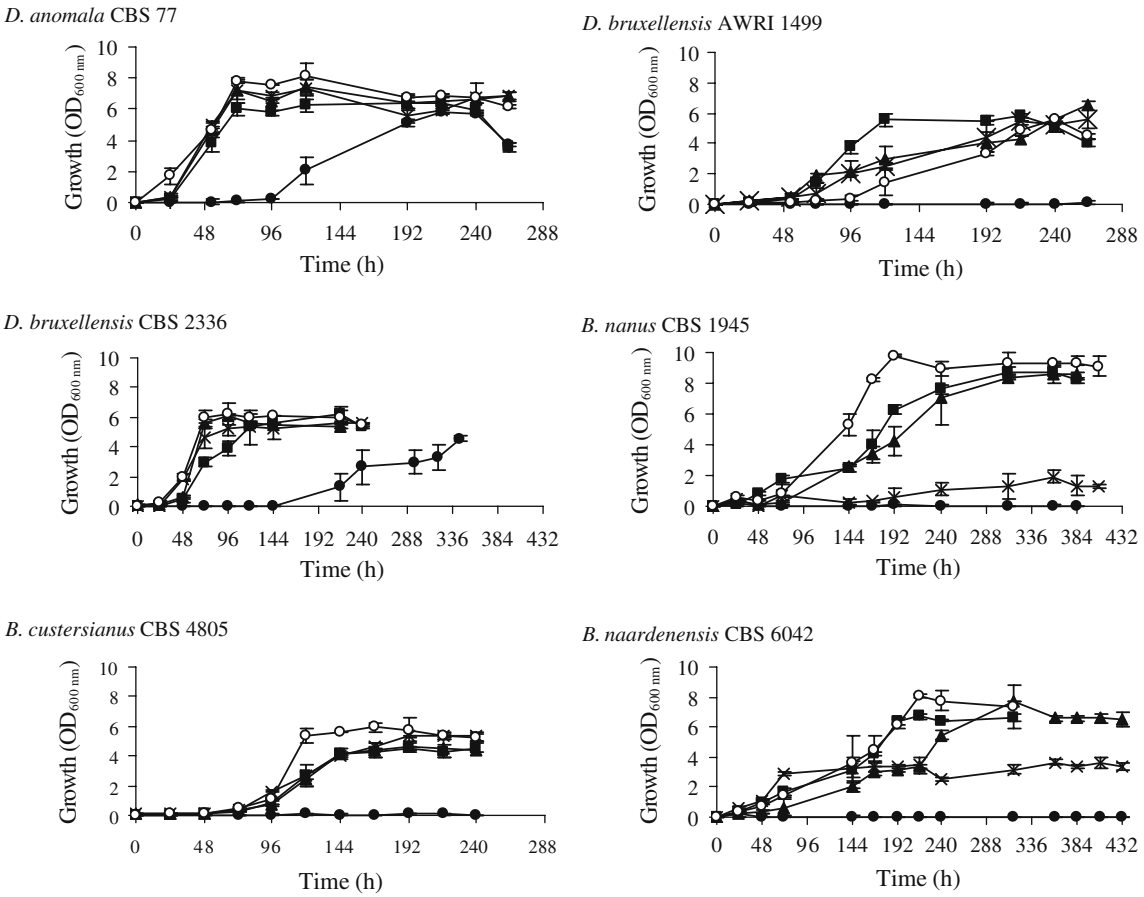

Fig. 1 *Dekkera* and *Brettanomyces* growth in the presence of 2 mM HCAs; *p*-coumaric (*filled square*), ferulic (*filled circle*) caffeic (*filled triangle*) and sinapic acid (*times symbol*) and control (*empty circle*) estimated by optical density ($OD_{600\ nm}$). Yeast strains CBS 77, AWRI 1499 and CBS 4805 were monitored over 288 h while CBS 2336, CBS 1945 and CBS 6042 were monitored over 432 h

tion. Both *Dekkera* species were inhibited by the addition of acids in combination. No 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 h. 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. 4). The final

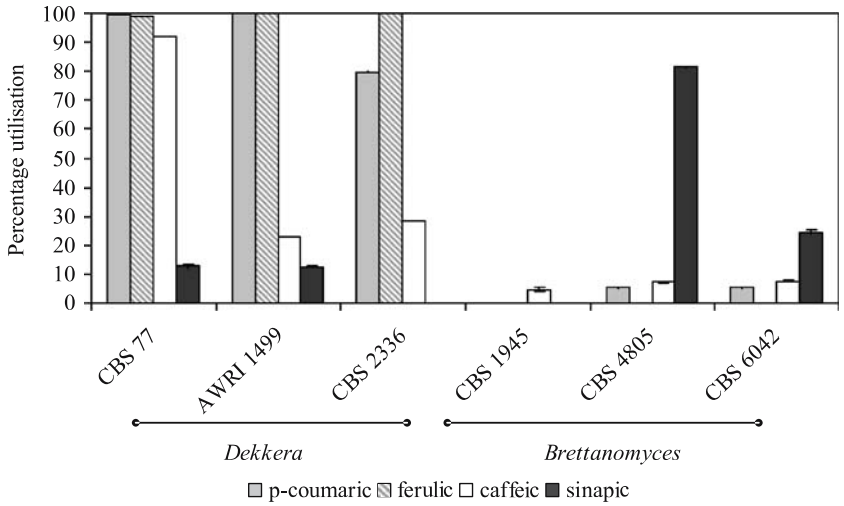

Fig. 2 The utilisation of HCAs by *Dekkera* and *Brettanomyces* yeast reported as the percentage of total supplied substrate (1 mM) utilised at the end point of fermentation. Fermentations were preformed in triplicate and HPLC analysis in duplicate. Standard deviations are included and were below 1%

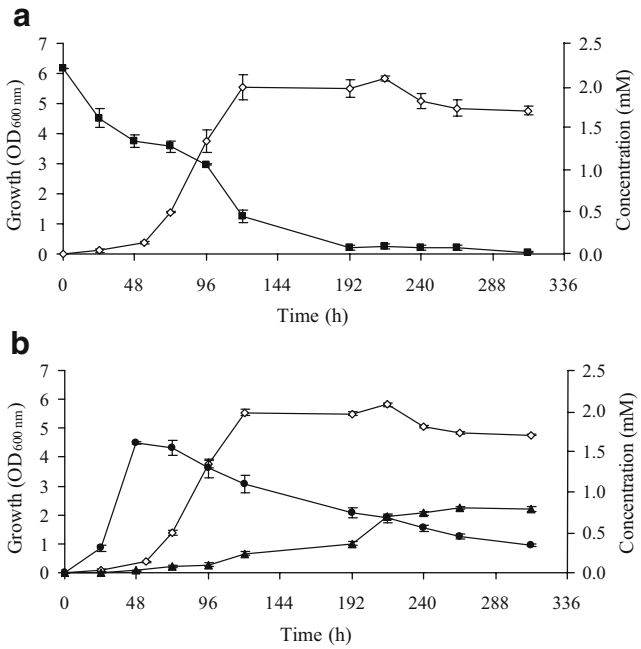

Fig. 3 **a** *D. anomala* (CBS 77) growth (*empty diamond*) as measured by optical density ($OD_{600\ nm}$) and utilisation of 2 mM *p*-coumaric acid (*filled square*) during fermentation. **b** Production of 4-vinylphenol (*filled circle*) and 4-ethylphenol (*filled triangle*) from *p*-coumaric acid during culture growth (*empty diamond*)

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. 4). Ferulic acid was removed at a rate lower than that of *p*-coumaric acid, such that ferulic acid was not completely depleted from the medium. High-performance liquid chromatography 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).

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 was examined by HPLC analysis (Fig. 5). *D. anomala* and *D. bruxellensis* utilised between 90 and 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 h, depending on the strain (data not shown). *Brettanomyces* species demonstrated an ability to utilise 4-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 and 30% of the supplied 4-vinylphenol (Fig. 5). The corresponding 4-ethylphenol product was not detected by HPLC.

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), 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 h (data not shown). 4-Ethylphenol was not metabolised during growth.

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,

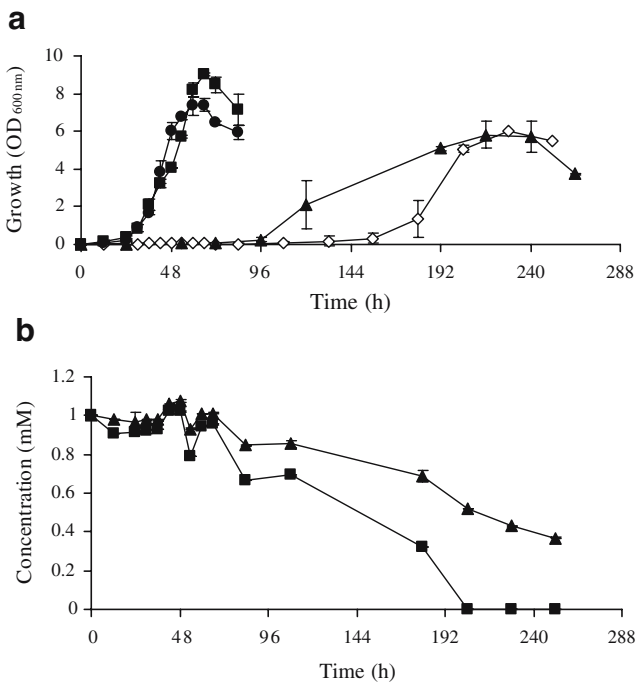

Fig. 4 **a** Comparison of *D. anomala* CBS 77 culture growth, estimated by optical density ($OD_{600\ nm}$), in the presence or absence (*filled circle*) of 2 mM *p*-coumaric (*filled square*) ferulic acid (*filled triangle*) or *p*-coumaric and ferulic acids in combination (*empty diamond*) each at a concentration of 1 mM. **b** The utilisation of *p*-coumaric (*filled square*) and ferulic acids (*filled triangle*) when combined was determined by HPLC analysis

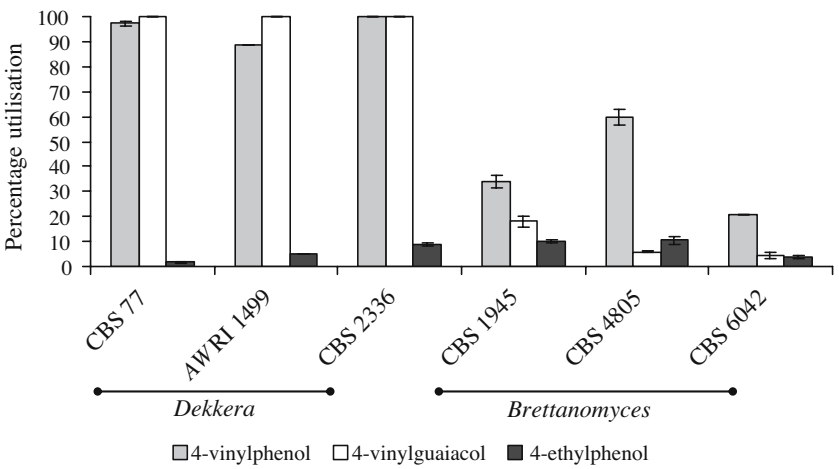

Fig. 5 Comparison of the utilisation of 4-vinylphenol, 4-vinylguaiacol and 4-ethylphenol by *Dekkera* and *Brettanomyces* yeasts during growth in CDM

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 *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. 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.

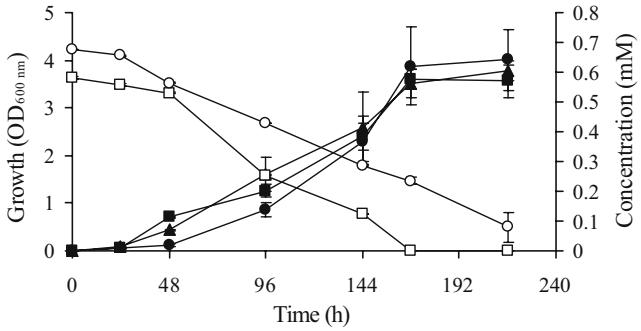

Fig. 6 Growth of *D. bruxellensis* AWRI 1499 in wine medium unsupplemented (*filled triangle*) and supplemented with 0.6 mM (100 mg/l) *p*-coumaric (*filled square*) and ferulic (*filled circle*) acids determined by optical density ($OD_{600\ nm}$). Uptake of *p*-coumaric (*empty square*) and ferulic (*empty circle*) acids from the medium was determined by HPLC analysis

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. 1). While these findings concur with other studies that have reported an inhibitory affect of some HCAs on microbial growth, this appears to be one of 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 the levels investigated to control other microorganisms (Stead 1995).

Strain variation was also seen with regard to the metabolism of HCAs (Fig. 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

being undertaken to isolate, 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 because Chatonnet et al. (1995) found *Brettanomyces* to be incapable of metabolising *p*-coumaric acid, these organisms would also lack the VPR enzyme. However, as 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 because of *Dekkera* yeasts absorbing the HCA into their cell walls as shown by Salameh et al. (2007). This absorption 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 beverages at the end of ferment through the action of other microbes could be acted on by *Dekkera* spoilage organisms to produce the highly undesirable 4-ethylphenol and 4-ethylguaiacol.

A semi-defined WM was developed to confirm the validity of results from CDM in an environment more analogous to that which occurs in an industry. Thus, CDGJM was pre-fermented to a model ‘wine’ using the commercial *S. cerevisiae* strain 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 because of the added presence of ethanol. Because 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.

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 medium and a wine-like condition. 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. Further investigation is required to fully understand the mechanism by which this occurs. The confirmation of possible enzymatic differences between *Dekkera* and *Brettanomyces* species are also under way not only to further characterise the organism but also to elucidate whether the addition of ferulic acid may allow inhibition of *Dekkera* and *Brettanomyces* in industrial situations.

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Chapter 3

Survey of enzymatic activity responsible for phenolic-off-flavour production by *Dekkera* and *Brettanomyces* yeast

Statement of authorship

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Survey of enzyme activity responsible for phenolic off-flavour production by *Dekkera* and *Brettanomyces* yeast

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Abstract Volatile phenols are produced by *Dekkera* yeasts and are of organoleptic importance in alcoholic beverages. The key compound in this respect is 4-ethylphenol, responsible for the medicinal and phenolic aromas in spoiled wines. The microbial synthesis of volatile phenols is thought to occur in two steps, beginning with naturally occurring hydroxycinnamic acids (HCAs). The enzyme phenolic acid decarboxylase (PAD) converts 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. Here, both steps of the pathway are 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 metabolise vinylphenols. *Dekkera* species form ethylphenols but the VPR enzyme appears to be highly unstable in cell extracts. A partial protein sequence for PAD was determined from *Dekkera anomala* and may indicate the presence of a novel enzyme in this genus.

Keywords Phenylacrylic decarboxylase · Hydroxycinnamic acid decarboxylase · Spoilage · 4-Vinylphenol · 4-Ethylphenol · 4-Vinylguaiacol · 4-Ethylguaiacol

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Introduction

Dekkera and *Brettanomyces* yeast have been isolated from a variety of foods (Fadda et al. 2001, 2004; Coton et al. 2006) and beverages (Kumara and Verachtert 1991; Lachance 1995; Coton et al. 2006) although the role they play, if any, in the final olfactory properties of these products is not necessarily established in detail. *Dekkera* and *Brettanomyces* belong to related but distinct genera based on phylogenetic analysis (Smith et al. 1990; Cai et al. 1996). There are five recognised species: *Brettanomyces custersianus*, *B. naardenensis*, *B. nanus*, *Dekkera anomala* and *D. bruxellensis* (Barnett et al. 2000).

