Physiology and metabolism of *Dekkera/Brettanomyces* yeast in relation to mousy taint production

by

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'a flavour often left on the tongue as an aftertaste, reminiscent of the odour from mice kept in a confined space' (Erckmann, 1898)

Table of Contents

Thesis Summary

Mousy taint is an insidious spoilage characteristic of wine and other fermented beverages. Its origin is reputedly due to the growth of certain strains of lactic acid bacteria and yeasts of the genus *Dekkera*, more often described by the asexual form, *Brettanomyces*. Tainted wines are rendered unpalatable and this is economically disastrous for the wine producer. The compounds considered responsible for mousy taint in wine are the tautomers 2-acetyl-1,4,5,6-tetrahydropyridine and 2-acetyl-3,4,5,6 tetrahydropyridine (described together as ACTPY).

The lack of a sensitive analytical method for the measurement of the compounds associated with mousy taint, which only occur at low concentrations (μg/L range), has constrained research in this area. In the present work a reliable analytical method was developed to examine the chemical basis of mousy taint and to investigate the role of *Dekkera/Brettanomyces* yeast in the formation of mousy taint.

A method for the extraction, analysis and quantification of ACTPY was developed, incorporating a continuous liquid/liquid extraction procedure and analysis by gas chromatography/mass spectrometry (GC/MS). The procedure was validated by a spiking trial and was shown to be reliable and highly sensitive. This enabled a detailed analysis of mousy taint compounds in wine and culture media extracts.

Detection and analysis of other compounds which reputedly had a mousy character was undertaken; 2-acetyl-1-pyrroline (ACPY) and 2-ethyl-3,4,5,6-tetrahydropyridine (ETPY) were identified as important constituents of mousy taint. The very sensorily potent ACPY was detected in mousy wines and in some experimental fermentations carried out by *Dekkera/Brettanomyces* species. This was the first report of the production of this compound by yeast. Its identity was confirmed by comparison of mass spectral data with

that of an authentic reference sample. The mousy impact of ACPY was confirmed by GC effluent sniffing (GC–sniff).

The compound ETPY was also detected in mousy wines and fermentation media, and synthesis of an authentic reference and comparison of mass spectral data confirmed its identity. Confirmation was also shown with co-injection analysis by GC/MS of an authentic sample and an extract containing tentatively identified ETPY. This analysis indicated the detection of ETPY by the symmetrical peak enhancement of the target compound with no significant change in relative ratios of the component ions. This compound was detected below its reported taste threshold in wine, indicating that it may have a limited impact on mousy taint in wine. However, it was always detected in association with ACTPY and to a smaller extent with ACPY. This suggests a possible route for its formation, but does not discount any additive sensory effect.

Two surveys of mousy taint production by the type strains of *Dekkera/Brettanomyces* cultured under different conditions were carried out. A rapid and simple method was used initially. The yeasts were grown in a grape juice medium and mousy taint detected by sensory evaluation. A large proportion of the yeasts, representing all species, produced taint under these conditions. A more detailed analytical study in which all the known type strains of *Dekkera/Brettanomyces* were cultured in a chemically defined medium was undertaken. This showed that all *Dekkera/Brettanomyces* tested, including the recently classified *B. nana,* could produce ACTPY and to a lesser extent ETPY. No ACPY was detected under these conditions.

A practical consequence of these data is that mousy taint formation in wine could arise from the presence in wine of any strain or species of these yeasts, depending on the conditions and availability of appropriate precursors. This work confirmed the previously reported observations that the amino acid L-lysine is an important precursor for the formation mousy taint, as a marked reduction in ACTPY and ETPY concentration was observed in its absence. This finding suggests that the intracellular pool of L-lysine is

enlarged by the presence of extracellular L-lysine, thereby increasing the flux of tetrahydropyridine formation. These data also indicated that L-lysine is not the precursor of ACPY. Substantial concentrations of ACPY were produced by the *Dekkera/Brettanomyces* species tested only when L-ornithine was the sole nitrogen source. The production of mousy taint compounds can therefore be considered a characteristic of the genera *Dekkera/Brettanomyces*.

The origin of the nitrogen heterocycle ACTPY (and ETPY) was investigated by the use of stable isotopes. In a feeding experiment, uniformly labelled L-lysine $^{13}C_6$-$^{15}N_2$ was found to be the primary precursor to ACTPY and ETPY, as labelled C and N were incorporated into these compounds. Further investigations using ^{15}N label at the either α-amino or ε-amino groups of L-lysine, showed that the ε-N contributed to ACTPY and ETPY. These data indicate a novel route of L-lysine catabolism in *Dekkera/Brettanomyces*.

The accumulation of ACTPY in culture media was strongly linked to the growth phase, with the amount of ACTPY increasing as the culture progressed from early to middle stationary phase. The formation of ACTPY coincided with the uptake of L-lysine from the medium, providing further evidence that the catabolism of this amino acid was important to tetrahydropyridine formation. In contrast to ACTPY, the production of ETPY was delayed until the middle stationary phase. This suggests that ETPY is a product of further metabolism of ACTPY. The strong link to growth indicates that mousy taint compounds are a product of general cell metabolism.

The role of oxygen in mousy taint formation was investigated in a grape juice medium. Air stimulated the production of both ACTPY and ETPY. The importance of air or oxygen to taint production was confirmed using a high-density whole cell incubation (WCI) technique. A strong repression of ACTPY formation was shown after anaerobically pre-cultured cells were incubated anaerobically in the high density WCI system. Aerobically pre-cultured cells, however, allowed ACTPY formation. This result explains the link between the high level of mousy taint production in wine exposed to oxygen in the

winery situation. A small *Dekkera/Brettanomyces* population growing or at least viable in a wine with limited air contact, then exposed to air via a processing or handling procedure is sufficient to induce taint production.

On the basis of the data reported in this thesis and previous investigations, a model of mousy taint production in wine by *Dekkera/Brettanomyces* is proposed (Figure 1). Exposure of a growing population of *Dekkera/Brettanomyces* yeast to oxygen in the presence of either L-lysine or L-ornithine will lead to the formation of ACTPY/ETPY or ACPY respectively.

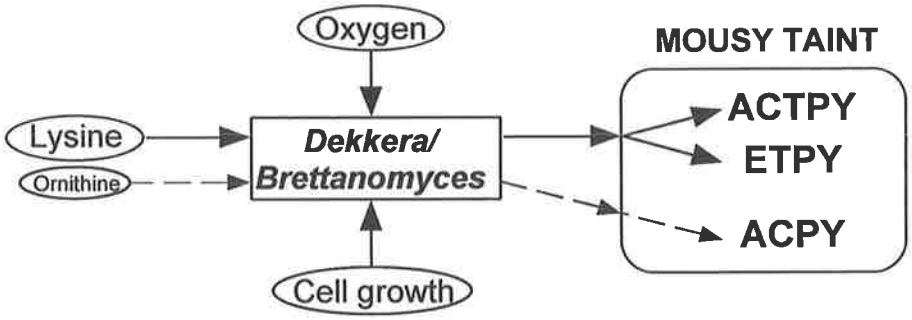

Figure 1. Proposed model for mousy taint production by *Dekkera/Brettanomyces* in wine.

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, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Paul R. Grbin

March 1998

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Finally, apologies to any I have missed, and thanks to the countless others who in some way contributed to the completion of this thesis.

Abbreviations

abs	Absorbance
ACPY	2-Acetyl-1-pyrroline
ACTPY	Tautomers of 2-acetyl-1,4,5,6-tetrahydropyridine (I) and 2-acetyl-3,4,5,6-tetrahydropyridine (II)
AWRI	The Australian Wine Research Institute
bp	Boiling point
ca.	Approximately
CBS	Centraalbureau voor Schimmelcultures
CDM	Chemically defined medium
cfu	Colony forming units
ETPY	2-Ethyl-3,4,5,6-tetrahydropyridine
FID	Flame ionisation detector
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
LAB	Lactic acid bacteria
LRI	Linear retention index
M^+	Molecular ion
MYPG	Malt and yeast extract, peptone and glucose
opm	Oscillations per minute
YPG	Yeast extract, peptone and glucose

CHAPTER 1 Introduction and Literature Review

1.1 Introduction

Mousy taint is an insidious spoilage characteristic of wine and other fermented beverages. The tainted beverage is rendered unpalatable and is therefore economically disastrous for the producer. No data exist as to the actual occurrence of this off-flavour in wine in either Australia or the rest of the world, partly because of the complex nature of the taint.

One of the earliest reports of this off-flavour in wine succinctly describes mousy taint as a 'peculiarly disagreeable flavour in wine, which is closely resembling to the smell of a residence of mice' (Thudichum, 1894 p 378). The taint was also originally described as resembling acetamide (Müller-Thurgau and Osterwalder, 1913). The use of acetamide to characterise mousy taint still persists in the literature (Peynaud and Domercq, 1956; Gilliland, 1961; Rankine and Bridson, 1971; Ribéreau-Gayon *et al.*, 1975; Bennett, 1996) even though in 1889, Mason had shown that pure acetamide was odourless (Tucknott, 1977). In fact it was an impurity of acetamide, identified as 2,4,6-trimethyl-1,3,5-triazine (Figure 1.1), that has a mousy aroma (Tucknott and Williams, 1972; Tucknott and Williams, 1973; Tucknott, 1977). However, this compound has never been isolated from any mousy beverage and is therefore not considered responsible for the off-flavour (Tucknott, 1977).

The nature and origin of mousy taint has been difficult to elucidate. An important factor affecting this is the wide range in sensory sensitivity of individuals to the taint (Müller-Thurgau and Osterwalder, 1913; Tucknott, 1977; Craig and Heresztyn, 1984; Heresztyn, 1986a). Detailed investigations have also been limited by difficulties associated with the chemical analysis of mousy taint, and no quantitative data has been reported (Tucknott, 1977; Strauss and Heresztyn, 1984; Heresztyn, 1986a).

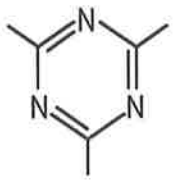


Figure 1.1. Structural formula of 2,4,6-trimethyl-1,3,5-triazine, identified as an impurity of acetamide with a mousy aroma (Tucknott and Williams, 1972;, 1973).

Several compounds have been found to be associated with this off-flavour, and a number of microorganisms have been described as causative agents (Tucknott, 1977; Craig and Heresztyn, 1984; Strauss and Heresztyn, 1984; Heresztyn, 1986a). Despite such efforts, questions still remain as to how and why this taint develops. The physiological basis for the production of these off-flavour compounds has not been examined in detail. Such information will lead to a better understanding of the problem and may offer strategies for its prevention. Analysis of the chemical nature of mousy taint will also contribute to this.

Tucknott (1977) comprehensively reviewed the literature that purported that mousy taint was purely the result of a chemical mechanism. It was suggested that mousy taint was related to high redox potential in wine and could be induced or removed by manipulating this factor. This critical review found the evidence for the chemical production of taint to be incomplete, due to lack of detail and inconsistent methodology. In addition, this re-examination revealed that mousy taint had not been induced by physio-chemical means in unfermented raw materials, thereby highlighting the importance of microorganisms.

Microorganisms were first suggested to produce mousy taint in wine (Thudichum, 1894). *Bacterium mannitopoem* was isolated from a mousy wine, and could produce a mousy character when inoculated and grown in a sound wine (Müller-Thurgau and Osterwalder, 1913). Douglas and Cruess (1936) isolated a mousy taint-producing bacteria from Californian wine, *Lactobacillus hilgardii,* and was the first report linking lactic acid bacteria (LAB) to this spoilage problem. The association of certain LAB with the production of mousy taint has since been confirmed (Tucknott, 1977; Heresztyn, 1986a). Acetic acid bacteria were also shown to be a causative agent of this off-flavour (Vaughn, 1938; Fornachon, 1943; Vaughn, 1955).

Yeast have also been reported to be capable of producing mousy taint. Recent work has provided confirmation of the role of *Brettanomyces* (the anamorph or asexual form of *Dekkera*) (Tucknott, 1977; Heresztyn, 1986a). It was Peynaud and Domercq (1956),

however, who isolated *Brettanomyces* species from wine and originally associated this genus with mousy taint. For the purpose of this thesis, the influence of only *Dekkera/Brettanomyces* will be considered in relation to mousy taint.

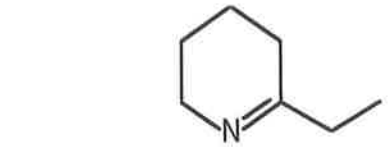
1.2 The chemical nature of mousy taint

1.2.1 2-ethyltetrahydropyridine

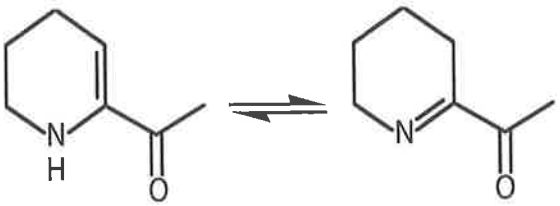
Tucknott (1977) suggested that the compound responsible for mousy taint in alcoholic cider, identified by gas chromatography and mass spectrometry (GC/MS), was the organic base 2-ethyl-3,4,5,6-tetrahydropyridine (ETPY) (Figure 1.2). ETPY was produced by *Brettanomyces anomalus* when grown in a chemically defined medium supplemented with L-lysine (Tucknott, 1977). This compound was not isolated from a mousy cider, possibly due to other interfering compounds present or the extraction technique and sensitivity of the analysis. ETPY was, however, detected in a commercial wine that was mousy (Tucknott, 1978). Further work attempting to detect ETPY in mousy wines was unsuccessful, although the compound(s) responsible were confirmed to be basic, as alkaline extracts of the mousy wines all had a mousy odour (Craig and Heresztyn, 1984; Strauss and Heresztyn, 1984). Spiking synthetic ETPY into wine did produce a mousy taint (taste threshold of 150 µg/L), and a solution of ETPY was found to have a mousy aroma by GC-effluent sniffing (GC-sniff) (Craig and Heresztyn, 1984).