Spoilage products synthesised by *Dekkera* yeast cause phenolic off-flavour, an undesirable characteristic in beer and wine, which is attributed to vinyl and ethylphenol production (Heresztyn 1986; Chatonnet et al. 1992; Vanbeneden et al. 2007). *Dekkera* is capable of producing quantities of volatile phenol compounds above the threshold limit of perception in wine and beer (Chatonnet et al. 1992; Vanbeneden et al. 2007). Vinylphenols are commonly associated with 'medicinal' aromas when present at concentrations above 725 μg/l in wine (Chatonnet et al. 1993) whilst 4-ethyl derivatives are associated with 'barnyard', 'animal' and 'Bandaid®' aromas (Licker et al. 1998; Coulter et al. 2003).

Dekkera yeasts decarboxylate hydroxycinnamic (phenolic) acids (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 (Dias et al. 2003;

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*.

The PAD enzyme and homologues have previously been characterised from bacteria and yeast (Clausen et al. 1994; Cavin et al. 1997b; Edlin et al. 1998; Barthelmebs et al. 2000a; Stratford et al. 2007). Bacterial species with characterised PAD enzymes include *Pseudomonas fluorescens* (Huang et al. 1994), *Lactobacillus plantarum* (Cavin et al. 1997a; Rodríguez et al. 2007), *Bacillus subtilis* and *pumilus* (Degrassi et al. 1995; Zago et al. 1995; Cavin et al. 1998), *Pediococcus pentosaceus* (Barthelmebs et al 2000b) and *Klebsiella oxytoca* (Hashidoko et al. 2001). PAD homologues are homodimers with a molecular weight of around 41–45 kDa (Huang et al. 1994; Degrassi et al. 1995; Cavin et al. 1998; Rodríguez et al. 2007) and are an inducible stress response to exposure to HCAs (Barthelmebs et al. 2000a). HCA substrates of bacterial PAD enzymes vary in a species- and strain-dependent manner. The amino acid sequences of these enzymes share around 64–84% similarity at the DNA level (Cavin et al. 1998; Barthelmebs et al. 2000a), but they do not have any sequence similarity to the PAD proteins in yeast (Cavin et al. 1998). In *Saccharomyces cerevisiae* and *Candida albicans*, there is a single copy of the *PAD1* gene (Clausen et al. 1994). The PAD enzyme has weak constitutive activity towards a number of HCAs.

An enzyme with decarboxylating activity towards HCAs has been partially characterised from *B. anomalus*, now classified as *D. anomala* (Edlin et al. 1998). This enzyme is constitutively expressed but its activity is induced in the presence of HCA substrates. The protein is a homodimer of approximately 39.8 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 pH 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 the molecular and biological functions are not yet known (*Saccharomyces* Genome Database, http://www.yeastgenome.org). *PST2* is involved in the response to oxidate stress and drug resistance (Karababa et al 2004). The ORF from *Saccharomyces* was cloned and expressed in *E. coli*, however, the protein 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 and beer production. Therefore, it is desirable to further characterise the enzymes involved in phenolic off-flavour production. Secondly, HCAs 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 control of *Dekkera* and *Brettanomyces* yeasts.

Materials and methods

Yeast strains

Dekkera yeasts used in this study were obtained from the Centraalbureau Voor Schimmelcultures (CBS), Netherlands and The Australian Wine Institute (AWRI; Table 1). Yeast were maintained on YPD agar [1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone, 2% (*w*/*v*) glucose and 2% (*w*/*v*) bacteriological agar] and stored at 4°C.

Cell extracts

Starter cultures (YPD broth, 50 ml) were grown from single colonies and used to inoculate 500 ml of YPD to 5×10^6 cells/ml. Cultures were grown with or without the addition of substrates and to a density of approximately 10^8 cells/ml. The cell pellet was then washed with 20 ml of 20 mM bis-Tris (pH 6) and resuspended in the same buffer then homogenised with zirconia/silica beads (0.5 mm, 10 min, MSK Braun Homogeniser). Beads and cellular debris were removed by centrifugation (10 min at 4,500×*g*) before clarification of the supernatant by ultra-centrifugation (30 min at 100,000×*g* and 4°C). The total protein concentration of extracts was determined using the modified Lowry method as per manufacturer's instructions (Pierce Biotechnology).

Enzymatic assays

All assays were performed in duplicate 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 40°C for 10 min and the reaction

Table 1 Yeast strains used during this investigation

Species	Strain
Dekkera anomala	CBS 77
Dekkera bruxellensis	CBS 2336; AWRI 1499
Brettanomyces naardenensis	CBS 6042
Brettanomyces nanus	CBS 1945
Brettanomyces custersianus	CBS 4805

was stopped by heating at 80°C for 10 min. Protein precipitate was removed by centrifugation (20,800×*g*, 1 min). The supernatant was analysed by high performance liquid chromatography (HPLC) in duplicate for a decrease in substrate concentration and/or product formation. A blank of 450 µl of buffer with 50 µl boiled extract was included and the mean of the obtained values subtracted from all assays. Activity was defined as units of activity per picomole of substrate removed per milligram of protein per second.

Purification of PAD enzyme

Purification was achieved using a modification of the protocol described by Edlin et al. (1998). Clarified cell extract (50 ml) obtained from a 5-l culture was loaded onto an anion exchange column (50 mm×10 mm; Q-sepharose resin, GE Healthcare), with a 12-ml column volume (CV). The column was equilibrated before loading with 10× CV buffer A (20 mM bis-Tris buffer pH 6, 0.01 mM DTT, 20% (*v*/*v*) glycerol) and washed after sample loading with 3× CV of buffer A 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 1 M NaCl. Fractions of 5 ml were collected using an automated fraction collector (BioRad) and protein elution was monitored by absorption at 280 nm. The protein concentration in each fraction was assayed using the modified Lowry method. Representative fractions were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) (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 sulphate 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× CV of buffer C (20 mM bis-Tris pH 6, 1.7 M $(NH_4)_2SO_4$) and washed after sample loading with 3× CV of buffer C 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. Active fractions were combined and analysed on a 12% SDS-PAGE gel (Fling and Gregerson 1986). A protein band was isolated and underwent trypsin digest sequencing at a commercial facility (Adelaide Proteomics Centre). Sequencing was achieved using a combination of ESI and MALDI MS/MS (Papayannopoulos 1995) and the results were analysed by MS-BLAST. Sequence alignment tools (Bioedit version 7.0.0) were used to align the *Dekkera* sequence to that of related protein sequences (EMBL-EBI tools, http://www.ebi.ac.uk/tools).

PAD enzyme stability

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

Survey of optimal conditions for enzymes involved in volatile phenol production

PAD activity was measured over a range of temperatures using the assay described previously, incubated at 30°C, 38°C, 39°C, 40°C, 41°C, 42°C, 45°C and 50°C. 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 40°C. 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 20–60°C at 10°C increments. A pH range between 3 and 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.

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.

HPLC analysis

HPLC analysis of assays was carried out in duplicate using the method described by Harris et al. (2008). In brief, samples were centrifuged at 10,000×g for 1 min and 200 µl of sample filtered through a 0.45-µm nylon filter before analysis. HPLC analysis was carried out on an Agilent 1100 series instrument fitted with a DAD detector. Samples

(5 μl) were injected onto a Phenomenex Synergi™ 4μ Hydro-RP 80A column (150 mm×2 mm). Peaks were separated over a 40-min gradient of 0–100% buffer B, where buffer A was 0.2% (*v*/*v*) phosphoric acid and buffer B was 80% acetronitrile with 20% buffer A. A flow rate of 0.2 ml/min was used and elution was monitored by absorbance at 254, 280 and 310 nm.

3′ RACE PCR

RNA was isolated from *D. anomala* CBS 77 using the RNeasy mini prep (Qiagen) as per manufacturer's instructions. cDNA was made from 2 μg of RNA, using superscript 3 reverse transcriptase (Invitrogen) as per manufacturer's instructions. Second strand synthesis was preformed with PFU DNA polymerase (Promega) using 2 μl of first strand reaction and 1 μl of 10 mM nucleotide mix (Roche). Oligo-dT (Invitrogen) was used to prime the reverse reaction. The sense primer (AAGGTCGCTATTA TYATCTAC) was designed from the back-translated N-terminal amino acid sequence (Edlin 1997). Reactions were preformed in an Eppendorf Mastercycler Gradient thermocycler for 35 cycles, denaturing at 95°C for 30 s, annealing at 54°C for 30 s, extending at 72°C for 30 s with a final 5-min 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 cleanup system (Promega) and 20 ng of the product was sequenced by the Australian Genome Research Facility (Brisbane) purified DNA sequencing service. Sequence analysis was preformed using the NCBI-BLAST 2 nucleotide tool, available from the EBI website (EMBL-EBI) and Bioedit (version 7.0.0).

Accession numbers

Sequences obtained from EMBL protein database for alignments *S. cerevisiae* accession number z74328 and *C. albicans* Q59y37.

Results

Storage of extract

The PAD activity in cell extracts from *D. anomala* and *D. bruxellensis* was examined for stability under different storage conditions to determine the optimum conditions for future protein investigations (Table 2). 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 between −80°C and 80°C. In general, the lower the storage temperature, the more enzyme activity was retained in the cell extracts, whilst the longer the extract was stored, the more activity was lost. When held at 40°C and above, the activity of the 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 (25°C) or with refrigeration (4°C) 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 −80°C all extracts retained between 71% and 86% of their original activity after 1 month.

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

Substrate specificity and induction of PAD enzyme activity

Dekkera and *Brettanomyces* cultures grown in YPD 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 HCAs (Fig. 1). No activity under any conditions was detected for any cell extracts

Table 2 Effect of storage on PAD activity over time

Storage (°C)	Percent 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

Assay were preformed in triplicate and analysed by HPLC in duplicate. Standard deviation for all values is below 5%

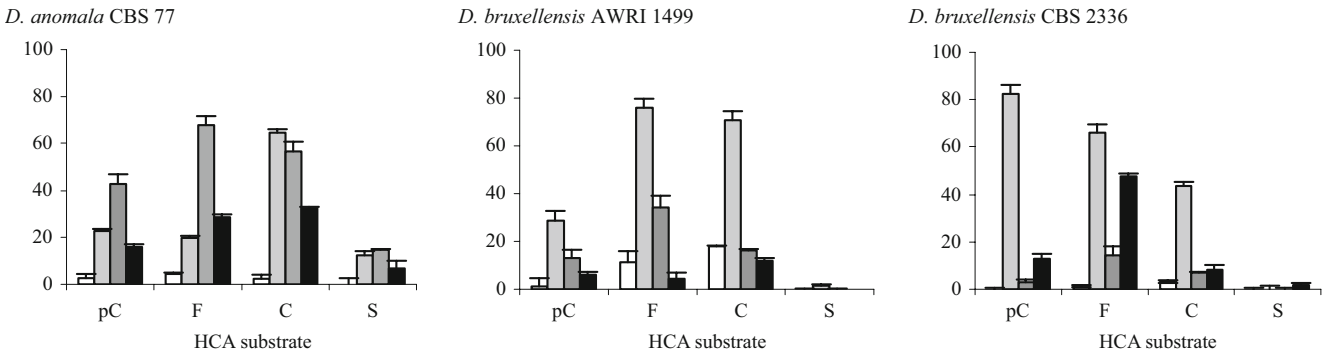

Fig. 1 Impact of preincubation of *Dekkera* cultures without *p*-coumaric acid (control, *white*) compared to grown with additions of *p*-coumaric acid (*grey*) ferulic acid (*dark grey*) and 4-vinylphenol (*black*) on the enzymatic utilisation of *p*-coumaric (*pC*) ferulic (*F*) caffeic (*C*) and sinapic (*S*) acids. Results are the mean of duplicate assays measured by HPLC in duplicate ± standard deviation, where 100% is equal to the initial assay concentration of 2mM

prepared from *Brettanomyces* species (data not shown). Preincubation in the presence of HCAs increased the activity of cell extracts from *D. anomala* and *D. bruxellensis*, and *p*-coumaric or ferulic acids induced activity by up to 64–80%, respectively (Fig. 1). The induction effect of ferulic acid was generally greatest for *D. anomala*, whilst *D. bruxellensis* induction was highest for *p*-coumaric acid.

D. anomala was the only yeast species investigated that was able to utilise sinapic acid (Fig. 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 whilst CBS 2336 removed over 80% when induced with *p*-coumaric acid; however, AWRI 1499 demonstrated higher utilisation of ferulic acid.

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 uninduced extracts, towards at least one vinyl compound (Fig. 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 of the order of 20% of the 4-vinylphenol but no 4-vinylguaiacol. *D.*

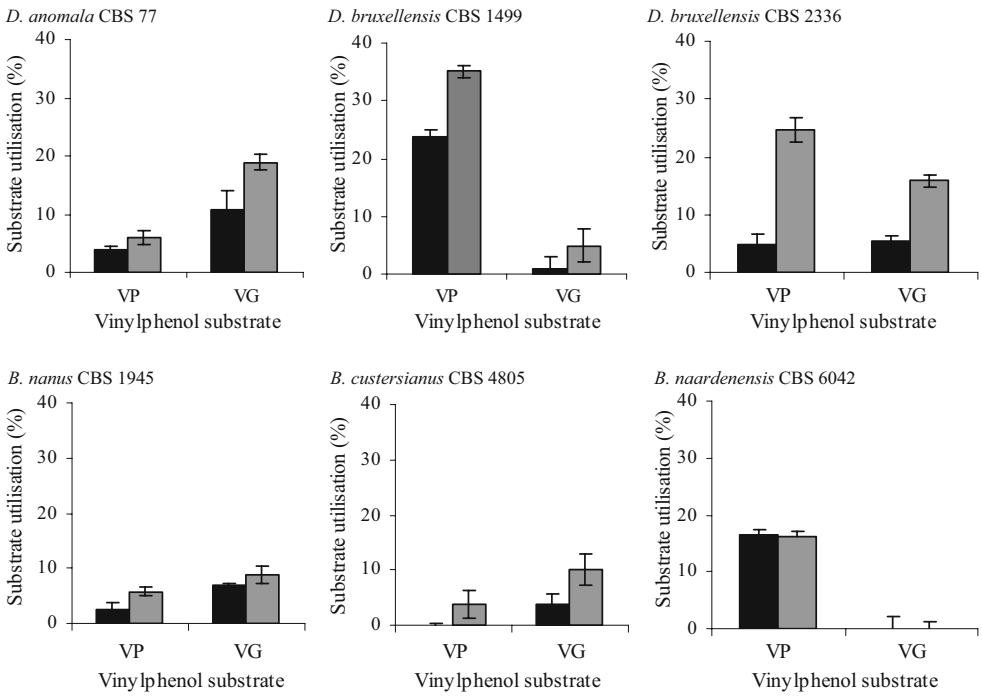

Fig. 2 Impact of preincubation with (*grey*) or without (control: *black*) 4-vinylphenol, on the utilisation of 4-vinylphenol (*VP*) or 4-vinylguiacol (*VG*) from assays, where 100% is equivalent to 1 mM initial concentration. Results are the means of duplicate assays measured by HPLC in duplicate ± standard deviation

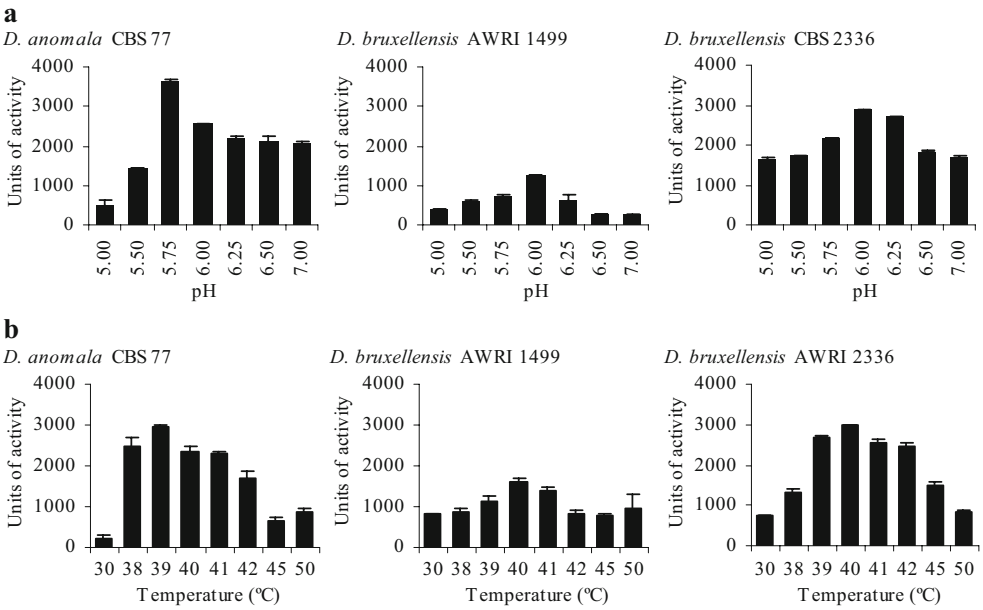

Fig. 3 Influence of pH (**a**) and temperature (**b**) on phenolic acid decarboxylase activity of *Dekkera* species. Units of activity are defined as picomole of *p*-coumaric acid removed from assay per milligram of protein in 1 s. Results are the means of duplicate assays analysed by HPLC in duplicate ± standard deviation

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. 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, whilst utilisation of both vinyl derivatives by *B. custersianus* increased 3-fold (Fig. 2). Extracts made from cultures induced with HCA did not influence the VPR activity of any yeast screened (data not shown).

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. 3a), whilst *D. anomala* had slightly lower pH optima of 5.75. Assays to ascertain the optimal temperature for PAD activity were conducted at temperatures ranging from 30°C to 50°C. The optimal temperature for the *D. bruxellensis* strains was 40°C and 39°C for *D. anomala* (Fig. 3b).

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 along with temperatures between 20°C and 70°C. The optimal pH for activity was pH 7 for all yeast strains except *D. anomala*, which had the highest activity at pH 6 (Fig. 4).

Temperature assays were performed at pH 6 and revealed minimal activity amongst any yeast at the extreme temperatures of 20°C and 70°C, but all yeast had an optimum of around 40–50°C (Fig. 5). *Dekkera* species in particular had activity over a large temperature range of 30–60°C.

PAD protein purification from *D. anomala*

In order to facilitate a more detailed characterisation, purification of the protein responsible for PAD activity in *D. anomala* was undertaken using the methods modified from Edlin et al. (1998). PAD protein was purified using FPLC from a crude cell extract made from 5 l of culture. Active fractions were combined and analysed by SDS-PAGE, with a protein band resolved at around 21 kDa. This protein band was isolated and sequenced. A partial sequence of 22 amino acid residues was obtained, YPLATTETLTAYDGVLFGVPTR (where L can be interchanged for I; Fig. 6).

The protein fragment obtained here aligned to the PST2 protein, from residues 60–81 of *Candida* and residues 58–79 of *Saccharomyces*, sharing 50% and 68% homology to *Candida* and *Saccharomyces,* respectively (Fig. 6). Additionally, the N-terminal sequence previously obtained from the same strain of *Dekkera* (Edlin 1997) was aligned with the PST2 sequences. This sequence aligns with around 50–56% homology to PST2. In comparison, the isolated protein only has around 20% homology to the *PAD1* gene from *S. cerevisiae*.

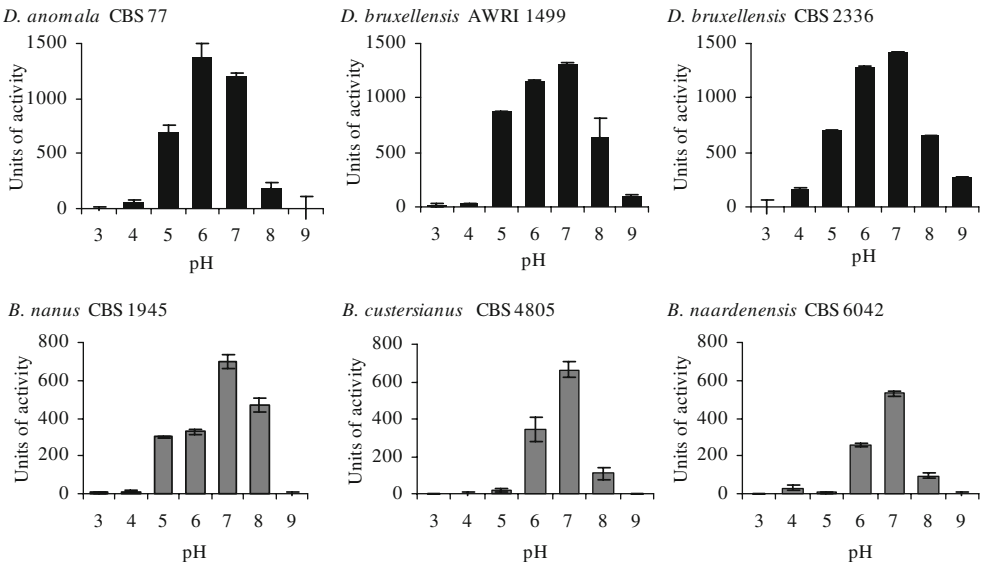

Fig. 4 Influence of pH on vinylphenol reductase activity of *Dekkera* and *Brettanomyces* species. Units of activity are defined as picomole of 4-vinylphenol removed from assay per milligram of protein in 1 s. Results are the means of duplicate assays analysed by HPLC in duplicate ± standard deviation

Sequencing of a 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 around 600 bp was isolated and sequenced. Sequence analysis was performed (BLASTN; EMBL) with the results indicating 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. 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.

Discussion

The metabolism of HCA acids by *Dekkera* can be important in sensory development of wine and beer. It is therefore vital to understand the metabolic processes that are involved in this flavour modification. This investigation

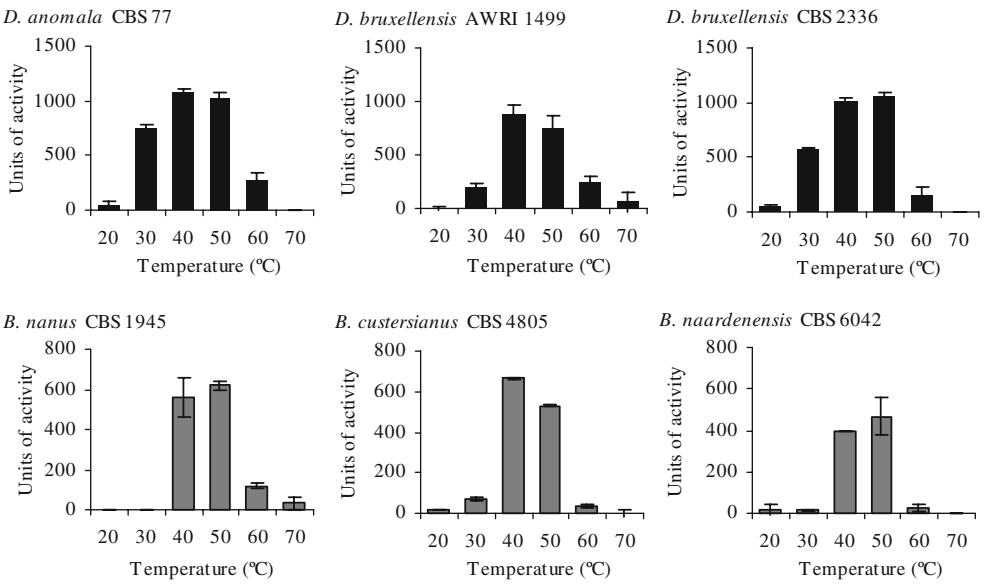

Fig. 5 Influence of temperature on vinylphenol reductase activity of *Dekkera* and *Brettanomyces* species. Units of activity are defined as picomole of 4-vinylphenol removed from assay per milligram of protein in 1 s. Results are the mean of duplicate assays analysed in duplicate by HPLC ± standard deviation

Fig. 6 Alignment of *Dekkera* protein sequence, obtained by tryptic digest during this investigation, to *PST2* sequences from *S. cerevisiae* and *C. albicans* (EMBL-EBI, Q12335 and Q59kv2)

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 alcoholic beverage production, and examines the closely related *Brettanomyces* genera.

The presence of *p*-coumaric or ferulic acid substrates in growth media augments the activity of *Dekkera* towards HCAs, 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 wines (Curtin et al. 2007), is most reactive to HCAs in growth media. The enzyme activity is greatly increased, and such induction could be due to an adaptation to growth in environments rich in the antimicrobial HCA compounds (Stead 1995; Hammond et al. 1999; Harris et al. 2008). *p*-Coumaric acid gave the greatest enzyme induction in both *D. bruxellensis* strains, which may explain why these yeasts are more resistant to *p*-coumaric acid than ferulic acid (Harris et al. 2008).

Previous research in this laboratory suggested that *Brettanomyces* species may be capable of utilising limited quantities of sinapic or caffeic acids (Harris et al. 2008). However, the production of vinyl and ethyl derivatives was not observed. The present study using crude cell extracts did not confirm the conversion of HCAs even when these

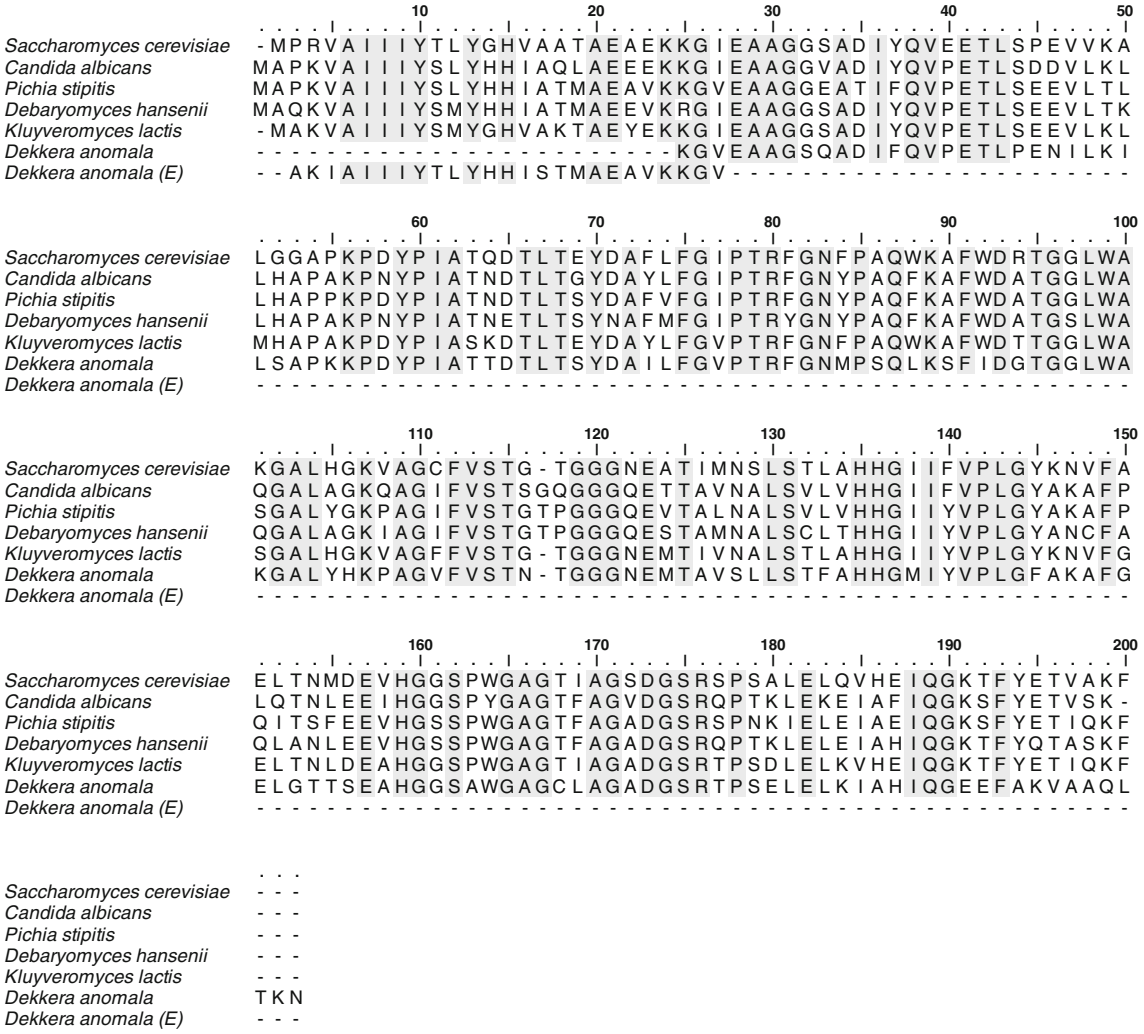

Fig. 7 Alignment of predicted protein sequence obtained by 3′ RACE from *D. anomala* CBS 77 to yeast proteins with high homology as determined using the NCBI-BLAST 2 protein program. Sequence for *Dekkera anomala* (E) is the previously reported N-terminal sequence from *D. anomala* CBS 77 (Edlin 1997)

activities were induced. Therefore, the *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 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 acids, confirming the 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 which occur. The activity of crude extract towards substrates does not reflect the results found in fermentation studies where *p*-coumaric was preferentially utilised (Harris et al. 2008). 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 optima 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 40°C. These parameters are similar to those determined for *Saccharomyces* and *Candida* yeast (Donaghy et al. 1999; Goodey and Tubb 1982).

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 *Dekkera* and *Brettanomyces* 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 h 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 (Harris et al. 2008) 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 HCAs 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) are 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′ rapid amplification of cDNA ends (RACE) with the N-terminal sequence obtained from the same yeast strain (Edlin 1997), an almost complete protein sequence has been determined (Fig. 7). Small differences in the sequences obtained by protein sequencing and 3′ RACE indicate that there may be at least two 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 indicates a 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. The translated protein sequence has a predicted flavodoxin mononucleotide binding site (InterProScan), indicating that 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 contain a flavin mononucleotide binding site. It is unlikely that PST2 from *Saccharomyces* or *Candida* is involved in HCA catabolism as PST2 is isolated from highly purified mitochondria and is induced by oxidative stress in a Yap1p-dependent manner (Karababa et al. 2004). However, PAD enzymes are induced in response to the weakly acidic antimicrobial HCAs that cause oxidative stress. Both PST2 and PAD are also involved in resistance to antimicrobial compounds (Clausen et al. 1994; Pardo et al. 2000). The protein with high homology to PST2 isolated from *Dekkera* has phenolic acid decarboxylase activity but the presence of conserved flavodoxin mononucleotide binding site and similar function may partially explain the observed homology to PST2.