1.2.2 2-acetyltetrahydropyridine

Subsequent work identified the organic base 2-acetyltetrahydropyridine (ACTPY) as responsible for mousy taint in wine (Strauss and Heresztyn, 1984). This was determined by analysis of mousy wines by GC-sniff and GC/MS. GC-sniff identified two important regions of the chromatograph producing a mousy taint sensory impression which corresponded to the two tautomeric isomers of 2-acetyl-1,4,5,6-tetrahydropyridine (I) and

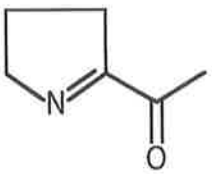


ETPY



I II

ACTPY



ACPY

Figure 1.2. Compounds purportedly responsible for mousy taint; ETPY, 2-Ethyl-3,4,5,6-tetrahydropyridine (Tucknott, 1977); ACTPY, tautomers of 2-acetyl-1,4,5,6-tetrahydropyridine (I) and 2-acetyl-3,4,5,6-tetrahydropyridine (II) (Strauss and Heresztyn, 1984); ACPY, 2-Acetyl-1-pyrroline (Seitz *et al.*, 1993).

2-acetyl-3,4,5,6-tetrahydropyridine (II) (Figure 1.2) (Strauss and Heresztyn, 1984). The two mousy regions identified by GC-sniff corresponded with the retention times and the mass spectra of an authentic reference sample of ACTPY tautomers. These compounds were also identified in mousy wines by Heresztyn (1986a). For the proposes of this thesis, these tautomers will be described together as ACTPY.

ACTPY could also be found in extracts of fermentations carried out by *Brettanomyces intermedius* in a chemically defined growth medium supplemented with L-lysine, and in extracts of *Lactobacillus brevis* fermentations [50% (v/v) grape juice, 0.5% (w/v) yeast extract, 5% (v/v) ethanol medium] (Heresztyn, 1986a). In this way both yeast and bacteria were implicated in the production of ACTPY. If L-lysine was not added to the yeast fermentation, ACTPY was not detected. The supplementation experiment demonstrated a possible role for L-lysine as a precursor to ACTPY in *Dekkera/Brettanomyces* fermentation.

Interestingly, ACTPY was originally identified as a primary odourant of bread crust (Hunter *et al.*, 1969) and has since been described as important to the odour of several other foods including rye and wheat bread crust (Schieberle and Grosch, 1983; 1984; 1985; 1987a; Schieberle, 1990a; c; Schieberle and Grosch, 1991), popcorn (Schieberle, 1990b; 1991; 1995), taco shells and tortilla chips (Buttery and Ling, 1995), and as an undesirable character in over-pasteurised wort and beer (Tressl *et al.*, 1981a; Helak *et al.*, 1989). ACTPY in these foods has been described as 'roasty' and 'cracker-like'. ACTPY is a relatively unstable compound (Hunter *et al.*, 1969; Büchi and Wüest, 1971; Buttery *et al.*, 1971; Helak *et al.*, 1989; Schieberle, 1990b), but is highly potent with an odour threshold of 0.06 ng/L in air (Schieberle and Grosch, 1991) and 1.6 µg/L in water (Teranishi *et al.*, 1975).

Early work on the production of ACTPY in bakery products investigated the thermal mechanism (Maillard reactions) whereby these compounds are generated (Hunter *et al.*,

1969; Hodge *et al.*, 1972). The mechanism relies on a source of the imino acid proline and a carbonyl compound, such as glucose, fructose or 2-oxopropanal (Tressl *et al.*, 1981a; b; Schieberle, 1990a; b; c; Rewicki *et al.*, 1992; Tressl *et al.*, 1993; Roberts and Acree, 1994; Stahl and Parliment, 1994). Schieberle (1990c) proposed that bakers' yeast cells were the source of the amino acid precursor in the formation of ACTPY in bread. This appears to be the only role in Maillard reactions for microorganisms in relation to the thermal production of ACTPY. L-Proline supplementation to a fermentation of chemically defined growth medium by *B. intermedius* did not induce ACTPY production. This suggests the mechanism for the biosynthesis of ACTPY is different from the Maillard process.

1.2.3 2-acetylpyrroline

The compound 2-acetyl-1-pyrroline (ACPY) (Figure 1.2) was recently reported as responsible for a mousy taint in wetted ground pearl millet (*Pennisetum americanum*) (Seitz *et al.*, 1993). ACPY, like ACTPY, is an impact component in bread crust and popcorn; it has a 'roasty', 'cracker-like' character and is structurally similar to ACTPY (Schieberle and Grosch, 1985; 1987a; Schieberle, 1989; 1990b; 1991; Schieberle and Grosch, 1991; Schieberle, 1993; Schieberle and Grosch, 1994; Gassenmeier and Schieberle, 1995; Schieberle, 1995). This compound was first identified as an impact component of the aroma of cooked rices (Buttery *et al.*, 1982; Buttery *et al.*, 1983b). It is especially important to the highly aromatic rices such as Basmati and Jasmin but is also detected in the non-aromatic types (Buttery *et al.*, 1983b; 1986; 1988; Lin *et al.*, 1990).

ACPY has also been identified in a variety of other sources such as the leaves of *Pandanus amaryllifolius* Roxb., a plant used to flavour non-aromatic rices (Buttery *et al.*, 1983a), in cooked meat (Gasser and Grosch, 1988), taco shells and tortilla chips (Buttery and Ling, 1995), other tortilla related corn products (Karahadian and Johnson, 1993), canned sweet corn (Buttery *et al.*, 1994), honey (Blank *et al.*, 1989), roasted sesame seeds (Schieberle, 1993), thermal extracts of cupuacu, chempedak fruit and jackfruit (Wong *et al.*, 1992;

Fischer *et al.*, 1993), and boiled trout (Milo and Grosch, 1993). ACPY has also been tentatively identified in tiger urine (Brahmachary *et al.*, 1990).

Recently, ACPY was detected in culture media of *Bacillus. cereus* strains previously isolated from cocoa fermentations (Romanczyk Jr *et al.*, 1995). This is the first report of microbial generation of ACPY. ACPY production was examined by culturing a *Bac. cereus* strain on ^{15}N labelled L-proline or L-glutamic acid. Incorporation of ^{15}N into ACPY was shown, indicating that either compound could act as the nitrogen precursor. The use of ^{13}C glucose also indicated that glucose was a carbon precursor to ACPY.

ACPY is more potent than ACTPY [cf. 0.6 ng/L in air and 1.6 µg/L in water], with an odour threshold of 0.02 ng/L in air (Schieberle and Grosch, 1991) and 0.1 µg/L in water (Buttery *et al.*, 1983b). ACPY, like ACTPY, is an unstable compound (Buttery *et al.*, 1983b; Schieberle and Grosch, 1992) and can be produced via a thermal reaction between amino acids and carbonyls (as per references for ACTPY). In bread flavour production, L-proline and L-ornithine can react with 2-oxopropanal (the thermal degradation product of dihydroxyacetone phosphate) to produce ACPY (Schieberle, 1989; 1990c; 1995).

1.3 *Dekkera/Brettanomyces* and beverage production

1.3.1 Taxonomy of *Dekkera/Brettanomyces*

Before any discussion of *Dekkera/Brettanomyces* can be made it is important to clarify the taxonomy. This yeast was first described by Claussen (1904) who isolated it from English stock beers (porter, ale and stout) and stated that "secondary fermentation effected by Brettanomyces (*sic*) is indispensable for the production of the real type of English beers." It was the work of Custers [(1940), cited by Henrici (1941), Skinner (1947), and van der Walt (1970b)], who carried out a detailed investigation of the morphology, physiology and metabolism of 17 strains of *Brettanomyces* yeast in order to classify the genus. Custers' classification was based on several criteria; a distinct ogival cell shape, the ability to

produce large amounts of acetic acid from glucose under aerobic conditions, the resultant short life span of the culture, slow growth in malt extract and on malt agar with a concomitant production of a characteristic aroma (no description made), and no ascospore production (Henrici, 1941; Skinner, 1947).

There are two further physiological characteristics important to *Dekkera/Brettanomyces*. The first was described for *Brettanomyces* by Custers as the stimulation of fermentation by oxygen; the Custers effect (Scheffers, 1966). The other characteristic is the presence of the respiratory ubiquinone system (coenzyme Q) which contains nine isoprene units. The coenzyme Q9 system is not unique to these genera, but occurs in all strains examined so far (Yamada *et al.*, 1980; Billon-Grand, 1987). *Dekkera/Brettanomyces* also contain other minor coenzyme Q systems, such as Q6, Q7 and Q8 (Billon-Grand, 1987), however, these appear to be of limited taxonomic importance.

Several reports describing yeast, including *Monilia vini* [Osterwalder (1912) cited by van der Walt and van Kerken (1958)] *Mycotorula intermedia* (Krumbholz and Tauschanoff, 1933), and *Torulopsis cylindrica* (Walters, 1943), have subsequently been shown to be *Brettanomyces* species (van der Walt and van Kerken, 1958; van der Walt, 1970b).

Ascosporogenous strains, *Brettanomyces bruxellensis* and *B. intermedius* were first described by van der Walt and van Kerken (1960), and new a teleomorphic genus, *Dekkera,* was proposed to account for this discovery (van der Walt, 1964). The genera only differ in that *Dekkera* shows alternation of generations with the formation asci and hat-shaped ascospores (van der Walt, 1964; van der Walt, 1970a). Table 1.1 summarises the many changes in the classification of these genera since 1970.

Table 1.1. Summary of publications since 1970 regarding the taxonomy and species relationships of *Dekkera/Brettanomyces,* including the basis of the description (in bold). Indent indicates synonym of species above.

(1) van der Walt (1970a; b)		**(2)** Kolfschten and Yarrow (1970), and Yarrow and Ahearn (1971)	**(3)** Campbell (1973)	
Morphology & physiology		**Morphology & physiology**	**Numerical analysis**	
Anamorph	Teleomorph	Anamorph	Anamorph	Teleomorph
B. bruxellensis	*D. bruxellensis*	*B. abstinens*	*B. abstinens*	
B. anomalus			*B. bruxellensis*	*D. bruxellensis*
B. claussenii			*B. anomalus*	
B. custersii			*B. claussenii*	
B. intermedius	*D. intermedia*		*B. custersii*	
B. lambicus			*B. intermedius*	*D. intermedia*
B. custersianus			*B. lambicus*	
		B. naardenensis	*B. custersianus*	
			B. naardenensis	

(4) Barnett *et al.* (1983)		**(5)** McArthur and Clark-Walker (1983)	
Morphology & physiology		**mtDNA restriction patterns**	
Anamorph	Teleomorph	Anamorph	Teleomorph
B. abstinens		*B. abstinens*	
B. custersii		*B. custersii*	
B. anomalus		*B. anomalus*	
B. claussenii		*B. claussenii*	
	D. bruxellensis	*B. lambicus**	*D. bruxellensis*
	D. intermedia		*D. intermedia*
B. lambicus			
B. custersianus			*B. custersianus*
B. naardenensis			*B. naardenensis*

(6) van der Walt (1984a; b)		**(7)** Smith and van Grinsven (1984), Lee and Jong (1985a; 1986a; b), and Jong and Lee (1986)	**(8)** Hoeben and Clark-Walker (1986), and Clark-Walker *et al.* (1987)	
Morphology & physiology		**Morphology & physiology**	**mtDNA gene mapping & sequencing**	
Anamorph	Teleomorph	Teleomorph	Anamorph	Teleomorph
B. abstinens		*D. abstinens*		
B. anomalus		*D. anomala*	*B. anomalus*	
B. bruxellensis	*D. bruxellensis*			*D. bruxellensis*
B. claussenii		*D. claussenii*		
B. custersii		*D. intermedia* **	*B. custersii*	
B. intermedius	*D. intermedia*			*D. intermedia*
B. lambicus		*D. lambicus*		
B. custersianus		*D. custersiana*	*B. custersianus*	
B. naardenensis		*D. naardenensis*	*B. naardenensis*	
			B. nana	

(9) Smith *et al.* (1990)		**(10)** Barnett *et al.* (1990)	**(11)** Molina *et al.* (1993)
Isoenzyme profiles & DNA-DNA homology		**Morphology & physiology**	**rDNA RLFP**
Anamorph	Teleomorph	Teleomorph	Teleomorph
B. anomalus	*D. anomala*	*D. anomala*	*D. anomala*
B. claussenii			*D. claussenii*
B. bruxellensis	*D. bruxellensis*	*D. bruxellensis*	*D. bruxellensis*
B. custersii			
B. abstinens			*D. abstinens*
B. intermedius	*D. intermedia*		*D. intermedia*
B. lambicus			*D. lambica*
B. custersianus		*D. custersiana*	*D. custersiana*
B. naardenensis		*D. naardenensis*	*D. naardenensis*

(12) Boekhout *et al.* (1994)		**(13)** Yamada *et al.* (1994)	**(14)** Cai *et al.* (1996)
Partial 26S rDNA sequences		**Partial 18S rRNA sequences**	**18 rRNA gene sequences**
Anamorph	Teleomorph	Teleomorph	Teleomorph
	D. anomala	*D. anomala*	*D. anomala*
	D. bruxellensis	*D. bruxellensis*	*D. bruxellensis*
B. custersianus		*D. custersiana* †	*D. custersiana*
B. naardenensis		*D. naardenensis*	*D. naardenensis*
B. nana			

*McArthur and Clark-Walker (1983) proposed that *B. lambicus* was a facultative synonym of *D. bruxellensis.*
**Lee and Jong (1985b) proposed *B. custersii* a facultative synonym of *B. intermedius,* imperfect form of *D. intermedia.*
† Yamada *et al.* (1994) proposed *D. custersiana* be transferred to a new genus, but not until ascospore production is confirmed.