In conclusion, this study 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 in both *Dekkera* and *Brettanomyces* species, and the results presented here confirm the presence of such an enzyme in these species.

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. It does however align 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.

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Chapter 4

Investigations into the use of ferulic acid as an antimicrobial against *Dekkera* yeast

Statement of authorship

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Investigations into the use of ferulic acid as an antimicrobial against *Dekkera* yeast

Abstract

Background and Aims: Hydroxycinnamic acids (HCAs) occur naturally in wine and are known to inhibit the growth of bacteria, fungi and yeast. This study sought to determine the potential of ferulic acid as an antimicrobial for the control of *Dekkera* yeast.

Methods and Results: The effect of ferulic acid additions on *Dekkera* growth was investigated under various parameters. *Dekkera* growth was inhibited by all HCAs, with ferulic acid being the most potent. Inhibition was enhanced by the presence of ethanol. When examined in a chemically defined medium, wine medium and wine, *Dekkera* yeasts were inhibited by successively lower concentrations of ferulic acid.

Conclusions: Ferulic acid was inhibitory to *Dekkera* yeast in various media, including a modified wine. Some wines may be naturally resilient to *Dekkera* spoilage due to the concentration of endogenous HCAs. The manipulation or addition of ferulic acid concentrations could be of industrial significance for the control of *Dekkera*. Further investigations are required to confirm this under typical winemaking conditions.

Significance of Study: This work highlights the potential of ferulic acid for controlling the growth of *Dekkera* yeast and suggests why some wines are less suscceptable to *Dekkera* spoilage.

Abbreviations:

HCAs Hydroxycinnamic Acids; **MIC** Minimum Inhibitory Concentration; **MIC_{90}** Minimum Inhibitory Concentration where 90% of growth is inhibited: **OD_{600}**, Optical

Density at 600 nm; **SEM,** Scanning Electron Microscopy; **w/v** weight/volume; **v/v** volume/volume

***Keywords*:** antimicrobial, *Brettanomyces*, spoilage, scanning electron microscopy

Introduction

Dekkera yeasts are often considered undesirable organisms leading to spoilage during wine production. They are recognised to impact the quality of other fermented beverages such as beer (Martens et al. 1997). *Dekkera bruxellensis* is of particular concern within the wine industry due to the variety and potency of negative impacts it imparts (van der Walt and van Kerken 1959, Grbin and Henschke 2000, Loureiro and Malfeito-Ferreira 2003, Silva et al. 2004). In addition, the yeast displays tolerance to low wine pH and high concentrations of ethanol and sulfur dioxide, which are usually sufficient to control other unwanted microorganisms (Silva et al. 2004, du Toit et al. 2005, Barata et al 2007). The majority of spoilage by *Dekkera* occurs after alcoholic fermentation, during delays before malolactic fermentation or upon ageing in oak barrels (Chatonnet et al. 1995, Rodrigues et al. 2001, Alexandre et al. 2004).

Strategies for limiting and preventing *Dekkera* contamination are important in controling spoilage of wines. Thus equipment that comes into contact with juice, musts and wine is ideally subjected to routine and rigorous sanitisation practices. Appropriate wine pH values and sulfur dioxide concentrations can also decrease the risk of *Dekkera* growth. Where these measures fail and the organism is detected, additions of sulfur dioxide can be made, however, they are not always successful (Barata et al. 2007). Concerns over the detrimental health effects of sulfur dioxide have led to reduced usage of this preservative by winemakers (García-Ruiz et al. 2008). The development of

alternate and ideally natural antimicrobials is therefore warranted to combat the potential for increasing susceptibility of wines to microbial spoilage.

Hydroxycinnamic acids (HCAs) are part of the phenolic composition of grapes and are considered natural food preservatives as they have antimicrobial activity (Smid and Gorris 1999, Ou and Kwok 2004). Ferulic acid (4-hydroxy-3-methoxycinnamic acid) has in fact been used to prevent spoilage of foods since 1975 (Ou and Kwok 2004). HCAs are known to have health giving properties. They are anti-inflammatory compounds that may decrease the risk of cancer, diabetes, cardiovascular and neurodegenerative diseases (Sakai et al. 1999, Ogiwara et al. 2002, Srinivasan et al. 2007). Given these purported health benefits and their antimicrobial properties against wine-related yeast and bacteria (Stead 1993, 1995, García-Ruiz et al. 2008), HCAs may have a future role in the control of *Dekkera*.

The total HCA content of wine primarily consists of caffeic, ferulic and *p*-coumaric acids. Caffeic acid has been reported at concentrations between 3.5–31 mg/L in red and white wines (Soleas et al. 1997, Kallithraka et al. 2006, Avar et al. 2007) while ferulic and *p*-coumaric acid concentrations are much lower, varying from undectable to 11 mg/L (Soleas et al. 1997, Kallithraka et al. 2006, Avar et al. 2007, Cabrita et al. 2008). These variations in part are due to the influence of viticultural parameters such as variety, berry size, ripeness, climate and geographical region impacting on synthesis and retention of HCAs during maturation (Nagel et al. 1979). However the presence of HCAs in wine is chiefly determined by the degree of extraction through skin contact (Oszmiansky et al. 1986, Macheix et al. 1990, Auw et al. 1996). Other factors such as malolactic fermentation, use of oak and storage conditions also alter the final HCA

concentration (Oszmiansky et al. 1986, Somers et al. 1987, Ibern-Gomez et al. 2001, Liu 2002, Matejicek et al. 2005, Morata et al. 2006, Recamales et al. 2006, Sartini et al. 2007).

HCAs have been reported to inhibit growth of a variety of organisms including plants, fungi and bacteria (Van Sumere et al. 1971, Ravn et al. 1989, Stead 1993, Campos et al. 2003, Walker et al. 2003). In particular a number of yeast species are inhibited by HCAs when present at upwards of 1 mM under laboratory conditions. Generally ferulic and *p*-coumaric acids are most inhibitory (Baranowski and Nagel 1980, Ravn et al. 1989, Edlin et al. 1995, Stead 1995, Walker et al. 2003, Harris et al. 2008).

Some microbes detoxify HCAs present in wine into vinylphenols, which at low concentrations smell smokey, sweet and fruity (Maga, 1978, Heresztyn 1986b, van Beek and Priest 2000), but at higher concentrations may have undesirable characteristics (Chatonnet et al. 1993). Vinylphenols can be further metabolised, predominantly by *Dekkera,* into ethylphenol derivatives. In this way *p*-coumaric acid is converted into 4-ethylphenol that has aromas reminiscent of Bandaid® and barnyards. Ferulic acid is metabolised into 4-ethylguaiacol which smells spicey and smokey (Chatonnet et al. 1992). Thus, while HCAs may act as precursors for ethylphenols, their use at fungicidal concentrations should eliminate the risk of this undesriable outcome.

This study expands on previous work (Harris et al. 2008; 2009) and examines a broader range of HCA concentrations and possible synergistic effects arising from combinations of HCAs, and other wine components. The objective of this work was to evaluate the extent of inhibition and the potential of ferulic acid for use as a novel antimicrobial

against *Dekkera.*

Methods

Yeast strains

Dekkera yeasts were obtained from the Centraalbureau Voor Schimmelcultures (CBS), The Australian Wine Research Institute (AWRI) or isolated directly from wine (Table 1). Wine strains were isolated on YPD agar (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) bacteriological agar) supplemented with 0.01% (w/v) cycloheximide. To confirm the identity of isolates, genomic DNA preparations were made (Adams et al. 1997) and the internal transcribed spacer (ITS) region was amplified by PCR (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 (NCIB-BLAST nucleotide; EMBL-EBI). All yeasts were routinely maintained on YPD agar stored at 4ºC.

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

Starter cultures were prepared from a single loop of freshly grown *Dekkera* (YPD plates) inoculated into 25 mL of YPD broth and grown at 30ºC with agitation (180 RPM) to a density of 1 x 10^8 cells/mL. Fifty mL of CDM in a 250 mL Erlenmeyer flask, supplemented with 0–2 mM ferulic acid, pH 3.8 (Harris et al. 2008) and inoculated with 5 x 10^6 cells/mL. Cultures were prepared in triplicate (unless otherwise stated) and flasks were incubated at 30ºC with lids fitted. 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

Table 1. Yeast strains used during this investigation

Species	**Strain**	**Original source**
Dekkera anomala	CBS 76	Beer
	CBS 77	Stout
	CBS 8139	Soft drink
Dekkera bruxellensis	CBS 2336	Wine
	AWRI 1499	Wine
	VH01†	Wine
	VH04†	Wine
	VH05†	Wine

†*Dekkera bruxellensis* strains isolated from a wine as part of this study

concentrations of up to 10% (v/v). Treatments included ferulic acid at a concentration of 0.5 mM. Culture growth was determined by optical density measurements at 600 nm (OD_{600}).

Minimum inhibitory concentrations in CDM, wine medium and wine

The minimum concentration at which HCA(s) addition significantly inhibited the growth of the *Dekkera* culture (i.e. the MIC) relative to a control with no addition was determined in triplicate using 10 mL of medium supplemented with HCA(s). Supplementation rates for CDM and wine medium (WM; Harris et al. 2008) totalled between 0–20 mM, comprising ferulic, *p*-coumaric or caffeic acids, singularly or in combination. For wine, a maximum supplementation of 8 mM ferulic acid was used.

An investigation into effects of ferulic acid addition on *Dekkera* growth was also performed in a modified red wine using the method identical to that used to obtain the other MIC values. Although the concentration of endogenous ferulic acid and other HCAs was not determined, the unsupplemented control wines contained the same basal amount of HCAs. The wine (Shiraz, 12.8% (v/v) ethanol, < 2 g/L residual sugar, pH 3.4) was centrifuged for 1 hour at 10,000 x *g,* filter sterilised (0.22 μm) and then adjusted to 10 g/L sugar (equimolar glucose and fructose), 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.

All media were inoculated with starter culture (prepared as detailed above) to 5 x 10^6 cells/mL and incubated at 30ºC. Initial OD_{600} measurements were recorded and used as a blank for subsequent measurements. Cultures in CDM, WM and wine were measured

after 1, 2 or 4 weeks, respectively. Growth was estimated by OD_{600} and results expressed as a percentage of the appropriate control (no HCA(s) added). The MIC was defined as the supplemented concentration of HCA(s) which resulted in a significant difference in growth as determined by Student's t-test with a probability value of $p < 0.05$. Further, the concentration of HCA supplemented to cultures, which displayed a 90% reduction in OD_{600} relative to the control was defined as the MIC_{90}. To establish the condition of cells so affected, an aliquot (1 mL) from the MIC_{90} cultures was then washed and re-inoculated into fresh CDM medium (9 mL). A control was also washed and inoculated for comparison. After two weeks OD_{600} was measured, with detectable growth being considered of indicative that the MIC_{90} was inhibitory to growth, whereas no growth suggested cells were killed or viable but non-culturable.

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

Cells of *D. bruxellensis* (AWRI 1499) and *D. anomala* (CBS 77) were grown (3 days, 30ºC) in CDM with the presence of ferulic acid (0, 0.5, 1 or 2 mM). *Dekkera anomala* (CBS 77) was also pre-grown in YPD before transfer and incubation for 16 hours at 37°C in PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.2) containing ferulic acid at up to 20 mM. All cells were harvested and washed in PBS buffer and then resuspended in PBS. One mL of each suspension was filtered through a 0.22 μm membrane and the harvested cells fixed to the membrane with aldehyde solution (4% (v/v) paraformaldehyde, 1.25% (v/v) glutaraldehyde and 4% (w/v) sucrose in PBS, pH 7.2) for 20 minutes. Membranes were washed in buffer (4% (w/v) sucrose in PBS) for 5 minutes before post-fixing in 2% (w/v) OsO_4 for 30 minutes. Cells were dried using a series of ethanol solutions (70%, 90%, 95% and 100% (v/v); twice for 10 minutes in each) before using a critical point dryer with liquid CO_2 at 95 bar pressure.

Filters were then mounted and coated with platinum (Adelaide Microscopy, South Australia) and examined using a scanning electron microscope (Philips XL20SEM).

Results

Growth of Dekkera *in the presence of ferulic acid*

Dekkera anomala and *D. bruxellensis* yeasts were grown in CDM with the presence of varying concentrations of ferulic acid (Figure 1). All strains examined were susceptible to inhibition by ferulic acid. In general, *D. bruxellensis* was more susceptible than *D. anomala*, with the growth of CBS 2336 and AWRI 1499 inhibited at a concentration of 2 mM. The *D. bruxellensis* strains isolated from wine (VH01, VH04, VH05) exhibited a longer lag phase (approx. 80 hr) compared to the other strains (up to 48 hr), even in the absence of ferulic acid. Further, wine isolates were less susceptible to higher ferulic acid concentrations compared with the culture collection isolates (Figure 1). The three wine isolates grew poorly in CDM with 2 mM ferulic acid, whereas, neither AWRI 1499 nor CBS 2336 grew.

Dekkera anomala (CBS 77) was sensitive to ferulic acid, its lag phase was four times that of the control and growth was reduced in the presence of 2 mM. The *D. anomala* strains CBS 76 and CBS 8139 were also inhibited by concentrations above 1 mM, although 2 mM of ferulic acid only increased the lag phase by 24 hours (Figure 1). In addition to the increased duration of culture growth for both *D. anomala* and *D. bruxellensis*, increasing concentrations of ferulic acid typically reduced the maximum OD_{600} achieved by the culture. Ferulic acid concentrations as low as 0.5 mM were sufficient for some strains (*D. bruxellensis* AWRI 1499 and VH05), to decrease their final culture density by approximately 25% relative to the control.

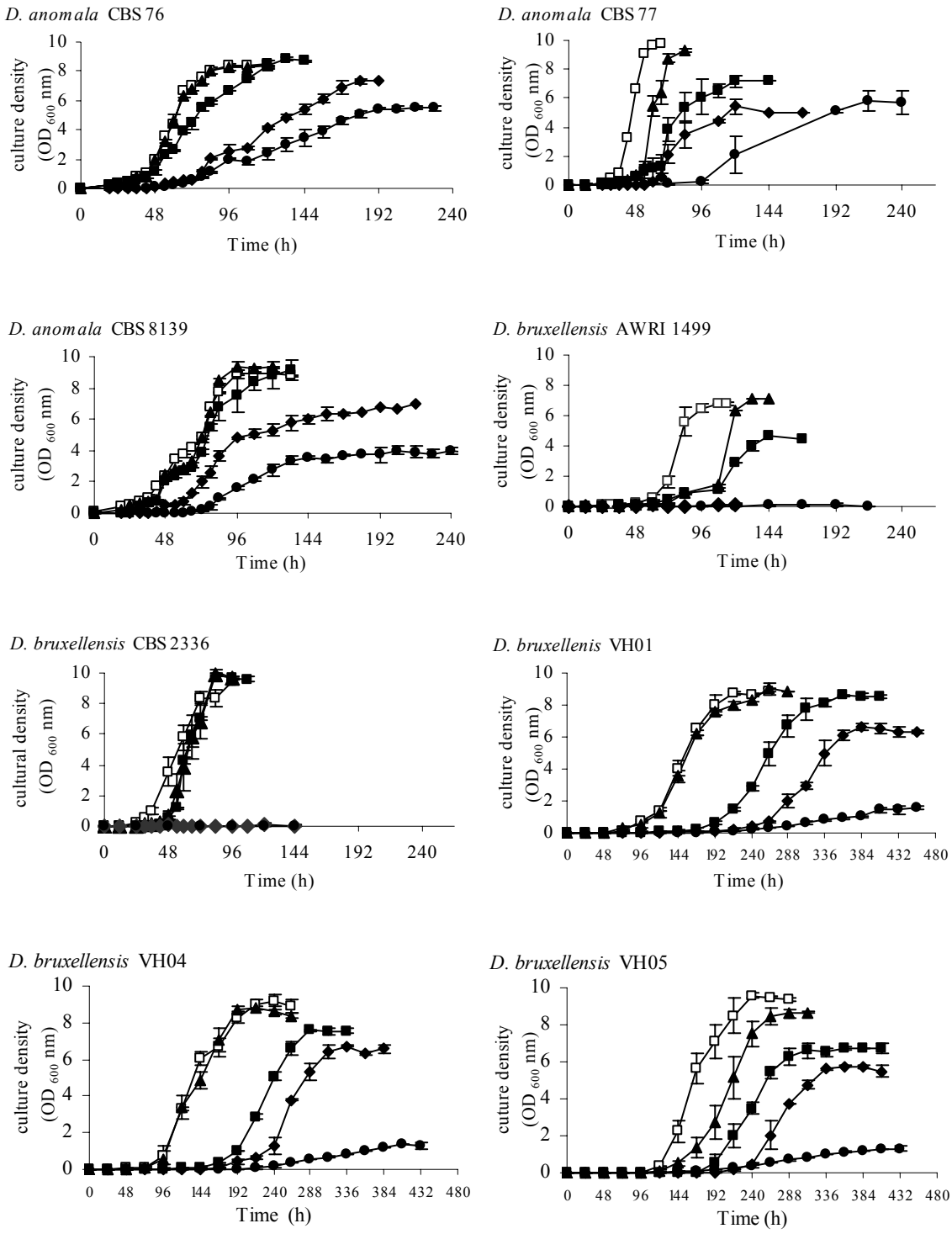

Figure 1. *Dekkera* growth in CDM in the absence (empty square) or presence of ferulic acid; 0.25 mM (triangle) 0.5 mM (filled square) 1 mM (diamond) and 2 mM (circle) estimated by optical density (OD_{600} nm). Yeast strains CBS 76, 77, 8138, 2336 and AWRI 1499 were monitored in duplicate over 240 hours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

The effect of ethanol, pH and ferulic acid on Dekkera *growth*

The growth of *Dekkera* yeast in CDM, in the presence of ethanol, alone or together with ferulic acid, was examined. In the absence of ferulic acid *D. bruxellensis* and *D. anomala* had a reduced growth rate when ethanol concentrations were above 5 and 7% (v/v), respectively (Figure 2). Additions of 10% ethanol delayed *D. anomala* growth for 14 days and reduced culture optical density by approximately a third. *Dekkera bruxellensis* (CBS 2336) showed limited growth in 5% ethanol and was inhibited by 7% ethanol. Ethanol in combination with 0.5 mM ferulic acid inhibited both species to a greater extent than ethanol alone, but of the two, *D. bruxellensis* was most susceptible. Furthermore, increasing ethanol concentrations enhanced this synergistic effect. The combined action of ethanol and ferulic acid resulted in stationary phase being delayed and maximum culture densities being reduced to a greater extent than with ethanol or ferulic acid additions alone (Figure 2).

Dekkera bruxellensis (CBS 2336) did not grow in CDM at a pH of 3, even in the absence of ferulic acid (Figure 3), whereas at pH values of 3.8, 4 and 5, cultures grew readily. The pH of the medium had little effect on the growth of *D. anomala* (CBS 77). The addition of 0.5 mM ferulic acid to the medium had no significant impact on growth of either strain with a decrease in cell density of only 5% being observed in cultures grown at pH 4 to 5 (Figure 3).

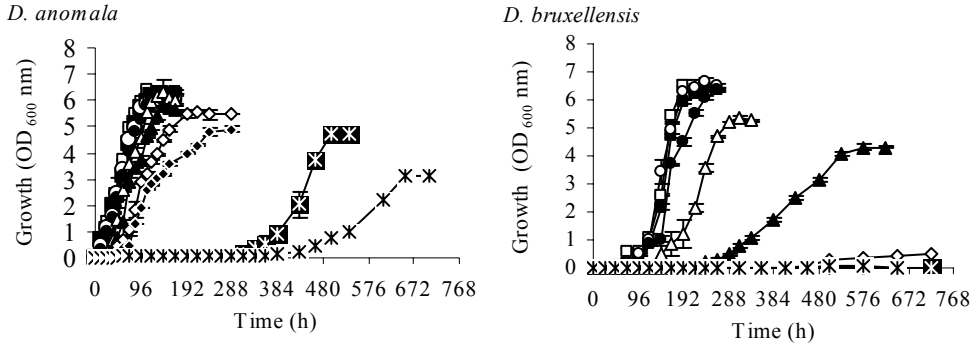

Figure 2. Growth of *Dekkera anomala* (CBS 77) and *Dekkera bruxellensis* (AWRI 1499) with (solid) or without (empty) 0.5 mM ferulic acid in media with 0% (square), 2% (circle), 5% (triangle), 7% (diamond) 10% (asterisk) ethanol. Growth was measured by optical density at OD_{600} nm ± standard deviation

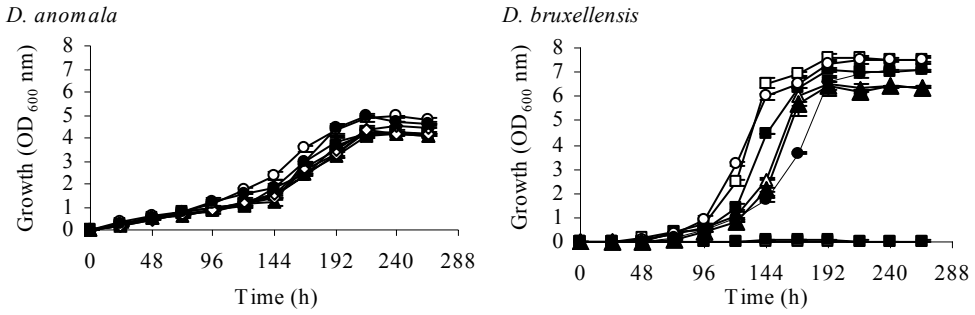

Figure 3. Growth of *Dekkera anomala* (CBS 77) and *Dekkera bruxellensis* (AWRI 1499) with (solid) and without (empty) 0.5 mM ferulic acid in CDM at pH 3 (diamond) pH 4 (square) pH 5 (circle) control pH 3.8 (triangle), growth was measured in duplicate by optical density at OD_{600} nm ± SD

Inhibitory activities of ferulic acid

The minimum concentration at which ferulic acid inhibited growth (MIC) of *Dekkera* yeasts was examined in CDM, WM and modified wine. Differences between the yeast were observed, however, a maximum of 2 mM was required to inhibit *Dekkera* with a MIC_{90} of 8 mM in CDM (Table 2). Cultures for which MIC_{90} values were observed (Table 2), were reinoculated into fresh media with no HCA additions. Neither *Dekkera* strains incubated with 8 mM ferulic acid or above and reinoculated as described had detectable growth under these conditions (data not shown).

The inhibitory effect of ferulic acid was enhanced in WM and modified red wine. In WM *D. bruxellensis* (AWRI 1499) growth was inhibited by over 90% by 1 mM. Further, cultures initially incubated with 2 mM and over failed to produce a detectable increase in culture optical density when re-inoculated into unsupplemented CDM (data not shown). In modified red wine *D. anomala* (CBS 77) was inhibited by 0.75 mM and *D. bruxellensis* (AWRI 1499) by 0.1 mM ferulic acid; a ten fold reduction to that required in CDM to achieve the same result (Table 2). When cultured with either 1 and 2 mM ferulic acid and then cells washed and reincoculated into unsupplemented CDM, no change in OD_{600} was observed after 2 weeks.

Determination of MIC values for combinations of common wine HCAs

To more closely reflect the typical HCA makeup of the wine environment, combinations of HCAs were investigated. Initially MIC values were determined for single additions of *p*-coumaric and caffeic acids. *Dekkera* yeasts were less sensitive to *p*-coumaric acid or caffeic acids than ferulic acid (Table 2). Growth of *Dekkera* tended to be stimulated by low concentrations of some HCAs (data not shown). *Dekkera*

Table 2. [illegible] *Dekkera* [illegible]

Dekkera st[illegible]		[illegible]	[illegible]	[illegible]	*p* [illegible]	[illegible]	[illegible] *p* [illegible]	[illegible]	[illegible] *p* [illegible]
D. anomala [illegible]	[illegible]	[illegible]	[illegible]	0.[illegible]5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible] $_0$	[illegible]	[illegible]	[illegible]	[illegible]0	[illegible]0	[illegible]	[illegible]	[illegible]
D. bruxellensis [illegible]	[illegible]	[illegible]	[illegible]	0.[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible] $_0$	[illegible]	[illegible]	0.[illegible]5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]es[illegible]ts p[illegible]ese[illegible]te[illegible] t[illegible]e [illegible]) [illegible]pe[illegible]ts pe[illegible] [illegible]te. [illegible]ts, t[illegible]e st[illegible] [illegible]s [illegible]ess t[illegible]. [illegible] *p* [illegible]. [illegible] t test, p≤ 0.05), [illegible] $_0$ [illegible]0[illegible] [illegible]0 [illegible]

bruxellensis (AWRI 1499) had a MIC value of 2 mM and a MIC_{90} of 8 mM for either compound (Table 2). Growth of *D. anomala* (CBS 77) was inhibited by 4 mM *p*-coumaric or 8 mM caffeic acid. Growth was detected in all cultures of *D. anomala* with caffeic acid when re-inoculated into fresh media. In comparison, subsequent growth of *D. bruxellensis* (AWRI 1499) from MIC_{90} cultures with *p*-coumaric or caffeic acids could not be detected.

The MIC for combinations of HCAs was below the MIC determined for *p*-coumaric and caffeic acids alone (Table 2). A combined total HCA concentration of 2 mM achieved the same inhibition for *D. anomala* (CBS 77) as the MIC of 4 or 8 mM for *p*-coumaric or caffeic acids individually. This represented a 2–4 fold reduction in total HCA concentration. Where all three acids were used in combination, a total MIC of 3 mM was observed for both *D. anomala* and *D. bruxellensis* strains (Table 2).

SEM observations of Dekkera *treated with ferulic acid*

Given that the addition of ferulic acid to culture media inhibited the growth of *Dekkera*, the effect of ferulic acid on cell morphology was investigated using scanning electron microscopy. Experiments were carried out with *D. anomala* (CBS 77) and *D. bruxellensis* (AWRI 1499). Electronmicrographs of *D. bruxellensis* (AWRI 1499) grown in the presence of 0.5 mM and 1 mM ferulic acid showed morphological differences compared to the control culture. Thus cells of the former appeared wrinkled and misshapen although budding was still evident (Figure 4A). *Dekkera anomala* grown in CDM without ferulic acid showed cell sizes ranging from 2 μm to greater than 20 μm in length (Figure 4C). Unlike the *D. bruxellensis* strain, cells of *D. anomala* grown with 0.5 mM of ferulic acid showed little wrinkling (Figure 4B), however, at 0.5 mM and

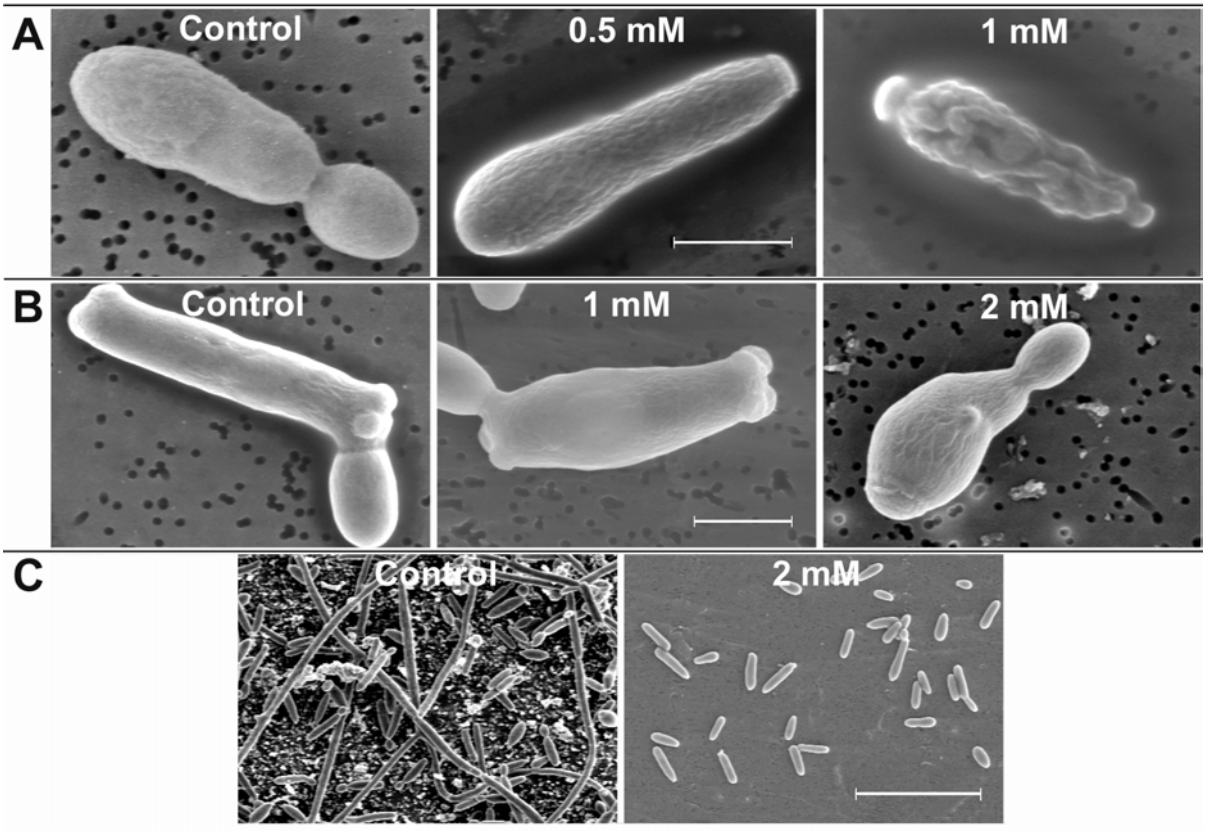

Figure 4. Scanning electronmicrographs of *Dekkera bruxellensis* AWRI 1499 (A) and *D. anomala* CBS 77 (B and C). Panel A; cultures were grown in CDM with additions of (from left to right) 0 mM, 0.5 mM and 1 mM ferulic acid, the scale bar is 2 μm. Panel B; cultures were grown in CDM with additions of (from left to right) 0 mM, 0.5mM and 2 mM ferulic acid, the scale bar is 2 μm Panel C; illustrates pseudohyphae formed by CBS 77 untreated cells compared to cells treated with 2 mM ferulic acid. The scale bar is 20 μm.

above, the majority of cells were reduced in size. Few were greater than 10 μm in length while the highly extended pseudohyphal growth observed in the control was also absent in the presence of ferulic acid (Figure 4C).