Electrophoretic comparison of enzymes and DNA–DNA reassociation (whole genome) were used in an attempt to explain the anamorphic-teleomorphic relationships between *Brettanomyces* and *Dekkera* (Smith *et al.*, 1990). This study demonstrated two teleomorphic *Dekkera* species (*Dekkera anomala* and *Dekkera bruxellensis*) and two anamorphic *Brettanomyces* species (*Brettanomyces custersianus* and *Brettanomyces naardenensis*); the synonyms for these species are detailed in Table 1.1.

The classification of *Dekkera/Brettanomyces* was examined by Barnett *et al.* (1990) using morphological, physiological and molecular techniques. The study described only four *Dekkera* species (no *Brettanomyces*) through reclassification of *B. custersianus* and *B. naardenensis* as *Dekkera* species, according to the results of Lee and Jong (1986a; b) who reported sporulation in these species. However, the classification of *Dekkera custersianus* and *Dekkera naardenensis* has been considered invalid. It was suggested that the ascospore formation observed by Lee and Jong (1986a; b) were artefacts due to the staining technique used (Smith *et al.*, 1990). Barnett *et al.* (1990) noted that sporulation in these species was not confirmed in this classification. A phylogenetic analysis of *Dekkera* also suggested that ascospore formation by these two species needed to be confirmed (Yamada et al., 1994).

Other molecular biological techniques have been applied in order to classify yeast. These include ribosomal RNA/ribosomal DNA (rRNA/rDNA) sequence comparison, restriction fragment length polymorphism (RFLP), and partial and full sequencing of ribosomal genes (Kurtzman, 1992; 1994). Both RFLP and sequencing techniques have been used to investigate the taxonomy of *Dekkera/Brettanomyces* (Table 1.1). The classification described by Smith *et al.* (1990) was confirmed by comparison of partial sequences of *Dekkera/Brettanomyces* 26S rDNA (Boekhout *et al.*, 1994). The full sequences of the 18S rRNA of *Dekkera/Brettanomyces* strains have also been compared and used to confirm the four stable and distinct species groups (Cai *et al.*, 1996).

The use of rDNA sequence comparison has provided evidence for the reclassification of the genus *Eeniella* to *Brettanomyces.* This yeast was initially invalidly classified as *Brettanomyces nanus* and then transferred to the new genus *Eeniella,* based on morphological and reproduction differences (Smith *et al.*, 1981). *Eeniella* shares many physiological characteristics with *Brettanomyces* (Scheffers, 1966; Yamada *et al.*, 1980; Smith *et al.*, 1990). Molecular mtDNA analysis confirmed that *Eeniella nana* was phylogenetically related to *Brettanomyces* (Hoeben and Clark-Walker, 1986; Clark-Walker *et al.*, 1987; Hoeben *et al.*, 1993). Partial 26S rDNA sequencing and a phylogenetic analysis showed that *Eeniella* was derived from within *Brettanomyces*, and was transferred to this genus as *Brettanomyces nana* (Boekhout *et al.*, 1994) (Table 1.1). However, a more recent report has suggested that *Eeniella* be maintained as a separate genus (Yamada *et al.*, 1995).

For the purposes of this thesis the taxonomy of *Dekkera/Brettanomyces* by Smith *et al.* (1990) and Boekhout *et al.* (1994) (see Table 1.1) will be used. While it is valid to adopt *Dekkera* to describe these yeast, as in Barnett *et al.* (1990), it would seem prudent while there is some discussion of the classification of *D. custersianus* and *D. naardenensis* to still maintain *Brettanomyces* for these species (Smith *et al.*, 1990; Yamada *et al.*, 1994). *Eeniella nana* will be accepted as *B. nanus*, and included in this work. The name *Brettanomyces* will still be used to describe strains, even though most have been assigned to *Dekkera.*

1.3.2 *Dekkera/Brettanomyces* spoilage other than mousy taint

Dekkera/Brettanomyces yeast have been isolated from wine in Australia (Heresztyn, 1986a) and in many other winemaking countries (Agostini, 1950; Schanderl and Draczynski, 1952; Barret *et al.*, 1955; Peynaud and Domercq, 1956; van der Walt and van Kerken, 1958; van Zyl, 1962; Wright and Parle, 1974; Arjun, 1980; Tucknott, 1983; Gaia, 1987; Ciolfi *et al.*, 1988; Froudiére and Larue, 1990; Larue *et al.*, 1991; Mateo *et al.*, 1991;

Chatonnet *et al.*, 1992; del Pino Salvadores *et al.*, 1993; Ibeas *et al.*, 1996). These yeast have been associated with wine spoilage other than mousy taint. The spoilage of German sparkling wines by *Brettanomyces* species was characterised by the production of a high concentration of acetic acid (Schanderl and Draczynski, 1952). Peynaud and Domercq (1956) found up to 4 g/L of acetic acid in wines containing *Brettanomyces* species, and Schanderl (1959) reported a concentration of 7.2 g/L acetic acid in wine after a 12-month growth period.

Of all the incidences of turbidity in 'dry' South African table wine caused by yeast, *Brettanomyces* species accounted for 50% of the total yeast population (van der Walt and van Kerken, 1958; 1959a). This was confirmed by another survey in South Africa which showed that *Brettanomyces* species were still largely responsible for turbidity in 'dry' wine (van Zyl, 1962). The survey also noted a strong mousy off-flavour produced by these yeast.

Production of volatile phenol compounds, vinylphenols (4-vinylguaicacol, 4-vinylphenol) and more importantly, ethylphenols (4-ethylguaicacol, 4-ethylphenol) (Figure 1.3), is an important spoilage characteristic of *Dekkera/Brettanomyces* yeast (Heresztyn, 1986b; Chatonnet *et al.*, 1992). Ethylphenols impart a phenolic off-odour to red wine, often described as 'animal' or 'stable' (Chatonnet *et al.*, 1992; 1995). Chatonnet *et al.* (1992) proposed that the formation of ethylphenols in wine by *Dekkera/Brettanomyces* was by a two step process, as detailed in Figure 1.3. The hydroxycinnamic acid precursors occurring in grapes, wine and oak wood are enzymatically decarboxylated, producing the respective vinylphenols which are further metabolised by a reductase forming the corresponding ethylphenols. Strains of *Saccharomyces cerevisiae* can produce vinylphenols in white wine, but not in red wine or in a chemically defined culture medium (Chatonnet *et al.*, 1992; 1993b; 1995). This yeast does not appear to be able to reduce vinylphenols to ethylphenols or produce any volatile phenols in red wines. Chatonnet *et al.* (1992) claims that wine spoilage by *Dekkera/Brettanomyces* is more often due to

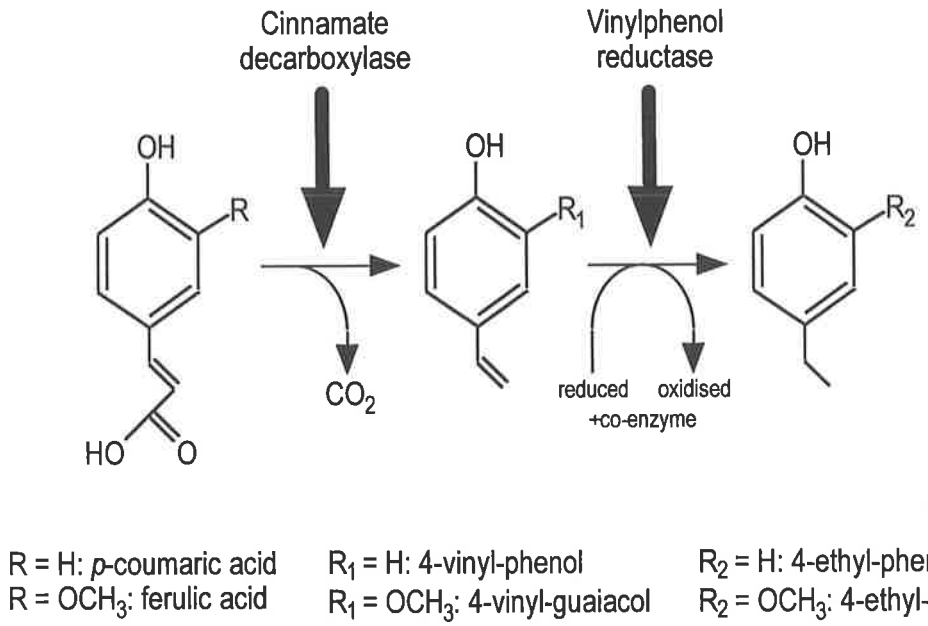


Figure 1.3. Production of volatile phenols by *Dekkera/Brettanomyces* [adapted from Chatonnet *et al.* (1992)].

ethylphenol production rather than mousy taint. This may in fact be correct, however, there are no data available as to the actual incidence of mousy taint spoilage.

Mousy off-flavour production is not discussed in several investigations of *Brettanomyces* spoilage. Van der Walt and van Kerken (1958; 1959a; 1959b; 1960; 1961) in their study of wine spoilage by *Brettanomyces* did not report any occurrence of mousy taint (or any other off-flavours). A survey of New Zealand wineries for the occurrence of *Brettanomyces* yeast did not indicate if any samples of spoiled wine were mousy (Wright and Parle, 1974). Why no discussion of mousy taint was made is unclear; perhaps the wines were not tasted, the researchers insensitive to the taint, or the wines were not mousy due to inappropriate conditions for the production of the off-flavour compounds.

Dekkera/Brettanomyces species are also associated with spoilage of beer (Shimwell, 1938; Smith, 1946; Gilliland, 1961; Rainbow, 1981; Smith *et al.*, 1981), cider (Beech, 1958; Tucknott, 1977; Cabranes *et al.*, 1990) and soft drinks (Kolfschten and Yarrow, 1970; Yarrow and Ahearn, 1971; Pitt, 1974; Put *et al.*, 1976; Smith and van Grinsven, 1984), through formation of sediment and production of acetic acid and off-flavours (not described).

1.3.3 Positive contributions of *Dekkera/Brettanomyces* in beverage production

In some cases *Dekkera/Brettanomyces* yeast are considered desirable for the manufacture of fermented beverages, for example, lambic and gueuze beers (van Oevelen *et al.*, 1977; Verachtert *et al.*, 1990). These beers are produced only in Belgium within a 16 km radius of Brussels and are obtained by spontaneous fermentation of wort by a wide variety of microorganisms (van Oevelen *et al.*, 1976). Van Oevelen *et al.* (1977) describes a mousy flavour that contributes to the typical taste of gueuze and observed that isolates of *Brettanomyces* yeast when grown in pure culture developed a strong mousy taste. Aroma components characteristic to lambic and gueuze beer produced by *Dekkera/Brettanomyces*

yeast include ethyl lactate, ethyl acetate and acetic acid (van Oevelen *et al.*, 1976; Verachtert *et al.*, 1990). *Brettanomyces* are also important to the flavour of English high gravity stock ales (Claussen, 1904; Andrews and Gilliland, 1952; Wiles, 1953). However, this style of beer is no longer common (Anonymous, 1952). *Brettanomyces* yeast have been isolated from Haipao, a fermented tea from Taiwan (Liu *et al.*, 1996), and from natural tequila fermentations (Lachance, 1995).

A role for *Dekkera/Brettanomyces* in wine production has been discussed by several authors (Hock, 1990; Fugelsang *et al.*, 1993; Kunkee and Bisson, 1993). Here the emphasis is on developing complexity in a wine style (particularly red wine), relying on the control of the production of characters such as ethyl-phenols (Section 1.3.1), which at low concentrations are suggested to be positive. This process may simply be an alternative method for managing an already existing *Dekkera/Brettanomyces* spoilage problem.

1.3.4 Incidence of *Dekkera/Brettanomyces* in wine production

Limited information is available on the ecology of *Dekkera/Brettanomyces* yeast. Davenport (1980) reported the isolation of *Brettanomyces* from barley grains, which suggests a possible source of the yeast for beer spoilage. There have been no reports of these yeast isolated from grape skins or vineyards, although *Dekkera/Brettanomyces* yeast were isolated from grape must, walls, floors and equipment in wineries (Peynaud and Domercq, 1956; van der Walt and van Kerken, 1961; Larue *et al.*, 1991; Longo *et al.*, 1991). A survey of New Zealand wineries failed to isolate *Dekkera/Brettanomyces* from winery fixtures, although *Brettanomyces* yeasts were isolated from commercial fermentations (Wright and Parle; 1974). These yeast are commonly isolated from barrel stored wines (Larue *et al.*, 1991; Chatonnet *et al.*, 1992; Chatonnet *et al.*, 1993a; Fugelsang *et al.*, 1993). Many strains of *Dekkera/Brettanomyces* have extracellular ß-glucosidase activity, an enzyme that breaks down cellulose, which is a major constituent of oak wood (Leclerc *et al.*, 1985; Freer, 1991; Spindler *et al.*, 1992). The products of this enzyme

complex are glucose and cellobiose. Cellobiose, which is also present in charred oak wood, can then be used as a carbon source by many strains of *Dekkera/Brettanomyces* (Barnett *et al.*, 1983; 1990). This indicates that barrels are a suitable ecological niche for *Dekkera/Brettanomyces* in wineries, although there are no reports of extracellular ß-glucosidase activity in *Dekkera/Brettanomyces* under winemaking conditions.