D. anomala (CBS 77) was also cultured in YPD with no addition of ferulic acid, then washed and incubated overnight in PBS buffer containing 0, 2 or 20 mM ferulic acid (Figure 5). Cells suspended in PBS containing no or 2 mM ferulic acid, closely resembled cells actually grown in the identical concentrations of ferulic acid (Figure 4A). Cultures incubated with 20 mM of ferulic acid were highly wrinkled (Figure 5), with a larger proportion of cells that appeared ruptured or damaged. Additionally there were indications that budding was affected, as new cell buds appeared deformed (Figure 5, arrow).

Discussion

Dekkera sp. play a role in the spoilage of wine and current control strategies are limited and often inadequate. This study examined the addition of phenolic grape components as a possible method to suppress *Dekkera* growth. Results presented here confirm previous reports that ferulic acid decreases the growth of *Dekkera* spp. (Edlin et al. 1995, Stead 1995, Harris et al. 2008) and indicates differences between *Dekkera* strains in resistance to ferulic acid. *Dekkera* can detoxify low concentrations of ferulic acid into 4-vinylguaiacol and 4-ethylguaiacol, which have aromas such as smoky, spicy, rum, roasted peanut (Chatonnet et al. 1992, Maga 1978). The aim of this work was to determine at which concentration *Dekkera* succumbed to the ferulic acid toxicity, preventing growth and therefore eliminating the risk of spoilage.

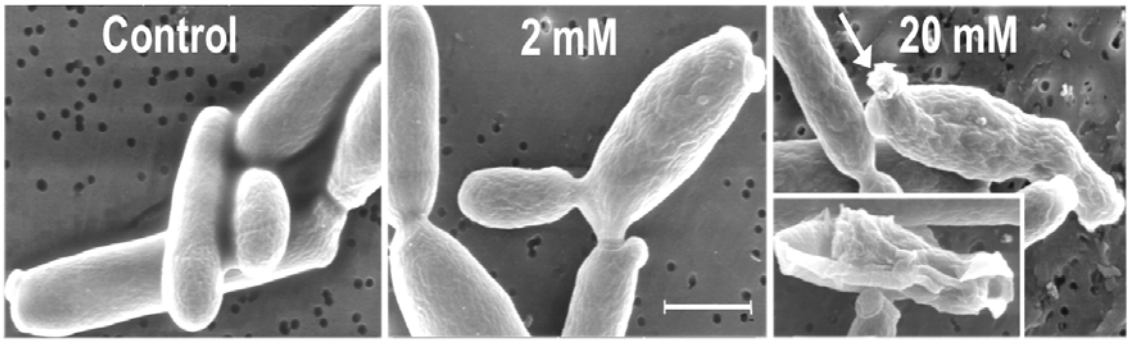

Figure 5. Scanning electronmicrographs of *Dekkera anomala* incubated overnight in PBS buffer with 0, 2 and 20 mM ferulic acid. The scale bar is 2 μm. The electronmicrograph of the 20 mM treatment has an insert showing a ruptured cell and the arrow indicates a budding cell, which appears to be misshapen.

Concentrations as low as 0.25 mM were shown to decrease culture density and increase lag times for growth of a range of species and strains. Concentrations of 2 mM ferulic acid strongly inhibited all *D. bruxellensis* strains. Wine isolates exhibited greater tolerance to ferulic acid, which may suggest that they were better adapted to this inhibitor, due to their isolation from or their relatively recent exposure to the wine environment which naturally contains ferulic acid. *Dekkera anomala* was shown to be more resistant to ferulic acid as previously reported (Harris et al. 2008). This increased survival is probably linked to this strain's greater ability to metabolise ferulic acid (Harris et al. 2008), however, concentrations above 8 mM completely inhibited growth and *D. anomala* are not considered to play an important role in wine spoilage. Studies on other microorganisms also found variations in the sensitivity to HCAs, where the inhibition depended on the organism and HCA under investigation (Van Sumere et al. 1971, Stead 1993, Campos et al. 2003).

Inhibition of *Dekkera* by ferulic acid was investigated in combination with a range of ethanol concentrations and pH values. Increasing ethanol concentrations suppressed *Dekkera* growth and this effect was augmented by the addition of ferulic acid. This suggests that at higher ethanol concentrations, less ferulic acid is required for a comparable effect on *Dekkera* growth. This finding has direct implications for the exploitation of ferulic acid as an antimicrobial during alcoholic beverage production. Many spoilage organisms can not grow in high concentrations of ethanol (Kalathenos et al. 1995), however, *D. bruxellensis* can grow in ethanol concentrations higher than 10% (Silva et al. 2004). An antimicrobial which acts in synergy with ethanol at such concentrations could offer distinct advantages in winemaking.

Previous studies on bacteria such as *Listeria, Escherichia coli, Staphylococcus* and *Bacillus* (Herald and Davidson 1983, Wen et al. 2003), found that reducing the pH of the growth medium from approximately 7 to 4.5 markedly increased the inhibitory properties of HCAs. In this study pH values closer to that of wine (pH 3–5) were examined. Where growth was observed, the inhibitory effect of ferulic acid was not significantly increased at lower pH values. That no significant effect of pH was observed may be due to the differences in the organisms and pH range examined.

Determination of MIC values for *D. anomala* (CBS 77) and *D. bruxellensis* (AWRI 1499) was made for three HCAs. Caffeic and *p*-coumaric acids were not as inhibitory towards *Dekkera* as ferulic acid. Inhibitory values recorded are determined by a decrease in turbidity from that of the control. MIC was calculated to be the concentration at which the optical density of the treated culture was significantly decreased from that of the untreated control. When treated cultures were recorded as having a MIC_{90}, i.e. an OD_{600} reduced by over 90% of that of the control, they were re-inoculated into fresh medium with no HCA additions in order to determine the condition of the remaining cells. Some of these cultures grew to detectable densities, indicating that they were merely inhibited. However at higher concentrations of HCAs no such growth was detected, thereby indicating possible death or non-culturability of cells exposed in this way. One limitation of the methods used here is the sensitivity of the techniques and the possibility that growth may occur at concentrations below that of the MIC and yet remain undetectable (Lopez-Malo Vigil et al. 2005). Future work should focus on determining whether ferulic acid at these concentrations is in fact fatal to *Dekkera* spp. For several other yeast including *Pichia* and *Saccharomyces,* ferulic acid is also a potent inhibitor (Baranowski et al. 1980, Stead 1980), but in the case of

the lactic acid bacteria, *Oenococcus oeni* and *Lactobacillus hilgardii, p*-coumaric and caffeic are more effective (Reguant et al. 2000, Campos et al. 2002).

Inhibition of *Dekkera* by ferulic acid has been investigated in various media, including a modified red wine. In general, *D. bruxellensis* was more sensitive to ferulic acid than *D. anomala* although only 1 strain of each was examined and a comprehensive survey is still required. In CDM *D. anomala* (CBS 77) required twice the concentration of ferulic acid as *D. bruxellensis* (AWRI 1499) to achieve a MIC_{90}. When cultured in modified red wine, as little as 0.1 mM (≈ 20 mg/L) of added ferulic acid significantly inhibited growth, while an MIC_{90} of 1 mM (≈ 200 mg/L) was determined for *D. bruxellensis* (AWRI 1499). Inhibition occurred in the modified wine at a lower concentration of ferulic acid than that of CDM and WM. This data illustrates that ferulic acid is in fact more effective in preventing growth of *Dekkera* under more wine-like conditions. The basis for the improved effectiveness of ferulic acid in wine may relate to the presence of naturally occurring ferulic acid, or else be a result of synergistic effects with other wine compositional parameters. Such parameters may include, ethanol, nutrients, SO_2 and of course the presence of other naturally occurring HCAs.

Surveys that quantify the phenolic content of wines are limited but indicate that individual HCAs can vary in concentration from undetectable to over 30 mg/L (Soleas et al. 1997, Kallithraka et al. 2006, Avar et al. 2007). The maximum ferulic acid concentration reported in wines is approximately 11 mg/L (Soleas et al. 1997) and is found to vary due to a range of viticultural and oenological factors (Oszmianski et al. 1986, Somers et al. 1987, Macheix et al. 1990, Auw et al. 1996, Morata et al. 2006). It is therefore feasible that an inhibitory concentration of >20 mg/L could be readily

achieved in wines either naturally or through supplementation. Additionally ferulic acid is not the sole HCA in wine and this report has demonstrated that combinations of HCAs also affect growth. The industrial potential of this antimicrobial should therefore be investigated further under more wine-related conditions as should the possibility that differences in the propensity of wines to become contaminated by *Dekkera* are, at least in part, due to their natural HCA content.

This is the first known investigation that demonstrates morphological changes caused by the action of ferulic acid on *Dekkera* cells. The morphology of the strain *D. bruxellensis* (AWRI 1499) was clearly and progressively altered with increasing concentrations of ferulic acid (Figure 4A), providing further evidence that this species is more sensitive to this HCA. Growth experiments and MIC determinations in CDM indicate that 2 mM ferulic acid inhibited growth but was not fungicidal to *D. anomala* cultures. The SEM images reflect these findings; while there are some morphological alterations, budding is still occurring (Figure 4B, 4C). Bennis et al. (2004) used a similar approach utilising SEM to investigate yeast cell surface alteration in response to simple phenolic compounds.

Attempts to induce morphological alterations in cells involved the addition of a high concentration of ferulic acid to *D. anomala* in PBS buffer. A concentration of 2 mM caused some modifications to *D. anomala* but at 20 mM cells were obviously altered, in that they appeared wrinkled and misshapen, and buds were deformed (Figure 5). This may suggest that modifications to *Dekkera* cells induced by ferulic acid affect both the cell membrane and vegetative growth. As yet it is unclear whether these alterations are fatal.

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 including 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 compounds are similar to ferulic acid and also cause modification and damage to the cell membrane, possibly during the cellular proliferation phase (Bennis et al. 2004). Further studies are required to determine the precise mechanism(s) by which ferulic acid adversely affects the cell.

The use of ferulic acid alone or in combination with other HCAs in wine may have a number of benefits over existing options for controlling *Dekkera* spoilage. Foremost is better antimicrobial control using reduced sulfur dioxide additions along with the possible health benefits associated with ferulic acid. Further, while more comprehensive analysis is required, informal sensory analysis revealed that at least no aromatic changes were detected in wine to which ferulic acid had been added at an antimicrobial concentration (data not shown).

These investigations although not utilising strict wine phyiso-chemical conditions show that ferulic acid has potential for use as an antimicrobial. Further research is required within an industrial setting to ascertain the effectiveness of using ferulic acid as an antimicrobial and its effect on wine quality. Ferulic acid, a natural component of grapes, is known to be metabolised by wine yeast into undesirable compounds, however, if the

yeast are inhibited and/or killed then no production of these off-flavour compounds will occur to cause spoilage. It is therefore vital to survey a large number of *Dekkera* wine isolates in order to ascertain at which concentration growth is completely suppressed, along with other impacts on wine quality before the use of ferulic acid as an antimicrobial additive to wine can be confirmed. The application of ferulic acid as an antimicrobial against other spoilage organisms and in non-wine matrices also warrants further investigation.

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Chapter 5

Discussion

5.1 Introduction

For over a century *Brettanomyces* yeast has been known to have organoleptic effects on alcoholic beverages (Claussen 1904). *Brettanomyces* has been isolated from other several sources where its role is less well defined. The related *Dekkera* spp. are frequently associated with spoilage of foods and beverages, most notably in red wines. While within the wine industry *D. bruxellensis* is of greatest concern (van der Walt and van Kerken 1959; Grbin and Henschke 2000; Loureiro and Malfeito-Ferreira 2003; Silva *et al.,* 2004). Spoilage characteristics of *D. bruxellensis* include, mousy-off-flavour, volatile acidity, haze and the production of volatile phenols (van der Walt and van Kerken 1959; Snowdon *et al.,* 2006; Grbin *et al.,* 2007). Many of the chemical compounds responsible have been identified and the biochemical pathways for their formation elucidated, nevertheless there remains significant gaps in knowledge. The pathway by which volatile phenols are biosynthesised consists of two enzymes (Chatonnet *et al.,* 1992; Edlin *et al.,* 1995). Prior to this study only one enzyme had been isolated and characterised from a single *Dekkera* strain (Edlin *et al.,* 1998). Existence of the second enzyme had only been hypothesised (Chatonnet *et al.,* 1992). There was also a dearth of information concerning the ability of the other four related species to produce VP. The aims of this research were to investigate volatile phenol metabolism and through this, to develop approaches to control or eliminate spoilage by these insidious yeasts.

Growth of yeasts in the presence of common grape HCAs along with some metabolic derivatives was investigated. The removal of these compounds and subsequent accumulation of products in the growth medium were determined for the five related species. Additionally the catabolic enzymes that produce VPs and EPs were

characterised resulting in the determination of a near complete sequence for the first of these, the HCD enzyme. Interestingly, physiological and biochemical investigations established that HCAs are toxic to *Dekkera* cells. This knowledge could in the future lead to the modification of grape growing and winemaking practices in order to modulate the phenolic content of juice and wine. In this way, infections might be controlled and the reliance on harsh antimicrobials reduced. For producers of organic or biodynamic wines or for those wishing to reduce chemical additives in response to growing consumer preferences, this would have significant importance.

5.2 Physiological investigations

The metabolism of *p*-coumaric acid by *Dekkera* cultures over time has been studied (Chatonnet *et al.,* 1992; Edlin *et al.,* 1995; Dias *et al.,* 2003b), however, *Brettanomyces* spp. have not been scrutinised in this manner. Breakdown of ferulic, caffeic and sinapic acids by either *Dekkera* or *Brettanomyces* spp. has thus far been ignored. Addressing these issues was therefore a priority.

Dekkera strains investigated effectively utilise *p*-coumaric and ferulic acids, and to a lesser extent, caffeic acid. One key departure from previously published studies was that *Dekkera* strains examined did not metabolise sinapic acid (Edlin *et al.,* 1995; Heresztyn 1986b). *Brettanomyces* spp. generally did not remove HCAs from the growth medium. A notable exception to this was the utilisation of sinapic acid by *B. custersianus* and *B. naardenensis*. This examination of all five species grown in the presence of each of the four HCAs is the most systematic to date and highlights potentially important strain and species divergences. The growth of *Dekkera* isolates in media supplemented with two or more HCAs was also investigated. In grape juice and wine, HCAs are present in

combination so this study more closely resembles wine environs. It appears that there is some synergy between the acids. Although the total concentration of HCAs remained constant isolates were less able to grow when more than one acid was present.

All acids metabolised were converted in a similar manner. During exponential phase VP production peaked first, followed by EP production. In some cases 100% utilisation of the HCA substrate was achieved, however, this did not give rise to an equivalent concentration of total VPs/EPs being produced. Compounds must therefore be lost from the HCA-VP-EP pathway either through absorption to cellular material (Salameh *et al*., 2008) or perhaps as substrates for other, as yet unknown, reactions.