The initial source of *Dekkera/Brettanomyces* in wineries is unknown. It has been proposed that insects are the likely vectors, particularly *Drosophila* species (van der Walt and van Kerken; 1961). *Dekkera intermedia* has been isolated from the honey stomachs of bees (Sandhu and Waraich 1985) indicating that insects are possible vectors.

1.3.5 *Dekkera/Brettanomyces* and mousy taint production

The role of yeast in mousy taint formation was confirmed in an investigation of the production of mousy taint in alcoholic apple cider (Tucknott, 1977). Tucknott showed that strains of *Brettanomyces* were able to produce the taint when grown under laboratory conditions. *Brettanomyces anomalus* (NCYC 449) produced taint (determined by tasting) when grown in apple juice or blackcurrant juice. A more detailed examination of mousy taint production was carried out for *B. bruxellensis*, *Brettanomyces claussenii* and *Brettanomyces. lambicus*, as well as *B. anomalus*. The yeasts were grown aerobically in three different media; apple juice plus 1% (w/v) yeast extract, or two different chemically defined media. All strains produced taint, but only in apple juice supplemented with yeast extract medium. It was concluded that medium composition was a critical factor. A synthetic medium (Wikén *et al.*, 1961) was modified (compounds potentially toxic were omitted as cultures were to be assessed organoleptically) and used as a base medium for further investigation. All strains were grown successfully in this medium, but only a slight taint was detected with *B. lambicus*. Cells from each culture were washed and inoculated into the apple juice plus 1% (w/v) yeast extract medium and all stains produced a mousy taint. This indicated again that a constituent of apple juice and/or yeast extract, while not

essential for growth, was acting as a precursor or activator for taint production. This same effect of yeast extract addition was also found for several *Lactobacillus* species co-inoculated with *S. cerevisiae* (taint was not produced by this yeast alone) (Tucknott, 1977).

The difference between the chemically defined medium and apple juice/yeast extract medium was nitrogen composition, specifically amino acids. A series of experiments (*B. anomalus*, grown in a chemically defined medium) were then undertaken to assess the potential of various amino acids to act as a mousy taint inducer. L-Lysine was found to be the only amino acid to induce off-flavour production, and amino acid analysis of the fermentation medium revealed that L-lysine had been removed (Tucknott, 1977). Heresztyn (1986a) confirmed that L-lysine could induce mousy taint production, using the strain *B. intermedius* (CBS 73) and culture conditions similar to those used by Tucknott. Analysis of a mousy fermentation extract by GC/MS showed that ACTPY, the compound reported to be responsible for mousy taint in wine, was only produced by *B. intermedius* if L-lysine was added to the chemically defined growth medium (Heresztyn (1986a).

1.4 Factors affecting mousy taint production by *Dekkera/Brettanomyces*

1.4.1 Lysine catabolism

That taint production does not occur without this amino acid indicates a possible role of L-lysine catabolism in mousy taint formation. L-Lysine occurs in the amino acid pool in both grape juice [1.2–23 mg/L (Ough and Amerine, 1988), 1–64 mg/L (Henschke and Jiranek, 1993)] and wine [0.0–35.7 mg/L (Ough and Amerine, 1988), 7.5–17 mg/L (Lehtonen, 1996)]. Therefore, grape juice and wine could act as the source of this potential precursor of mousy taint compounds. However, nothing is known of the specific action of L-lysine in mousy taint production. A better understanding of the catabolism of L-lysine by yeast may provide a clue as to the role of this amino acid in the formation of mousy taint compound(s) by *Dekkera/Brettanomyces*.

Many pathways of L-lysine degradation have been proposed and at least nine different initial steps in the catabolism of this amino acid have been described in nature (Fothergill and Guest, 1977; Misono and Nagasaki, 1982; Hammer *et al.*, 1991b). In mammals and some fungi, L-lysine can be degraded via saccharopine (Higashino *et al.*, 1965; Wickwire *et al.*, 1990). The other initial steps in microbial degradation of L-lysine so far described include: lysine decarboxylase (Soda and Moriguchi, 1969; Sabo, 1974); α-oxidase (deaminating) (Kusakabe *et al.*, 1980); monooxygenase (decarboxylating) (Takeda and Hayaishi, 1966); mutase (Chirpich *et al.*, 1970); racemase (although activity of this enzyme is inadequate to support growth) (Ichihara *et al.*, 1960; Chang and Adams, 1971; Chang and Adams, 1974); ε-dehydrogenase (deaminating) (Misono and Nagasaki, 1982; Hammer *et al.*, 1991a); ε-N-acetylation (Schweet *et al.*, 1955; Rothstein, 1965; Guenegerich and Broquist, 1976); and ε-N-transamination (which is atypical in nature) (Soda *et al.*, 1968; Kern *et al.*, 1980; Kinzel *et al.*, 1983).

Lysine catabolism in yeast was reviewed by Large (1986). The first pathway postulated for yeast was via ε-N-acetylation in *Hansenula saturnus* (Rothstein, 1965), *S. cerevisiae* (Guenegerich and Broquist, 1976) and *Yarrowia lipolytica* (Gaillardin *et al.*, 1976). The enzyme L-lysine ε-N-acetyltransferase is responsible for the acetylation of lysine (Schmidt *et al.*, 1988b) and the structural gene (*LYC 1*) encoding this acetyltransferase has been identified in *Y. lipolytica* (Bode *et al.*, 1993). Activity of this enzyme has been reported for several other yeasts including *Candida* species, *Metchnikowia pulcherrima*, *Rhodotorula aurantiaca* and *Saccharomycopsis capsularis* (Hammer *et al.*, 1991b). The pathway postulated for the degradation of L-lysine commencing with ε-N-acetylation is shown in Figure 1.4. An L-lysine induced δ-aminovalerate, α-ketoglutarate aminotransferase, has been described in yeast (der Garabedian, 1986b; der Garabedian and Vermeersch, 1989). This enzyme may be the second transaminase involved in the acetyl-lysine pathway (Figure 1.4). The acetylation of L-lysine stops the formation of cyclic intermediates (Paik, 1962; Paik and Kim, 1964; Rothstein, 1965). This suggests that acetylation pathway is not

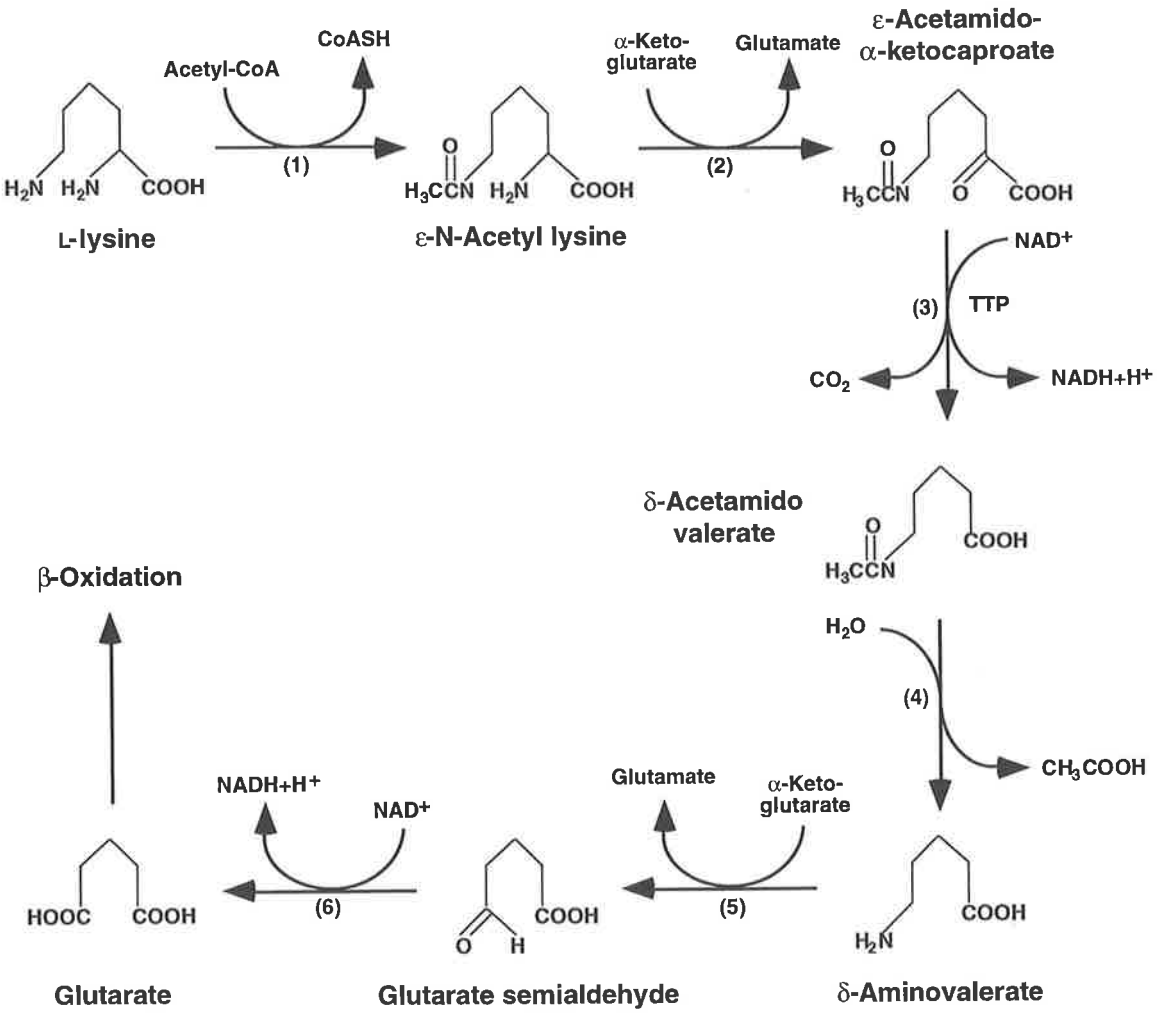


Figure 1.4. Proposed pathway of L-lysine catabolism in yeast via ε-N-acetylated intermediates (Rothstein, 1965; Gaillardin *et al.*, 1976; Guenegerich and Broquist, 1976; Large, 1986). Enzyme details: (1) Acetyl-CoA: L-lysine ε-N-acetyltransferase (Schmidt *et al.*, 1988b; Bode *et al.*, 1993), (2) ε-N-acetyl lysine: α-ketoglutarate aminotransferase, proposed by Guenegerich and Broquist (1976), (3) α-keto-ε-acetamidohexanoate dehydrogenase, proposed by Gaillardin *et al.* (1976) and Large (1986), requiring thiamine pyrophosphate (TPP) (Guenegerich and Broquist, 1976), (4) δ-acetamidovalerate deacetylase, proposed by Large (1986), (5) δ-aminovalerate aminotransferase, proposed by Gaillardin *et al.* (1976) and Large (1986), (6) glutarate semialdehyde dehydrogenase, proposed by Large (1986).

involved in the biosynthesis of the mousy taint compounds, as each contain a ring structure.

A second pathway of L-lysine catabolism described for yeast is via oxidative deamination by lysine ε-aminotransferase (Figure 1.5). This enzyme was first described from *Rhodotorula glutinis* using α-ketoglutarate as the amino group acceptor and accumulated α-aminoadipate-δ-semialdehyde and glutamate as products (Kinzel *et al.*, 1983). In solution, α-aminoadipate-δ-semialdehyde primarily exists in its dehydrated form, Δ^1-piperideine-6-carboxylate (Basso *et al.*, 1962; Soda *et al.*, 1968) (Figure 1.5). The importance of the ε-aminotransferase in L-lysine catabolism in *R. glutinis* was demonstrated through the use of a mutant strain blocked for formation of α-aminoadipate-δ-semialdehyde by an alternative step, the simple reversal of the L-lysine biosynthetic pathway (Kinzel and Bhattacharjee, 1979). A significant derepression of ε-aminotransferase activity (determined by α-aminoadipate-δ-semialdehyde concentration) in L-lysine grown wild type and mutant cells was observed, verifying the role of this enzyme in L-lysine catabolism.

This α-ketoglutarate dependant ε-aminotransferase has been detected in other yeast including *B. anomalus, Debaryomyces hansenii, Hansenula fabianii, Cryptococcus uniguttlatus* and *S. cerevisiae* (Hammer *et al.*, 1991b; Thomas and Ingledew, 1994). Pyruvate and oxaloacetate dependant ε-aminotransferases have been described for L-lysine catabolism in yeast (Schmidt *et al.*, 1987; 1988a; Hammer *et al.*, 1991b).

The further breakdown of α-aminoadipate-δ-semialdehyde is probably via α-aminoadipate as has been identified in *Pseudomonas* species (Fothergill and Guest, 1977) (Figure 1.5). Some evidence for the existence of this pathway in yeast has been found. The enzyme glutamate-α-ketoadipate transaminase, which catalyses the conversion of α-aminoadipate to α-ketoadipate (Figure 1.5), has been characterised from a *Saccharomyces* species and *Schizosaccharomyces pombe* (Matsuda and Ogur, 1969; Ye *et al.*, 1991). Thomas and

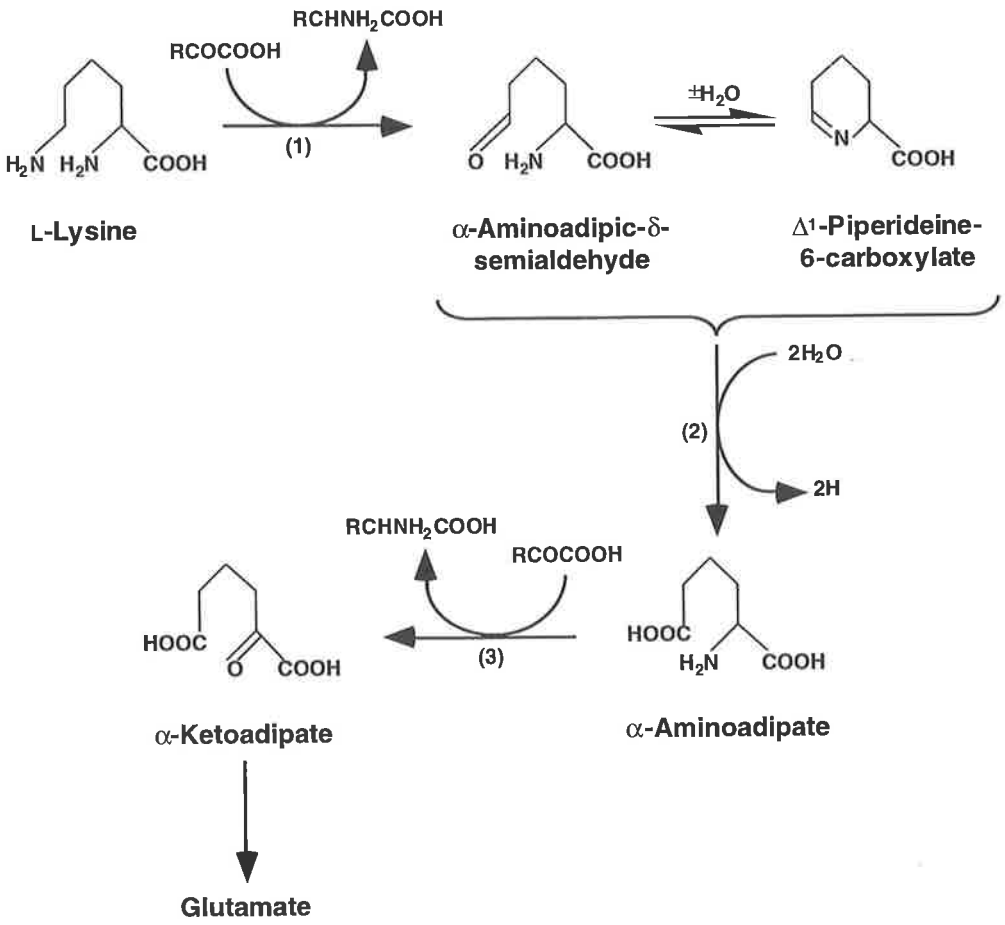


Figure 1.5. Proposed pathway of L-lysine catabolism in yeast via lysine ε-aminotransferase (1) (Kinzel *et al.*, 1983; Hammer *et al.*, 1991b; Thomas and Ingledew, 1994). The pathway probably progresses in a similar way to that proposed in *Pseudomonas* (see Fothergill and Guest, 1977). Other enzymes involved: (2) L-α-aminoadipate δ-semialdehyde oxidoreductase (Calvert and Rodwell, 1966) which has not been described in yeast, (3) L-α-aminoadipate aminotranferase from *Pseudomonas* (Hartline and Rodwell, 1971), yeast enzymes with similar activity have been reported (Matsuda and Ogur, 1969; Ye *et al.*, 1991). For the remaining steps to glutamate see Reitz and Rodwell (1969), and Hartline and Rodwell (1971). R: α ketoglutarate, pyruvate, or oxaloacetate.

Ingledew (1994) provided further evidence for the *Pseudomonas* pathway in yeast. They showed that L-lysine-grown *S. cerevisiae* which accumulated α-ketoadipate from α-aminoadipate, was not the result of the reversal of the L-lysine biosynthetic pathway. The reversal of the biosynthetic route potentially is an additional L-lysine degradation pathway in yeast. This process as a catabolic route has been described in mammals and other fungi (Higashino *et al.*, 1965; Wickwire *et al.*, 1990). L-Lysine biosynthesis proceeds via α-ketoadipate, α-aminoadipate, α-aminoadipate-δ-semialdehyde and saccharopine to L-lysine (Kinzel and Bhattacharjee, 1979; Zaret and Sherman, 1985).

A third variation in L-lysine catabolism by yeast is via L-lysine ε-dehydrogenase, that was found to catalyse the initial degradation step in *Candida albicans* (Hammer *et al.*, 1991a). Activity of this enzyme corresponded with an intracellular accumulation of α-aminoadipate-δ-semialdehyde (Δ^1-piperideine-6-carboxylate) (Figure 1.6). Further catabolism would probably proceed via α-aminoadipate and the *Pseudomonas* pathway, as described with the ε-aminotransferase system detailed in Figure 1.5. L-Lysine dehydrogenase, however, does not appear to be common in yeast, and has only been reported in one other genus, *Kluyveromyces marxianus* (Hammer *et al.*, 1991b). Both the ε-aminotransferase and the ε-dehydrogenase pathway produce N-heterocyclic intermediates. These cyclic compounds potentially could be involved in mousy taint biosynthesis.

Apparently, some yeast have multiple routes for the catabolism of L-lysine. For example, *S. cerevisiae* possesses at least two pathways, one via L-lysine ε-N-acetylation (Guenegerich and Broquist, 1976; Bode *et al.*, 1993) and another with L-lysine ε-aminotransferase (Thomas and Ingledew, 1994). This may be strain specific as other investigations found no L-lysine ε-aminotransferase activity in *S. cerevisiae* (Large, 1986; Hammer *et al.*, 1991b). By comparison, other yeast may only have one route of L-lysine degradation. *Brettanomyces anomalus,* when tested for all three pathways described above only contained the α-ketoglutarate specific ε-aminotransferase (Hammer *et al.*, 1991b). It

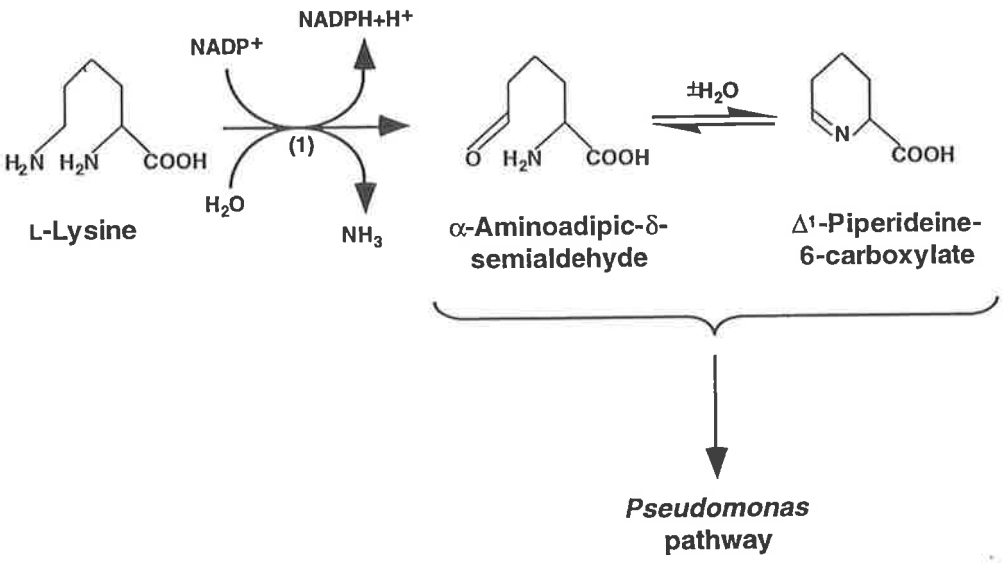


Figure 1.6. Proposed pathway of L-lysine catabolism in yeast via lysine ε-dehydrogenase (1) Hammer *et al.* (1991a; b). The proposed catabolism progresses via the *Pseudomonas* pathway as described for the ε-aminotransferase in yeast (see Figure 1.5).

is tempting to attribute this variation to differences in the ability of yeast to use lysine as a sole nitrogen and/or carbon source and yeast vary widely in this characteristic (Schultz and Pomper, 1948; Walters and Thiselton, 1953; Hedrick and Dupont, 1968; Hammer *et al.*, 1991b). While *B. anomalus* can utilise L-lysine as a sole nitrogen and carbon source (Walters and Thiselton, 1953; Hammer *et al.*, 1991b), *S. cerevisiae* can only use L-lysine as a sole carbon source. However, in *S. cerevisiae* L-lysine degradation can proceed via acetylation, which does not occur with *B. anomalus* (ε-aminotransferase activity in *S. cerevisiae* was described when lysine was not the sole source of nitrogen). In order to understand why this variation exists, further investigation of L-lysine metabolism and its regulation in yeast is required.

It is unclear if differences in L-lysine catabolism between genera can be used to account for mousy taint production. A better understanding of the role L-lysine plays in the formation of the compounds responsible is necessary before this distinction can be made. The pathway described for the catabolism of L-lysine by ε-aminotransferase in *Dekkera/Brettanomyces*, if related to mousy taint formation, would result in the incorporation of the α-amino group of L-lysine into the mousy taint compound(s). However, the fact that multiple pathways for the degradation of L-lysine can occur within one yeast strain suggests that the primary pathway of L-lysine catabolism is not necessarily the same route as employed for mousy taint biosynthesis.

1.4.2 Ethanol and mousy taint

Ethanol also appears to be a critical agent for mousy taint biosynthesis. It is particularly important for LAB, which do not produce a significant level of mousy taint without it (Tucknott, 1977; Heresztyn, 1986a). Tucknott (1977) described a symbiosis between a *Lactobacillus* and a *S. cerevisiae* strain for the production of mousy taint in an apple juice medium (containing 1% (v/v) yeast extract). To determine if a component produced by the yeast was acting as a mousy taint precursor, the *Lactobacillus* was grown in apple juice

medium supplemented with individual alcohols. Sensory evaluation of the culture media for mousy taint was then carried out. Only ethanol produced a strong response; a slight taint was detected with the addition of *n*-propanol, but not with *iso*-propanol, *n*- or *iso*-butanol, 2- or 3-methyl-butanol and *n*-hexanol (Tucknott and Davies, 1975; Tucknott, 1977).

This work was confirmed using *L. brevis* grown in 50% (v/v) grape juice medium supplemented with 0.5% (w/v) yeast extract (Heresztyn, 1986a). The mousy taint compound, ACTPY was detected only when 5% (v/v) ethanol or *n*-propanol were added to the growth medium. Further evidence for the importance of these alcohols was provided by GC/MS detection of the propionyl analogue of ACTPY (Figure 1.7). When *n*-propanol was added to the growth medium 2-propionyltetrahydropyridine was produced by both *L. brevis* [50% (v/v) grape juice, 0.5% (w/v) yeast extract, 5% (v/v) *n*-propanol) and *B. intermedius* (chemically defined medium, supplemented with 5% (v/v) *n*-propanol). This indicates that ethanol also influences taint production by yeast.

The formation of 2-propionyltetrahydropyridine suggests that the role of ethanol in mousy taint biosynthesis is to provide the acetyl side chain of ACTPY. Therefore, L-lysine serves as the precursor to the tetrahydropyridine ring.

1.4.3 The role of air/oxygen

The work carried out by Tucknott (1977) and Heresztyn (1986a) has investigated mousy taint production by *Dekkera/Brettanomyces* only in the presence of air (aerobic or air-saturated conditions). Anaerobic treatments were not considered. Anecdotal evidence has suggested a possible effect of air on mousy taint formation. This effect on taint production by air or oxygen must therefore be considered.

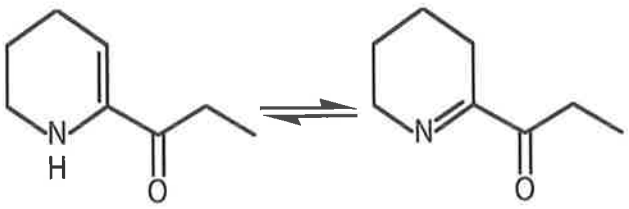


Figure 1.7. 2-Propionyltetrahydropyridine; propionyl analogues of 2-acetyltetrahydropyridine.

A characteristic of the genera *Dekkera/Brettanomyces* is the 'negative Pasteur effect', that is, alcoholic fermentation is inhibited under strict anaerobic conditions but stimulated by the presence of molecular oxygen. The negative Pasteur effect was re-named the 'Custers effect' to avoid confusion with the Pasteur effect (Scheffers, 1966). The Custers effect has been shown to occur in all strains of *Dekkera/Brettanomyces* species examined but also occurs in a limited number of other genera (van der Walt and van Kerken, 1959b; Wikén *et al.*, 1961; Scheffers, 1966; Scheffers and Wikén, 1969).

Air or oxygen could play a role in mousy taint production by *Dekkera/Brettanomyces*, perhaps by directly stimulating fermentation via the Custers effect. These yeast may be present at low levels in wine stored under anaerobic conditions and given a small air addition, through a standard wine processing treatment, with the resultant increase in cell number and fermentation activity inducing mousy taint production. At this stage the influence of growth and fermentation stimulation on taint production has not been investigated.

The Custers effect was attributed to the characteristic production of acetic acid by *Dekkera/Brettanomyces* resulting in an initial shortage of nicotinamide adenine dinucleotide (NAD^+) on the addition of glucose (Scheffers, 1961; Scheffers and Misset, 1974). This shortage can be alleviated by a minute concentration of oxygen or organic hydrogen acceptors (for example, acetoin, acetone, acetaldehyde or dihydroxyacetone), whereby NADH is oxidised by the respiratory chain to restore the redox balance and drive glycolysis at the level of glyceraldehyde 3-phosphate (Scheffers, 1961; 1963; 1966; Scheffers and Nanninga, 1977; Carrascosa *et al.*, 1981; Gaunt, 1988).