Variations in the yeasts ability to grow when exposed to simple phenolics and the production of organoleptic compounds were pronounced. The addition of HCAs, particularly ferulic acid, decreased the growth rate of all yeasts examined. This phenomenon has been previously noted (Edlin *et al.,* 1995; Couto *et al.,* 2005) but no significance was attached. Divergence in HCA metabolism could reflect genetic modifications of the HCD enzyme or differences in a strain's ability to uptake HCAs from media. These differences could be related to environmental adaptations for survival under the conditions from which they were isolated, i.e. a broad range of HCA concentration.

Previous experimental work examining the HCA-VP-EP pathway has been conducted in many different media, varying from defined media and grape juice to wine (Chatonnet *et al.,* 1992; Dias *et al.,* 2003b; Edlin *et al.,* 1995). The former does not closely resemble natural conditions while the latter are undefined and growth

parameters such as pH, composition and HCA content vary greatly, making comparison of these studies difficult. The development of a semi-defined wine medium (WM) provides a compromise between these methods, while enabling the study of *Dekkera* spp. under more defined wine-like conditions. WM is a useful tool in the laboratory however final confirmation must still be completed in wine.

This study substantially increased the current knowledge and adds cohesion to already published reports. It was also instrumental for the design of subsequent experimentation. For example, observed inhibition of *Dekkera* and *Brettanomyces* by some HCAs inspired further evaluation of this area with the aim to determine whether this could lead to a novel method of antimicrobial control. Physiological investigation also led to the further purification of the enzymes involved in the HCA-VP-EP pathway.

5.3 Biochemical and molecular characterisation of HCA decarboxylase and VPR enzymes

he breakdown of HCAs to 4-vinyl and 4-ethyl derivatives by *Dekkera* agreed with previous reports of *p*-coumaric catabolism (Chatonnet *et al.,* 1992; Edlin *et al.,* 1995; Dias *et al.,* 2003b). Hence further characterisation of HCA decarboxylation was undertaken with the aim of purifying the enzyme responsible and elucidating the gene sequence. Biochemical characterisation included defining key parameters for activity and subsequently, protein isolation was performed. Sequence data determined from the isolated protein was fundamental for continued molecular investigations.

Dekkera spp. examined had constitutive activity towards all HCAs. This activity was enhanced by pre-growth in the presence of any HCA examined. This fact suggests that

Dekkera spp. have a single enzyme which catabolises multiple HCAs rather than multiple enzymes expressed in response to different acids, such as seen in *L. plantarum* (Barthelmebs *et al.,* 2000a). Confirmation of this notion could by achieved by the production of a HCD-knockout strain of *Dekkera.* However, while such manipulations are routine in *S. cerevisiae* they are yet to be developed for this yeast.

The HCD enzyme was isolated and purified, and from this a near complete putative *HCD* gene sequence was derived. Despite some *Dekkera* genome sequencing data becoming available recently (Woolfit *et al*., 2007), the region obtained did not have any homology to the reported sequences. This is unsurprising, as only approximately 40% of the *Dekkera* genome has been sequenced to date (Woolfit *et al*., 2007). In fact, analysis conducted here demonstrated that the protein fragment and partial gene sequences are not similar to any previously described *PAD* genes. Instead, it aligns with a putative flavodoxin-like protein (*PST2*) from *S. cerevisiae* and *C. albicans*. PST2 has no reported activity towards HCAs (Edlin 1997). However, the translated sequence does align with the N-terminal sequence reported for the enzyme characterised by Edlin (1997). When these sequences are combined, with reference to overlapping homologue, all but the first couple of amino acids can be determined. This fact supports the contention that *Dekkera* spp. possesses a novel enzyme responsible for HCA decarboxylation.

The isolated HCD also has an amino acid sequence predicted to be a flavodoxin mononucleotide binding site (InterProScan, 2009), indicating it is a member of the 'flavodoxin-like' family to which PST2 and PAD proteins belong (Grandori and Carey 1994; Rangarajan *et al.,* 2004). However, we suggested that the protein isolated from

Dekkera and having activity towards HCAs, be described as HCA decarboxylase rather than phenylacrylic acid decarboxylase (*PAD*) as it has substantial variations to other previously characterised enzymes.

Dekkera are potent spoilage organisms partly due to their capacity to produce EPs under wine conditions (Chatonnet *et al.,* 1992). As stated, the enzyme responsible for this had previously only been postulated (Chatonnet *et al.,* 1992). The ability of *Brettanomyces* to metabolise VPs into EPs had never been described. For the first time, this work examined VP utilisation *in vitro* and *in vivo* resulting in the partial characterisation of VP activity of all *Dekkera* and *Brettanomyces* spp. All species removed VPs from medium without previous exposure to HCAs and extracts had constitutive activity towards at least one VP compound. This activity could be enhanced by exposure to VPs but not HCAs. This is the first report of the capacity of *Brettanomyces* yeast to remove any VP from the growth media. It had been presumed that a lack of HCA activity precluded them from any subsequent reactions in the HCA-VP-EP pathway. Whether the removal of VPs by *Brettanomyces* occurs because of the involvement of a VPR mediated reaction or by a unique pathway has yet to be established.

At the time this work commenced, there were no published reports characterising VPR activity. Recently two research papers (Godoy *et al.,* 2008; Tchobanov *et al.,* 2008) have been published which describe isolation and characterisation of the VPR enzyme from *D. bruxellensis* strains. Tchobanov *et al.* (2008) also identified the VPR ORF. Both papers examined the reduction of 4-vinylphenol but Tchobanov *et al.* (2008) also confirmed the enzyme was active against 4-vinylguaiacol in accordance with results presented here. These papers present strikingly different results in relation to the VPR

protein isolated. Godoy *et al.* (2008) determined the protein to have a molecular mass of 37 kDa combined as a tetramer with a total size of 118kDa, while Tchobanov *et al.* (2008) reported a protein band of 26 kDa and suggest that VPR is a monomeric protein. It is possible that *Dekkera* actually has 2 enzymes that can reduce VPs and thus both studies are correct. Further examination is required to obtain a definitive answer.

As all 5 species of *Dekkera* and *Brettanomyces* are examined, the research presented here is the most comprehensive investigation of the reduction of 4-vinylphenol and 4-vinylguaiacol by these organisms. The optimum pH for vinylphenol reduction to occur was pH 7 for all species except *D. anomala,* which had a pH optimum of 6. This and the optimal temperature are slightly higher than that described elsewhere (Tchobanov *et al.,* 2008), although the VPR enzyme retains almost 100% activity at the temperature optimum found here. Observed differences may be explained by the use of different strains or other factors. Cultures were investigated under different conditions, the biggest variation being whether or not cells were induced before harvest. Additionally characterisation was carried out using extracts of varying purity (Tchobanov *et al.,* 2008; Godoy *et al.,* 2008). Further work will clarify the findings made across these three reports.

5.4 Antimicrobial action of HCA and possible mechanisms

Two seemingly contradictory observations relate to ferulic acid and *Dekkera*. Firstly, as is commonly accepted, *Dekkera* removes ferulic acid and other HCAs from growth media catabolizing them into VP and EP compounds that can have negative implications for foods and beverages. Secondly, a new concept has arisen, that is that HCAs, particularly ferulic, are toxic to *Dekkera* cells and thus could be used as a

method to control unwanted *Dekkera* growth. This method may not be universally applicable, however, in wines with an initially high content of HCAs, a small addition of one or more simple phenolics may confer resistance to *Dekkera* growth with little or no impact on the sensory properties of the wine. These findings may also go someway to explaining, why, some wines are not prone to spoilage.

The physiological studies indicated that *Dekkera* and *Brettanomyces* populations were reduced when the medium was supplemented with certain HCAs. Although this effect had previously been noted (Edlin *et al.,* 1995; Couto *et al.,* 2005) no in-depth study had been undertaken to determine whether this inhibition could be developed as a control strategy. The primary aim of work conducted here was to resolve what concentrations were required to markedly inhibit *Dekkera* spp. This was achieved by defining the minimum concentration of HCA that inhibited culture growth significantly, as shown by Student t-test = $p \leq 0.05$; the minimum inhibitory concentration (MIC). The MIC for ferulic, caffeic and *p*-coumaric acids in CDM varied from 1 mM of ferulic acid to 8 mM caffeic acid. In addition, concentrations at which growth was decreased by 90% were defined as the MIC_{90}. In general the MIC_{90} was higher than the MIC and varied by up to 5-fold depending on the strain and acid used. Less *p*-coumaric or caffeic acids were required when added in combination with ferulic acid. In the wine environment HCAs are always present in combination therefore this has important implications that require further investigation.

Subsequently cultures with a MIC_{90} were re-inoculated into fresh CDM to determine whether the inhibited yeast were still viable and thus able to re-establish growth when no longer inhibited by HCAs. *Dekkera* strains investigated were unable to recommence

growth, even in media without HCA additions when grown initially grown in CDM in the presence of 8 mM and above of ferulic acid. This inhibitory concentration was decreased when yeast were examined in wine medium and wine. *Dekkera anomala* (AWRI 1499) was rendered non-culturable by 4 mM and 1 mM of ferulic acid in WM and wine respectively. This suggests that the cells were incapable of reproduction (i.e. were viable but non-culturable) or dead. Scanning electron microscopy images indicated that HCAs caused cell damage. At high concentrations this damage included wrinkling and rupture as well as alterations to budding cells.

Initial MICs were determined in CDM which, as discussed previously, serves as a guide to what may occur in the wine environment. Thus the MIC and MIC_{90} of *Dekkera* cultures were also determined in WM and a modified red wine. This is the first report of *Dekkera* being inhibited in modified wine by a compound found naturally in grape juice and wine. Wine parameters such as ethanol content and endogenous HCAs probably act in synergy with ferulic additions thereby reducing the required supplementation. The synergy of ferulic acid and wine components has a direct implication for its use in industry. It is posited that in normal wine without the modifications made here to encourage *Dekkera* growth, the concentration of ferulic acid required could be further reduced. These findings further support the proposal for using HCAs to supplement naturally occurring phenols as a method to control unwanted *Dekkera* growth.

SEM analysis of ferulic acid treated cells appears to show physical alterations to the cell membrane. It is not clear if this is the primary cause of growth inhibition or symptomatic of the exposure. Possible methods of antimicrobial action by similar compounds on bacteria have been suggested. These include inhibition of enzymes and

action against the cell membrane that allows leakage of K^+, glutamate and intracellular RNA, among others (Campos *et al.,* 2003). The related phenolic compounds sorbic and gallic acids have also been shown to cause wrinkling and cracking of yeast cells (Bennis *et al.,* 2004). Ferulic acid could be acting directly on *Dekkera* cells i.e. by absorbing to the cell wall (Salameh *et al.,* 2008) or acting on membrane proteins. The observed results could also be attributed to cell death caused by other mechanisms such as intracellular acidification.

Until now, there has been no explanation as to why *Dekkera* causes spoilage in some wines but not others. The natural HCA content of grape juice and wine may be sufficient in some cases to prevent growth and hence spoilage. Concentrations of ferulic acid and related simple phenolic compounds reported in grape juice are below those required, to inhibit or kill the *Dekkera* spp. examined. However the total phenolic content is dependant on many factors, some of which could be modified to maximise concentrations of free HCAs and hence produce juice/wine, which is resilient to *Dekkera* growth. The fact that ferulic acid is naturally present in grapes also means that it has the potential for use as a wine additive as an alternate natural antimicrobial. Further investigation of many *Dekkera* wine isolates needs to be undertaken to determine optimal concentrations of HCAs needed to fully inhibit growth. This is of critical importance before any recommendations be made, however, these findings could impact both viticulture and viniculture practices in the future.

The potential of phenolic compounds for the management of unwanted lactic acid bacteria in wine has previously been suggested (García-Ruiz *et al.,* 2008) but no laboratory or industrial experimental work have been preformed to evaluate the

practicalities. The use ferulic acid or a combination of HCAs has the potential to restrict growth of deleterious wine organisms such as *Dekkera* or to control other organisms such as lactic acid bacteria. This combined with the development of technologies that extract phenolics from grape seeds (García-Ruiz *et al.,* 2008) as an acceptable source of additives for the wine industry could be the next generation of antimicrobials for development and application in this industry. The use of naturally extracted phenolics could provide a viable alternative to sulfur dioxide in the prevention and treatment of *Dekkera* contamination. Also, these methods might not be limited to the wine industry. Beer, dairy and a number of other sectors affected by unwanted *Dekkera* or *Brettanomyces* could also benefit from these findings. Auxiliary research is, however, essential to minimize the potential for development of rebarbative qualities if HCA additions were made at an inappropriate level or time.

In conclusion, significant advances have been made to the current perception and understanding of wine spoilage as caused by *Dekkera* spp. The lesser known *Brettanomyces* genera have also been investigated adding greatly to the body of knowledge for these yeast. The aims of this work were accomplished, the biological pathway by which EPs are produced have been scrutinized in detail and an alternative method for the control of unwanted *Dekkera* spp. in wine has been proposed. A range of questions has arisen from these results and some future research directions are discussed below.

5.5 Future direction

The current body of knowledge concerning *Dekkera* and *Brettanomyces* yeast has been substantially increased. Yet there are a number of questions highlighted which require

further exploration. The removal of HCAs and VP from media has been examined in depth over the time course of fermentation, however, the mechanism by which the HCAs are taken into the cell is not known. Additionally, VPs are detected in fermentation medium after the catabolism of HCAs. Whether these compounds are released due to cell death or actively transported out of the cell before being reabsorbed and further catabolised is unclear. The use of labelled compounds could clarify these points and could lead to new targets by which uptake of HCAs and therefore VP and EP production could be prevented thus avoid spoilage.

Physiological studies combined with enzymatic characterisation using cell extracts provided a comprehensive comparison between the species. Representatives of all *Dekkera* and *Brettanomyces* spp. have been examined; however, expanding this work could identify strains that are highly sensitive or resistant to HCA compounds. A survey of this nature would also determine the range of HCA concentrations required to inhibit growth. Utilisation of newly identified gene sequences, biochemical and other molecular methods would elucidate key deviations. Combining this information could highlight specific sequence alterations responsible, thus going some way to explaining the variability observed in wines affected by *Dekkera* contamination. Practical advantages may include the ability to identify which *Dekkera* isolates pose greatest risk when found in the winery environment or isolates that may be advantageous for brewing. Identification of novel control methods or modulation of aromatic compounds utilising *Dekkera* may result from such research.

Comparisons of *HCD* and *VPR* gene sequences from multiple isolates will greatly increase the current knowledge and is only now becoming possible. It is essential to

finalise the *HCD* gene sequence. Once completed, experiments such as over-expression or disruption could become viable. The ability to clone the gene and express a functional protein would make enzymatic study simpler and the creation of a knockout strain would determine if *Dekkera* has only one gene capable of metabolizing HCAs. The sequence will also enable characterisation and comparison that would determine any molecular basis for phenotypic variations between species and strains of *Dekkera* and may explain why *Brettanomyces* spp. are not as capable as *Dekkera* at HCA metabolism. These findings could have broader significance outside food and beverage industries. *Dekkera* in particular produces high quantities of VPs and as such the *HCD* gene is a candidate for cloning into a microorganism suitable for the production of VP, which could be used in pharmaceutical, agricultural, flavour, fragrance or chemical industries (Mathew and Abraham 2006).

To understand VPR action it is essential that the enzyme be fully characterized. Currently two contradictory descriptions have been put forward. One is that the protein is a monomer, the other a tetramer. This may indicate that in fact two enzymes capable of producing EPs are active in *Dekkera*. In order to confirm this, knockout strains could be produced. Although, as previously discussed, this is not a method routinely used with *Dekkera* spp. alternatively, the crystalline structure of the protein could be elucidated.