1.5 Proposed pathway for mousy taint formation

Figure 1.8 describes postulated pathways for the formation of mousy compounds by *Dekkera/Brettanomyces*. Previous observations indicate that *Dekkera/Brettanomyces* can

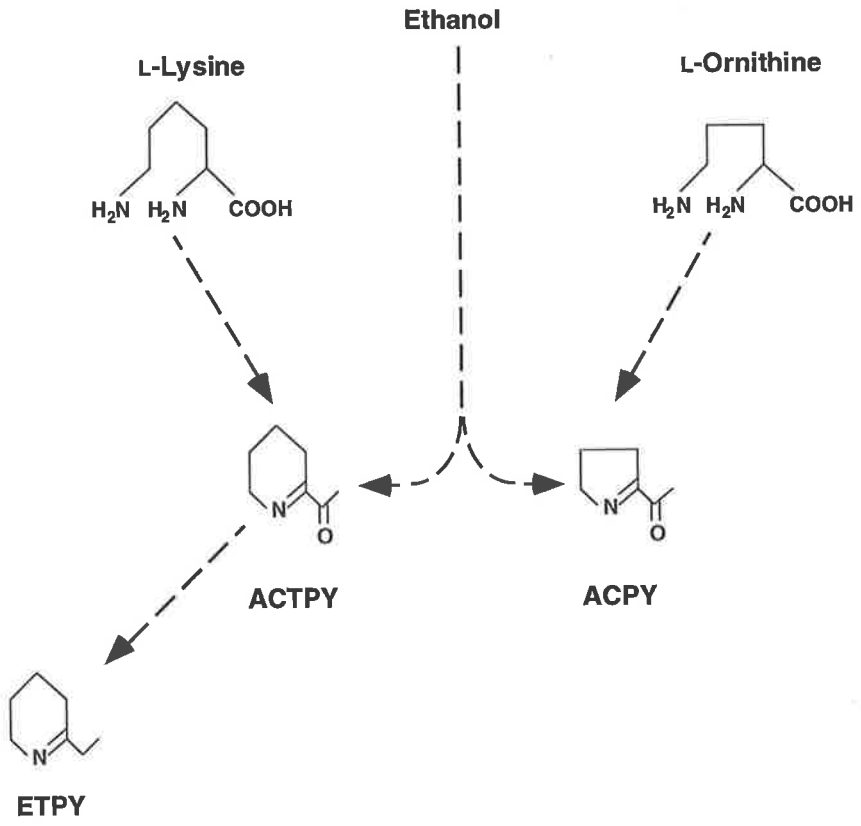


Figure 1.8. Postulated pathways of mousy compound formation from the amino acids L-lysine and L-ornithine, and ethanol. ACTPY, tautomers of 2-acetyltetrahydropyridine; ACPY, 2-acetylpyrroline; ETPY, 2-ethyltetrahydropyridine.

produce mousy taint. The most important mousy taint compound so far identified in wine is ACTPY. The C_5N backbone of L-lysine provides C_5N ring of ACTPY, and the acetyl group of ACTPY is probably provided by carbon metabolism, in particular via ethanol. That L-lysine is a precursor rather than L-proline, as is the case for the thermal production of ACTPY, may indicate that the mechanism of formation in the yeast system is different to that of the Maillard system. The catabolism of L-lysine in relation to ACTPY biosynthesis has not been investigated. ETPY is considered to be of limited importance to mousy taint, although it can be produced by *Dekkera/Brettanomyces*. The formation of ETPY, which has also been related to L-lysine, may proceed via ACTPY or by some other means.

In view of the similarity in the structure, and the sensory characteristics of ACPY and ACTPY, ACPY must be considered as a possible contributor to mousy taint in wine. Therefore, the C_4N backbone of the amino acid L-ornithine would be the likely precursor to the C_4N ring of ACPY. Further, it is proposed that the acetyl group of ACPY would be provided by the same mechanism as for ACTPY.

1.6 Research aims

The predications made in the proposed pathway were considered further. Characterisation of the three compounds proposed to contribute to mousy taint in wine and their biosynthesis by *Dekkera/Brettanomyces* was undertaken.

The lack of a sensitive chemical method for the quantification of mousy taint compounds has always limited research in this area. Both Tucknott (1977) and Heresztyn (1986a), while able to detect ETPY and/or ACTPY, were not able to measure and compare the concentration of these compounds. The development of a reliable analytical method was carried out. The implementation of this method was beneficial in enabling the identification of the compound related to mousy taint, allowing for the first time the

reporting of quantitative data of mousy taint in wine, as well as serving as a tool for a detailed examination of mousy taint production by *Dekkera/Brettanomyces*.

There are only limited data available on the conditions that are conducive to taint production by *Dekkera/Brettanomyces*. More information on the growth and biochemistry of *Dekkera/Brettanomyces* in relation to the production of mousy taint in wine was required. Initial investigations focused on whether mousy taint production was a strain/species specific effect, or a characteristic of these genera. Screening of the type strains of *Dekkera/Brettanomyces* in relation to mousy taint production was therefore carried out.

Based on this investigation a more detailed analysis of selected type strains was performed and involved an examination of the growth of these strains under defined conditions. The precursors of mousy taint compounds were examined, and the catabolism of L-lysine by *Dekkera/Brettanomyces* with respect to the biosynthesis of ACTPY was investigated. An assessment of other physiological factors was carried out, including how growth of these yeast related to taint production, and if air or oxygen was important to the ability of the selected strains to produce mousy taint compound(s).

The data produced from these investigations was used to propose a pathway(s) for the biosynthesis of mousy taint compounds, which was then incorporated into a model for the production of mousy taint by *Dekkera/Brettanomyces*. The model may then be used to develop strategies to limit or abolish the formation of mousy taint spoilage in wine.

CHAPTER 2 Method Development for the Analysis of Mousy Taint Compounds

2.1 Introduction

Research investigating the nature and origin of mousy taint in fermented beverages has always been limited by the lack of a reliable analysis procedure. It is the physico-chemical properties of the compounds involved that has made the development of an analytical technique difficult. This chapter describes the development of a highly sensitive method for the extraction, analysis and quantification of mousy taint compounds. The work described in this chapter was carried out jointly by the author and fellow post graduate student Mr Peter J. Costello, supervised by Dr Markus Herderich.

Of the compounds so far described as responsible for mousy taint, ETPY and in particular the ACTPY tautomers, are both labile (Hunter *et al.*, 1969; Büchi and Wüest, 1971; Buttery *et al.*, 1971; Tucknott, 1977; Craig and Heresztyn, 1984; De Kimpe and Stevens, 1993; De Kimpe and Stevens, 1995), volatile [*i.* ACTPY bp *ca.* 35 °C, 0.1 mm, Büchi and Wüest (1971), 23–29 °C, 0.01 mm, De Kimpe and Keppens (1996), *ii.* ETPY bp 42 °C, 20 mm, Tucknott (1977), 50 °C, 30 mm, Craig and Heresztyn (1984)] and very potent. The odour threshold of ACTPY in water has been reported as 1.6 µg/L (Teranishi *et al.*, 1975). Therefore any analytical procedure has to be highly sensitive and the technique must be free from any potential to produce artefacts. No quantitative data has been reported in relation to these compounds in mousy tainted beverages.

The work described in this chapter focused on the analysis of ACTPY, which is the most important compound in relation to mousy off-flavour in wine. The study also investigated ETPY and ACPY, which have been described as having mousy characteristics, to gain a better understanding of the nature of mousy taint and the particular contribution of these compounds.

Several extraction techniques that could provide a reliable analysis of mousy taint compounds in wine and microbiological culture media were considered. These procedures included ion exchange, which had been previously described in mousy taint analysis (Tucknott, 1977; Strauss and Heresztyn, 1984; Heresztyn, 1986a), simultaneous distillation and extraction, a technique commonly used in the analysis of food and beverage volatiles (Schultz *et al.*, 1977; Buttery *et al.*, 1983; Schieberle and Grosch, 1987b; Gasser and Grosch, 1988; Blank *et al.*, 1989; Herderich and Winterhalter, 1991) and continuous liquid/liquid extraction which has been previously used for the analysis of grape juice, wine and other alcoholic beverages (Clutton and Evans, 1978; Wilson *et al.*, 1984; Marais, 1986). Continuous liquid/liquid extraction has been used in mousy taint analysis (Craig and Heresztyn, 1984). All analysis was undertaken with capillary GC and/or GC/MS to provide qualitative and quantitative data of the volatile compounds extracted.

An important analytical technique for describing the sensory characteristics of volatiles is GC–sniff. This combination separates individual compounds from the background matrix and allows aroma assessment of the GC column effluent (Belitz and Grosch, 1987). GC–sniff has been used previously to identify mousy taint compounds in wine and cider (Tucknott, 1977; Strauss and Heresztyn, 1984; Heresztyn, 1986a) and was used further as part of this work to investigate the mousy character of ACPY and ETPY.

2.2 Materials and methods

2.2.1 Chemicals and reagents

All reagents, solvents and gases used in this study were of analytical grade. Solvents were redistilled prior to use. The internal standards 4-N,N-dimethylaminopyridine, 2-, 3- and 4-acetylpyridine were obtained from Aldrich Chemicals (Castle Hill, NSW, Australia). All water used was ultra-pure (18 kM?) produced by a Milli-Q reagent system (Millipore, Bedford, MA, USA).

2.2.2 Mousy wine samples

The wine samples used in these investigations had been submitted to The Australian Wine Research Institute Technical Services Group for analysis, during the period of 1993–1996. The wines were described as mousy after sensory evaluation by a panel of judges familiar with the characteristics of this off-flavour. All wine samples were 0.2 μm membrane filtered prior to extraction.

2.2.3 ACTPY

2.2.3.1 Synthesis

The tautomers of ACTPY, 2-acetyl-1,4,5,6-tetrahydropyridine (I) and 2-acetyl-3,4,5,6-tetrahydropyridine (II) were synthesised according to the method of Hunter *et al.* (1969). Analysis was carried out by GC/MS (see Section 2.2.3.3B). A standard solution of ACTPY was prepared by dissolving a known amount of the synthetic product in dichloromethane. The remaining ACTPY was stored as a dilute solution in ethanol or as a bisulphite adduct. The bisulphite adduct was prepared by dissolving the synthetic ACTPY (90 mg) in dichloromethane (20 mL). It was then washed with $NaHSO_3$ (0.5% (w/v), 4 x 5 mL), all glassware was then rinsed with $NaHSO_3$ (20 mL) and the fractions combined and stored overnight at –20 °C. The frozen pooled fractions were then lyophilised (16 hr), and the resulting white powder was stored at –20°C. Fast atom bombardment mass spectral analysis of the powder was used to confirm if the ACTPY–$NaHSO_3$ adduct was obtained (see Section 2.2.3.3C). ACTPY could be recovered by redissolving the adduct in ethanol and filtering off the precipitated bisulphite.

2.2.3.2 Continuous liquid/liquid extraction

This procedure was carried out using Freon F11 as the organic solvent as detailed in Figure 2.1. The initial manual liquid/liquid extraction at pH 2.5 removes acid and neutral volatiles prior to the overnight continuous extraction. The first internal standard was 4-

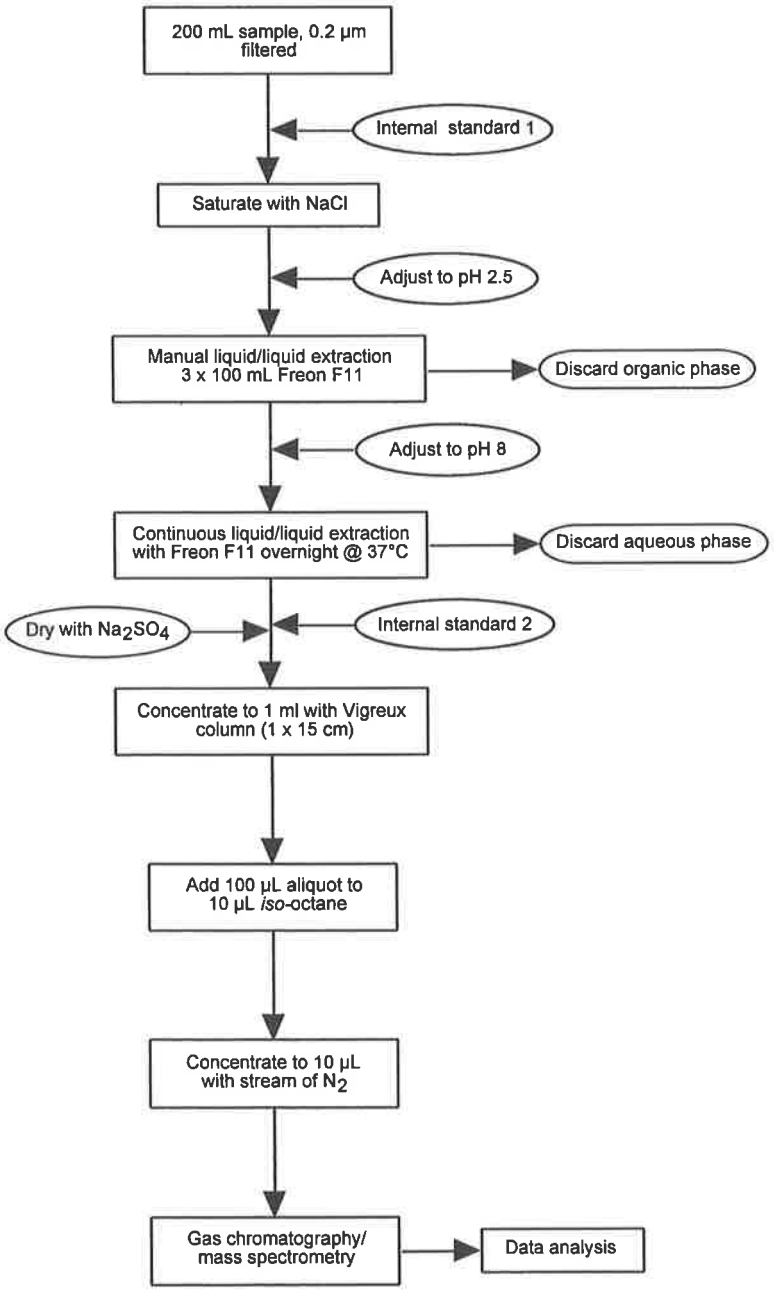


Figure 2.1. Extraction protocol combining manual liquid/liquid extraction and continuous liquid/liquid extraction of mousy taint compounds. Internal standard 1 was 4-acetylpyridine (100 µg/L) and internal standard 2 was 3-acetylpyridine (100 µg/L).

acetylpyridine. The addition of 3-acetylpyridine, the second internal standard, allowed monitoring of the extraction efficiency. All wine and culture media extractions were then carried out as described in Figure 2.1.