A possible approach to control or eliminate the problem of spoilage of wine by these yeasts has been suggested. It has been proposed that the naturally occurring HCAs could be manipulated or supplemented to prevent growth of *Dekkera* in wine. This proposal is based on laboratory MIC observations. This area requires further investigation. Primarily it is important to screen a larger number of wine isolated

Dekkera sp. to determine the concentration that will inhibit all *Dekkera* growth in wine and hence prevent unwanted production of VP and EP compounds.

It is essential to consider microbial action and interactions of the HCAs with wine components such as proteins, sugars or oxidants. For example the increased HCA content of juice could, in theory, lead to the formation of vinylphenol adducts by chemical means (Schwarz *et al.,* 2003) that would deplete total free HCA concentrations and antimicrobial effectiveness. Under such conditions, inhibited, viable *Dekkera* cells may recommence growth utilising remaining HCAs for volatile phenol production. Studies, considering all these factors, are required for establishing the possible application in relation to winemaking. Experiments to evaluate the repercussions of HCA additions, such as monitoring spiked wines for chemical, aromatic and flavour alterations under different conditions, would be beneficial. Work also is required to ascertain the positive and negative impact of increased HCAs on other wine microorganisms.

The development of a rapid test for MIC determination would be of benefit to the wine industry. In this way wineries could determine accurately the concentration of HCAs required to inhibit or kill *Dekkera* resident within there winery. This is in a similar fashion to that currently undertaken in the health sciences area for antibiotic susceptibility determination. Sensitivity could be enhanced by using samples of the actual base juice/wine to allow for oeneological factors that may influence ferulic acid toxicity. Winemakers could then make appropriate HCA additions to their wines, depending on both strain requirements and wine composition, thus eliminating the risk off over- or under-supplementation. It would also be of interest to determine whether

appropriate antimicrobial concentration of HCAs could be achieved by manipulating viticultural and other oenological conditions such as grape ripeness and time on skins.

Outside of the wine industry, this research is applicable in food, as well as non-alcoholic and alcoholic beverage industries where *Dekkera* or *Brettanomyces* has been identified as a spoilage organism. There might be a benefit as well in beer production where VPs may be desirable. Additionally VP derivatives produced biologically could be of high commercial value. Isolating and cloning a functional *HCD* gene could be of industrial significance outside of the wine industry for the production of these VP compounds.

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Appendix 1

Attempts at molecular cloning of the *HCD* gene from *Dekkera* and *Brettanomyces*

A.1 Attempts at molecular cloning of the hydroxycinnamic acid decarboxylase gene from *Dekkera* and *Brettanomyces* species

The aim of this research was to characterise the HCD enzyme at the molecular level, however there is a dearth of genetic information available for *Dekkera* and *Brettanomyces* yeasts. When this study commenced, information in sequence databases was limited to a partial RAD4 gene sequence and the internal spacer region of rRNA, both used for molecular diagnostics (Ibeas *et al*., 1996; Guillamón *et al*., 1998). No DNA sequence of the *HCD* gene had been determined. Edlin *et al.* (1997). At that time HCA metabolism by *Dekkera* was thought to be meditated by phenylacrylic acid decarboxylase, encoded by a putative gene, *PAD,* it was thought that the gene from *Dekkera* would share homology with *PAD1* gene isolated from *S. cerevisiae* and related yeasts. The complete genome sequence of *S. cerevisiae,* including, the *PAD1* gene is available (*Saccharomyces* Genome Database). Conducting BLAST similarity searches with this sequence revealed a number of homologous sequences to which primers were designed (Table A1). PCRs were carried out under an array of conditions, whereby temperature and magnesium gradients were modified in order to optimise the reactions. However the orthologue of *PAD1* was not identified by homologue cloning.

In addition to PCR methods, the *PAD1* gene was amplified from *S. cerevisiae*, modified with a Dig label and used as a probe in Southern blot hybridisations against *Dekkera* and *Brettanomyces* spp. Although the probe hybridised to *S. cerevisiae* control DNA (Figure A1), no binding was observed for any *Dekkera* or *Brettanomyces* yeast even when reaction specificity was low (Figure A1).

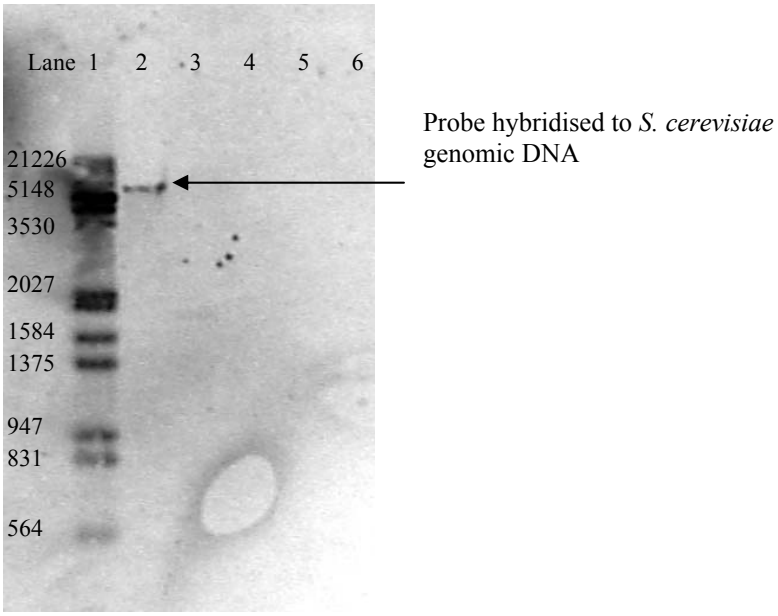

Figure A1. Southern blot analysis with Dig-labelled *PAD1* probe under the following conditions: Genomic digest performed with *EcoR*I overnight at 37˚C and run on a 1% agarose gel at 80 volts for 1 hour. DNA was transferred overnight to a nylon membrane and UV cross-linked. The membrane was incubated with Dig-Easy-Hyb (Roche) at 37°C for 2 hours and then in a pre-warmed probe solution (10 mls Dig-Easy-Hyb and 25 µl Dig-labeled probe) at 40°C overnight. The membrane was washed in 2% saline-sodium citrate (SSC) buffer, 0.5% SSC and then a blocking reagent (Roche) applied for 45 mins. Dig-specific antibody was applied for 1 hour. The membrane was washed with 1 x maleic acid buffer and the equilibrated in detection buffer (10 mM Tris, 10mM NaCl pH 9.5). Final incubation was carried out overnight with 150µl of detection reagent. Chemiluminescence was then detected with the BioRad gel doc system. Lane 1, Roche Dig-labelled marker III; lane 2, *S. cerevisiae* positive control; lanes 3-6, *D. anomala* CBS 77, *D. bruxellensis* AWRI 1499 and *Brettanomyces custersianus* CBS 4805

Through personal communications (Edlin *pers. comm.* 2005) during this work it was discovered that reported in a PhD thesis, there was a partial amino acid sequence of a protein from *D. anomala* with HCA decarboxylase activity. Edlin *et al*. (1998) partially purified a protein with HCD activity and from this short amino acid sequence corresponding to the N-terminal was established, but only reported in a PhD thesis (Edlin 1997). This partial sequence had no homology to any known PAD enzymes from yeast or bacteria but did align with an unrelated gene, now identified as *PST2*, from *S. cerevisiae*. Forward primers were reverse engineered from the amino acid sequence and the reverse primers had homologue to yeast *PAD1*or *PST2* genes (Table A1). No significant orthologues of the *HCD* gene were identified.

A Dig-labelled probe (Sigma Proligo; Figure A2) for Southern hybridisation was designed based on the N-terminal amino acid sequence. In bacteria the PAD enzyme is known to have the greatest sequence variation in the C- and N-terminal regions. In order to compensate for this a BLAST search was performed using the N-terminal sequence and related sequences compared to determine the section with least variation and a short degenerate probe designed. Despite this, no reaction was observed for *S. cerevisiae*, *Dekkera* or *Brettanomyces* yeasts. This was most likely due to the short length and degenerate nature of the probe. Therefore it was decided that protein isolation would be carried out in order to obtain further sequence information, as described in Chapter 3.

Whilst undertaking protein purification the partial genome sequence of *D. bruxellensis* CBS 2499 was released (Woolfit *et al.*, 2007). It was reported that the *PAD* gene was not identified amongst the obtained sequence (Woolfit *et al.*, 2007) however, it was

Table A1. Primers used during molecular investigation of the *HCD* gene from *Dekkera* and *Brettanomyces* Spp.

Primer	Sequence
PADOUTFWD[1]	TGCTCCAAAAGGAAAGTTGGC
PADOUTREV[1]	GTACATGTGTAAATATCTGGGTTCA
*PAD1*FWD[2]	ATGCTCCTATTTCCAAGAAGAACTAATATAGCC
*PAD1*REV[2]	TTACTTGCTTTTTATTCCTTTTCCCAACG
PAD70FWD[3]	AGAACCATTACRACTTSAMCAKSTTTCC
*PAD1*56FWD[3]	CAAGACCWAAGAGAATWGTYGTSGCAATWACTGG
PAD277FWD[3]	GGGGTRYRGCAACAATGAAATATG
PAD497FWD[2]	CAAAATGGGGTGCAGCAA
PAD721REV[3]	CCTTCCCAACGWGGRAAAGT
EDLIN1[4]	GCTAAGATTGCTATTATTATTTACACTTTGTAC
EDLIN2[4]	GCNAARATHGCNATHATHTAYACNTTNTAYCAYCA
EDLIN3[4,5]	AAGGTCGCTATTATYATCTAC
EDLIN4[4]	TGGCTGAAGCTGTTAAGAAGG
PST2REVa[6]	CGTTTCGTAGAAAGTCTT
PST2REVb[6]	AATTCCAAGGCGGAAGGAGA

[1] Primers designed with homologue to the non-coding region up and down stream of *PAD1* in *S.* cerevisiae.[2] Primers designed with homology to the *PAD1* gene of *S. cerevisiae.* [3] Degenerate primers designed with homologue to *PAD1* gene aligned yeast sequences allowing for base changes between sequences. [4] Primers reverse engineered from the N-terminal amino acid sequence of an enzyme with HCA decarboxylase activity (Edlin 1997). [5] Primer was used during 5'RACE PCR as described in Chapter 3. [6] Primers designed with homologue to the *PST2* gene of *S. cerevisiae.*

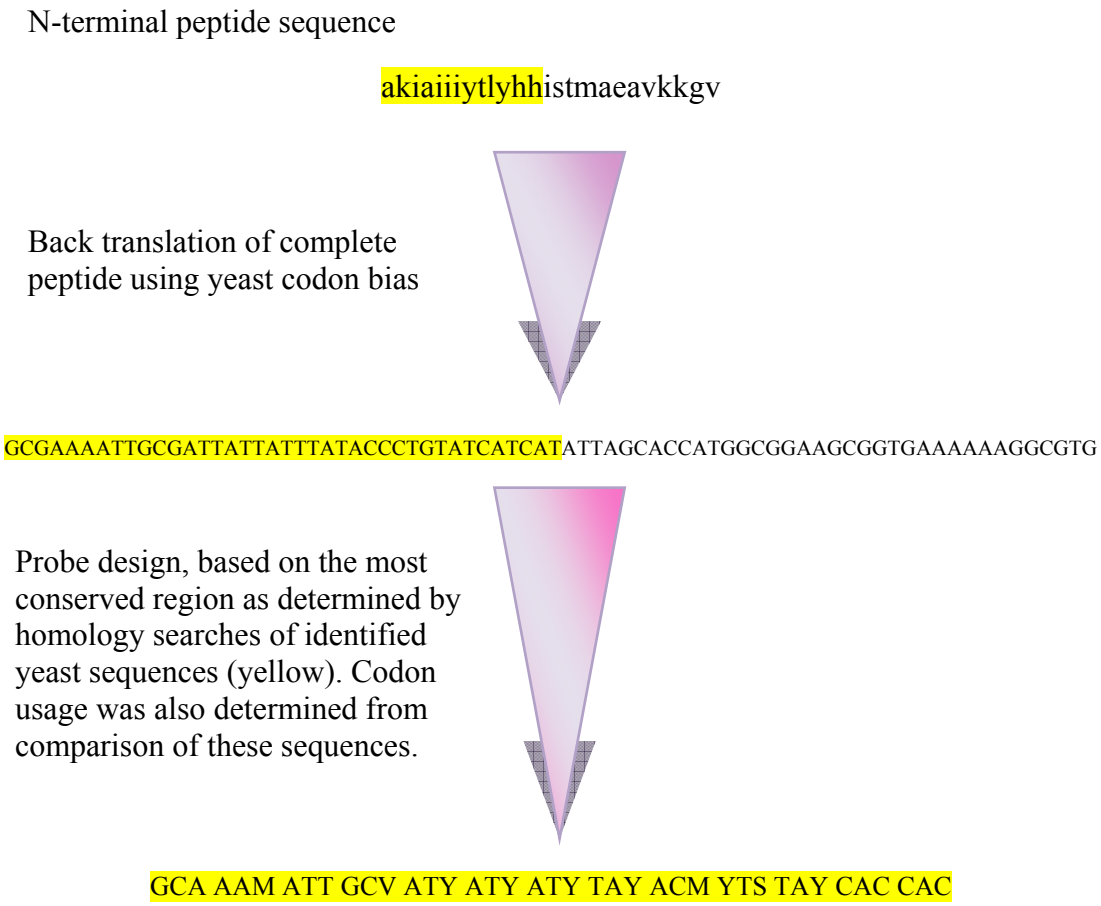

Figure A2. Dig-labelled Southern blot probe design. The N-terminal peptide sequence as determined by Edlin (1997) was translated using the yeast codon frequency. Additionally the peptide was used to search for similar yeast sequences by BLAST similarity searches (EMBL). These sequences could be compared to the known DNA sequence for these enzymes and thus an actual codon usage as applied to this enzyme determined. The back-translated sequence did not compare favourably with known sequences, mainly in the third base position. Therefore the probe was designed to the region with highest conservation (highlighted) and with reference to related yeast sequences.

unlikely that the authors were aware of the unpublished peptide sequence. Therefore the released genomic information was searched (EMBL) for the back-translated HCD fragment. No orthologs were found. When further protein and DNA sequence data was determined (Chapter 3) it was also compared to the known genome with no homology identified.

A partial DNA sequence has now been elucidated for a candidate *HCD* gene utilising protein purification and reverse engineering primers for 3'RACE PCR (Chapter 3). The sequence obtained has no homologue to the *PAD1* gene of *S. cerevisiae* (EMBL) and has subsequently been named hydroxycinnamic acid decarboxylase (*HCD*). This gene has no similarity to either the PAD enzymes of yeast or bacteria. It is therefore proposed that the *HCD* of *Dekkera* may belong to a new class of phenolic acid decarboxylase enzymes (Chapter 3). Although primer design using *PAD1* sequence information at the time was the most logical approach it is not surprising that, in light of current results, attempts to clone the gene using primers designed to amplify the *PAD1* were unsuccessful. Future work should focus on cloning the complete *HCD* gene for over-expression studies to confirm its identity. Screening of a number of *Dekkera* and *Brettanomyces* spp. should also be carried out to determine if sequence variations account for the variations in enzyme activity observed.

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