2.2.3.3 Analysis

A. Capillary gas chromatography (GC). Analysis was carried out using a Varian 3300 gas chromatograph with a flame ionisation detector (FID) coupled with a Milton-Roy C1–10 integrator. The chromatograph was equipped with either a 30 m J & W DB1701 fused silica column, 0.32 mm i.d. and 0.25 μm film thickness or a 30 m J & W Carbowax 20 CAM fused silica column, 0.25 mm i.d and 0.25 μm film thickness. Split injections were made with a split ratio of 1:25, and the carrier gas (helium) flow rate was 25 mL/min. The GC temperature program for the DB1701 column was 60°C for 3 min, to 120°C at 5°C/min, and to 220°C at 20°C/min, injector temperature was 240°C and detector 260°C. For the Carbowax column, which is temperature sensitive, injector and detector temperature was 220°C. The column temperature program was as for DB1701.

B. Capillary gas chromatography/mass spectrometry (GC/MS). Analysis was undertaken with a Finnigan TSQ 70 mass spectrometer directly coupled to a Varian 3400 gas chromatograph. The chromatograph was equipped with a 30 m J & W Carbowax 20 CAM fused silica column, 0.25 mm i.d and 0.25 μm film thickness. Injections were made with (1) a split injector at 200 °C and a split ratio of 1:25, (2) splitless (0.5 min), or (3) on-column injection (temperature program as for column oven). The column was held at 60 °C for 3 min, programmed at 5 °C/min to 220 °C and held at 220 °C for 5 min. Electron impact mass spectra (EIMS) were taken at an ionisation voltage of 70 eV and the scanned mass range was *m/z* 40–200.

C. Fast atom bombardment mass spectrometry (FAB MS/MS). Analyses were carried out using a triple stage quadrapole Finnigan TSQ 70 mass spectrometer. Xenon was the

bombardment gas, the ionisation voltage was 8 KeV, ion current < 0.5 mA and the collision cell pressure 1.800 millitorr. The matrix used was glycerol.

2.2.3.4 Quantification

Quantification was carried out for GC analyses by comparison of the integrated area under the peak of interest with the integrated area of a known amount (amt) of internal standard (IS) added as described by the following equation;

$$\text{amt ACTPY} = (\text{amt IS} \times \text{area ACTPY})/\text{area IS}$$

The assumption was made that the relative responses for the target compound and the internal standard is unity (see Section 2.3.1.2).

Quantification of the GC/MS analysis used the same equation, however, area integrations of target compounds and internal standard were determined using an auto-quantification system (Finnigan ICIS software). This program was set up to integrate the area of a peak after first identifying it as the target or the internal standard by comparison of mass spectral and retention time data of an authentic reference sample previously analysed. As for GC analyses the response factor was assumed to be 1.

For trace concentrations of target compounds, quantification was carried out by measuring the abundance of selected ions; *m/z* 111 for ACPY, *m/z* 110 for ETPY and *m/z* 125 for ACTPY. By determining the area of these ion trace peaks and that of the internal standard ion trace peak (*m/z* 121), and assuming the response factor of the selected target ion with respect to internal standard ion was 1:1, trace concentration of the target compounds could be calculated using the formula detailed above.

2.2.3.5 Protium/deuterium exchange

To D_2O (1 mL), ACTPY/ACPY (0.1 mL) reference material in ethanol was added and mixed thoroughly for approximately 1 min. This solution was then immediately extracted

with diethyl ether (3 x 0.5 mL). The extracts were pooled and analysed by GC/MS (Section 2.2.3.3B, split injections).

2.2.4 ETPY

2.2.4.1 Synthesis

2-Ethyl-3,4,5,6-tetrahydropyridine was synthesised using a modified method of Tucknott (1977). Diethyl ether was dried over NaOH (20 g/L) for 1 hr prior to redistilling. N-chlorosuccinimide (26.7 g) was added to diethyl ether (200 mL). To this slurry, 2-ethylpiperidine (19.6 mL) was added and stirred for 15.5 hr at ambient temperature under N_2. This reaction step was monitored by GC/MS. Water was then added (200 mL Milli-Q) and the diethyl ether layer removed. The aqueous layer was extracted with 2 x 20 mL diethyl ether and all ether fractions combined, filtered (Whatman No. 4) and then dried over Na_2SO_4 for 3 hr. The combined diethyl ether fractions were then filtered, MeOH (200 mL) added and the diethyl ether removed by rotary evaporation.

To this 1-chloro-2-ethylpiperidine solution (20 mL, 93% purity by GC/MS), ground KOH pellets (3 g) were added and stirred for 4 hr. This slurry was then filtered and passed down a K_2CO_3 column (containing *ca.* 5 g K_2CO_3, dried for 1 hr at 75°C). The column eluent was then filtered, and dried over Na_2SO_4 (1 hr), and stored at –20°C. A sample was analysed by GC/MS for mass spectral comparison with published data (Tucknott, 1977; Craig and Heresztyn, 1984).

2.2.4.2 Identification of ETPY in mousy samples

Identification of ETPY was determined by comparison of retention time and mass spectral data of the target compound with that of synthetic ETPY and published data (Tucknott, 1977; Craig and Heresztyn, 1984). The identity of ETPY in mousy samples was also confirmed by a coinjection analysis. This was undertaken by spiking synthetic ETPY into a sample known to contain the target compound (ETPY produced by fermentation), and

monitoring for an increase in the intensity of the target compound by GC/MS (Section 2.2.3.3B, splitless).

2.2.5 ACPY

2.2.5.1 Reference material

An authentic sample of ACPY was kindly donated by Dr Ron Buttery of the Agricultural Research Service, Western Regional Research Center, U.S. Department of Agriculture, Albany, CA. Identification was made on the basis of comparing mass spectral data of this reference sample and published data (Buttery *et al.*, 1982; 1983).

2.2.5.2 Identification of ACPY in mousy wine

An unknown compound in mousy wine extracts was identified as ACPY by comparison of retention time and mass spectral data with that of the authentic reference sample.

2.2.5.3 GC/MS analysis

See 2.2.3.3.B, splitless injections.

2.2.5.4 Capillary gas chromatography-sniff (GC–sniff)

A Varian 3300 gas chromatograph equipped with FID and modified with an external Teflon sniff cup was used. The same column was used as described in Section 2.2.3.3B. An SGE Vitreous Silica outlet splitter separated the sample between the FID and the sniff cup. The FID and sniff cup were connected to the splitter via 2 x 30 cm, 75 µm deactivated fused silica columns (LC Packing, Switzerland) with a split ratio of 1:1. Splitless injections were performed (0.5 min). The temperature program was 80 °C for 3 min, then 10 °C/min to 180 °C. The helium carrier gas flow rate was 25 mL/min. Water saturated air at 100 mL/min was passed through the sniff cup. The column temperature was maintained from the GC to the sniff cup at 150 °C by a heated transfer line.

2.3 Results and Discussion

2.3.1 ACTPY

2.3.1.1 Synthesis

By comparison with published mass spectral data, synthesis of ACTPY was confirmed (Strauss and Heresztyn, 1984; Heresztyn, 1986a; De Kimpe and Stevens, 1993; Roberts and Acree, 1994; Buttery and Ling, 1995; Schieberle, 1995). Direct retention time comparison could not be carried out as there was no published data for the capillary GC column used in this study (J&W Carbowax 20 CAM). Data for a similar column, Chrompack CP-Wax/Amine [Kováts index: ACTPY I 1433 and ACTPY II 1635, Schieberle, (1991)], was in close agreement. The mass spectral data of ACTPY I *m/z* (% relative intensity): 125 molecular ion (M^+) (88), 124 (7), 110 (4), 97 (30), 83 (87), 82 (92), 55 (79), 54 (69), 43 (100), 41 (23); the mass spectral data of ACTPY II *m/z* (%): 125 (M^+ 84), 124 (22), 110 (8), 83 (11), 82 (100), 55 (19), 54 (47), 43 (29), 41 (7). A linear retention index (LRI) was determined by comparison with an *n*-alkane series, that was analysed under the same GC/MS conditions and temperature program as the target compounds (Section 2.2.3.3B, splitless injection) (Ettre, 1993). The LRI for ACTPY I was 1439, and 1665 for ACTPY II.

The unstable nature of ACTPY is widely reported (Hunter *et al.*, 1969; Büchi and Wüest, 1971; Buttery *et al.*, 1971; De Kimpe and Stevens, 1993; 1995). It degrades rapidly on exposure to air and forms a brown polymeric material that is insoluble (slightly soluble in dichloromethane). Buttery *et al.* (1971) have reported that 2-acetylpyridine was a degradation product of ACTPY. Some supporting evidence for this was found after a compound was observed by GC in a sample of dilute solution of ACTPY stored for 31 days (–20°C) in ethanol as having the same retention time as spiked 2-acetylpyridine (Table 2.1). This tentative identification suggested it would be prudent not to use 2-acetylpyridine as an internal standard. Further to this work, 2-acetylpyridine was

identified by mass spectral data when the sample was reanalysed 29 months later by GC/MS (Table 2.1), although it was not the primary degradation product. A deposit of insoluble brown polymeric material was observed in the sample, which probably accounts for the balance of the degradation. Neither ACPY or ETPY were detected in the 29 month stored sample, indicating that these compounds are not produced by the chemical degradation of ACTPY under these conditions.

Table 2.1. Degradation of ACTPY during storage in ethanol at –20°C. 2-Acetylpyridine is one of the products of this process. The concentration of ACTPY is expressed as the sum of the 2 tautomers.

Sample	ACTPY (µg/mL)	2-Acetylpyridine
0 days	300[a]	Not detected
31 days	223[a]	Identified by retention time
29 months	10[b]	Identified by retention time and mass spectrum

[a] Determined by GC peak area.
[b] Determined by GC/MS peak area

A. Bisulphite adduct. The labile characteristics of ACTPY and a need to have an easily handled form of this compound, necessitated an attempt to produce a bisulphite adduct. The stabilisation of ACTPY by the formation of a bisulphite complex was first described by Hunter *et al.* (1969) and carbonyl–bisulphite adducts have been reported as important to flavour stabilisation (Kandeda *et al.*, 1994). The successful production of the adduct was monitored by FAB MS/MS (Figure 2.2), although HSO_3 was in excess of the ACTPY–HSO_3 adduct. This made simple determination of the actual amount of ACTPY by weight impossible. The adduct, however, was useful for the preparation of reference samples and the concentration of ACTPY could be determined by GC. The fact that ACTPY readily complexes with sulphite may influence the sensory perception or degree of mousy taint in

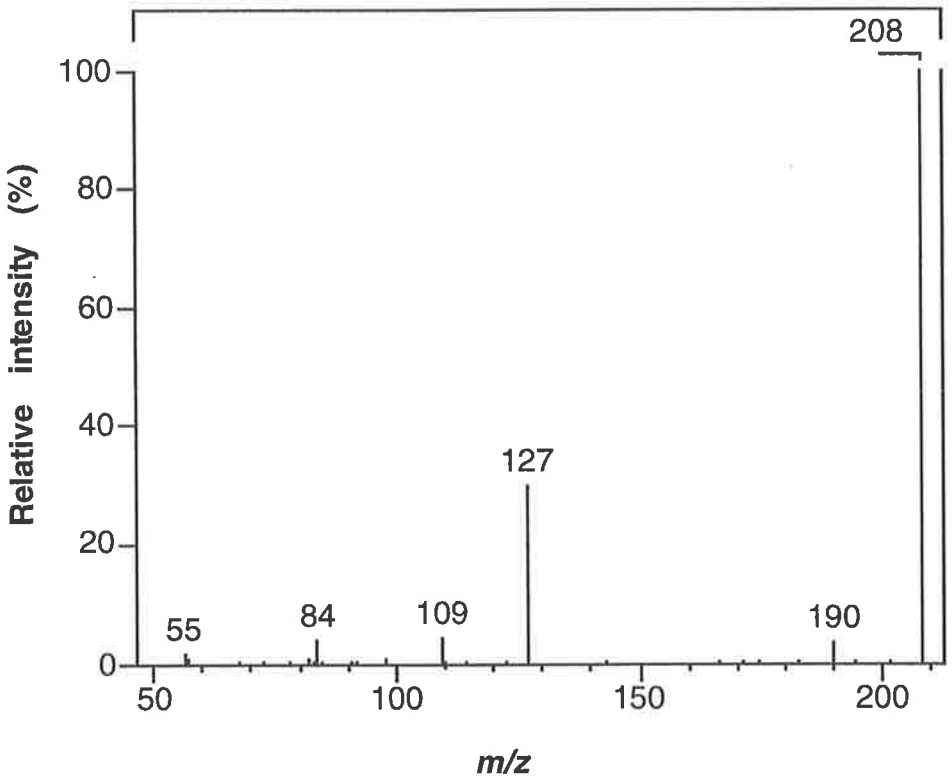


Figure 2.2. Mass spectrum of ACTPY–HSO_3 adduct. FAB MS/MS, positive daughter ion of *m/z* 208, in a glycerol matrix.

wines. Sulphite is a common preservative used in the production of all wine styles and there is some anecdotal evidence that addition of sulphite can mask mousy taint in wine, although large additions do not completely remove it. The influence of ACTPY adducts on taint perception has not been investigated and may warrant investigation, but was beyond the scope of this thesis.

2.3.1.2. Quantification

The stability of ACTPY influenced the quantification of this compound. Attempts to determine response factors for ACTPY versus various internal standards (2, 3, 4-acetylpyridine and 4-N,N-dimethylaminopyridine) with either GC or GC/MS were problematic. Virtually instantaneous browning was observed when ACTPY was microdistilled (final purification step in the synthesis procedure), indicating degradation via polymerisation was occurring, and therefore rendering the accurate weight determination required suspect. Other endeavours to purify ACTPY by vacuum distillation and silica gel fractionation, were also affected by this process when weight measurements were carried out. In light of this, response factor estimations were not used to quantify ACTPY, and the assumption was made that the relative responses for the target compounds and the internal standards was unity.

Response factors for ACPY and ACTPY have been determined by other authors, who reported 1.05 and 1.10 for these compounds respectively (Karahadian and Johnson, 1993; Schieberle, 1995). These studies must make the assumption that the reference material has not degraded while carrying out this analysis. In our attempts to determine a response factor for ACTPY we could not be satisfied of this. No response factors for ACPY and ETPY were determined, as sufficient pure reference material was not available and these compounds which are as labile (Tucknott, 1977; Buttery *et al.*, 1983; De Kimpe *et al.*, 1993; De Kimpe and Keppens, 1996), would also be affected by the problems we encountered in attempts to determine a response factor for ACTPY.

A response factor of 0.92 for 4-acetylpyridine quantification using 3-acetylpyridine as the internal standard was calculated after analysing 2 samples (3 injections, by GC, Carbowax 20 CAM column) of known amounts of these 2 compounds. These compounds are stable and therefore this was a simple procedure.

2.3.1.3 Internal standards

The use of an internal standard is common in analysis by GC and GC/MS. Several were trialed for use in analysis of ACTPY. Two of these, 4-N,N-dimethylaminopyridine and 2-acetylpyridine were found to be unsuitable. 4-N,N-Dimethylaminopyridine coeluted with the second tautomer of ACTPY (II) when the Carbowax 20 CAM column was used. 2-Acetylpyridine was found to be a degradation product of ACTPY even when stored as a dilute solution, although 2-acetylpyridine was never detected in any mousy wines, fermentation samples or microbiological culture media analysed as part of this study. The compounds 3 and 4-acetylpyridine were chosen as the internal standards for the final method development work. The mass spectral data for 2-acetylpyridine *m/z* (%): 122 (6), 121 (M^+ 73), 106 (6), 93 (39), 80 (9), 79 (100), 78 (82), 52 (30), 51 (31), 50 (11), 43 (26);. 4-acetylpyridine *m/z* (%): 122 (9), 121 (M^+ 100), 107 (7), 106 (87), 79 (25), 78 (89), 52 (10), 51 (40), 50 (19), 43 (36); and 3-acetylpyridine *m/z* (%): 122 (5), 121 (M^+ 71), 107 (7), 106 (96), 79 (15), 78 (100), 52 (11), 51 (34), 50 (15), 43 (17). The LRIs for 2-, 4- and 3-acetylpyridine respectively were 1577, 1756, and 1786. 4-Acetylpyridine was the first choice internal standard as it has a closer retention time to ACTPY (II) than 3-acetylpyridine.

The best internal standard, in theory, is the target compound itself labelled with an appropriate stable isotope that is not affected during the analytical work-up. Methods for the quantification of ACTPY and ACPY (in popcorn and bread crust respectively) by a stable isotope dilution assay have been described (Schieberle and Grosch, 1987b; Schieberle, 1995). These procedures use deuterium labelled ACTPY and ACPY to quantify the target compounds. This was carried out by determining the protium to

deuterium ratio by GC/MS. The amount of deuterium/protium exchange during the analytical work-up was monitored by model experiments and reported to be 10% or less, which was within acceptable analytical parameters (Schieberle and Grosch, 1987b; Schieberle, 1995). We observed that ACTPY readily exchanges protons with deuterium. In a simple experiment of just shaking ACTPY with D_2O and subsequent GC/MS analysis, up to four protons (possibly five) were exchanged, and a similar effect was observed with ACPY easily exchanging three protons (Figure 2.3). It would have to be confirmed whether this exchange occurs in the matrix of the samples (wine and culture media), and during the analytical work-up applied in this work before the stable isotope dilution assay could be used for the quantification of mousy taint.

Further to this, synthesis of labelled material would be subject to the unstable nature of these compounds. Any weight determination must assume the initial material is pure, but as we have observed, this is not always a valid assumption (see Section 2.3.1.2). For these reasons this methodology was not pursued for this work, although it would be an area worth investigating further if the issue of stability was resolved.

2.3.1.4 Instrumentation

GC/MS was adopted as the analytical method of choice, due to the nature of the compounds involved and the matrix of the samples analysed. While GC coupled with FID was ideal for reference samples which have only a small number of impurities present, wine and microbiological media samples are totally different. Wine is a very complex medium containing a large number of volatiles. Even the extraction procedure used in this work, which removes neutral and acid volatiles prior to analysis, still isolates a large number of basic volatiles, and for this reason analysis by GC with FID alone is not discriminating enough. It was difficult to separate out the peaks of the low concentration of mousy compounds from this background. This made GC/MS the ideal analytical tool.

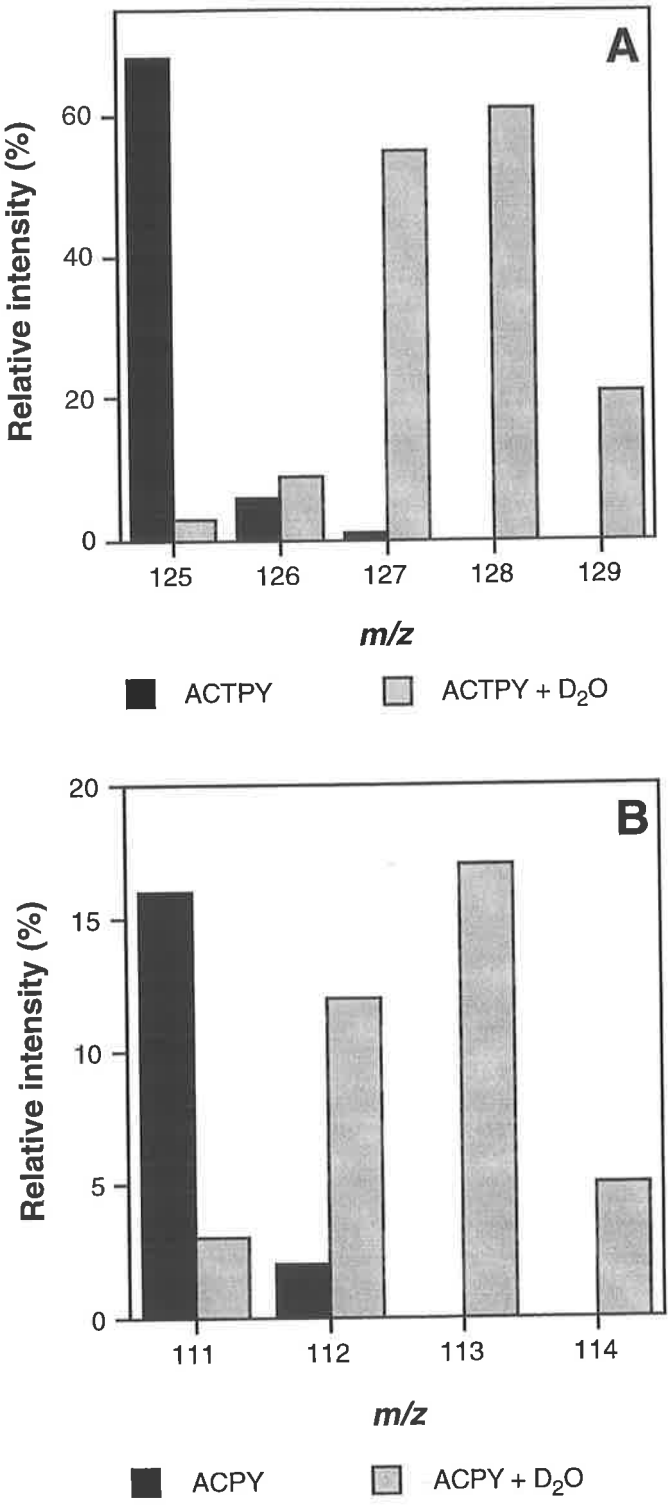


Figure 2.3. Molecular ion protium/deuterium exchange in mousy taint compounds, (A) 1st tautomer of ACTPY, (B) ACPY.

To exclude the possibility of formation or degradation of the target compounds during analysis at the GC injector (which is held at 200°C), a comparison was made between standard split injections and on-column injections, whereby the sample is loaded directly onto the top of the capillary column. This cold on-column injection technique should reduce or eliminate any possibility of artefact formation due to the temperature of the split/splitless injector block (Schomburg, 1990; Ueda *et al.*, 1990). No difference between split and on-column injections of the target compounds (including internal standards) were observed.

2.3.1.5 Column type

The introduction of fused silica capillary columns has dramatically improved analysis by GC, increasing separation efficiency, resolution and sensitivity over packed and SCOT type columns (Schomburg, 1990; Ueda *et al.*, 1990). Previous investigations of mousy taint used GC and GC/MS instruments equipped with packed and SCOT type columns, rather than capillary columns (Tucknott, 1977; Craig and Heresztyn, 1984; Strauss and Heresztyn, 1984; Heresztyn, 1986a).

The capillary column of choice in this study was the J & W Carbowax 20 CAM. This amine-specific column could resolve the 2 tautomers of ACTPY completely by either GC or GC/MS. The limitations of this type of column were the sensitivity to temperature and oxygen. The other capillary column used, DB1701, did not perform as well, especially resolving of the tautomers of ACTPY, and with use, the chromatography deteriorated significantly.

2.3.1.6 Extraction technique selection

Three types of extraction techniques were considered in the development of a reliable procedure for ACTPY analysis; simultaneous distillation and extraction, ion exchange and continuous liquid/liquid extraction. Preliminary investigations of simultaneous distillation and extraction using a modified Likens-Nickerson apparatus (Schultz *et al.*, 1977)

indicated there was a potential to produce artefacts. In several studies investigating Maillard type reactions, ACTPY and ACPY were produced by the heating of a model solution of the imino acid proline and a carbohydrate (Hodge *et al.*, 1972; Tressl *et al.*, 1981a; b; Schieberle, 1990a; b; c; Roberts and Acree, 1994). Schieberle (1990b; c) found that boiling proline with fructose specifically produced ACTPY, while a mix of ornithine (or citrulline) and fructose resulted in ACPY. Labelling studies showed the incorporation of C1 of glucose (labelled with ^{13}C) into the methyl group of ACTPY, when boiled together with proline (Rewicki *et al.*, 1992; Tressl *et al.*, 1993). These data are particularly important to this work as proline, ornithine, fructose and glucose all naturally occur in grape juice and wine. This indicates that the simultaneous distillation and extraction technique, which requires boiling, has the potential to produce both ACTPY and ACPY by the Maillard reaction mechanism, and so this extraction procedure was not further developed for wine analysis.

A method for the extraction of ACTPY by ion exchange has been described (Heresztyn, 1984; 1986a). This technique employed the cation exchange resin Dowex 50W. However, the use of ion exchange as a technique for mousy taint analysis was also discounted. The harsh conditions required, specifically the use of high pH to elute and extract mousy taint compounds, may cause degradation or further reactions. ACTPY readily reacts with aldehydes, such as acetaldehyde, at high pH to produce 7*H*-cyclopenta[*b*]pyridin-7-ones, which have negligible odour and taste (Helak *et al.*, 1989). This degradation of ACTPY could occur under the conditions required for the ion exchange extraction of mousy taint compounds.

The method adopted for analysis of mousy taint was a continuous liquid/liquid extraction. A preliminary investigation using dichloromethane as the organic phase indicated that ACTPY could be successfully extracted from a model solution. The non-volatile organic solvent Freon F11 was used, replacing dichloromethane in the further development of the extraction technique. Freon F11 is an ideal solvent for the analysis of trace flavour

compounds in alcoholic beverages, as it does not extract ethanol, has a low boiling point and is stable (Clutton and Evans, 1978). This solvent has been previously reported in the analysis of ACTPY by manual liquid/liquid extraction (Heresztyn, 1986a) and continuous liquid/liquid extraction of ETPY added to wine (Craig and Heresztyn, 1984). Saturation with NaCl of the sample prior to extraction was adopted in an attempt to reduce emulsion formation throughout the extraction procedure. The use of two internal standards and the final concentration step into *iso*-octane (2,2,4-trimethylpentane) were incorporated into the process (Figure 2.1). The addition of the second internal standard (3-acetylpyridine) after the continuous liquid/liquid extraction process allowed monitoring of the extraction efficiency. This was carried out by determining the recovery of the first internal standard (4-acetylpyridine) relative to the second. The recovery data indicated whether any problems occurred during the analytical work-up. The relatively high boiling point (99 °C) and low vapour volume of *iso*-octane produced the benefits of a greater ease of handling of the extracted sample once concentrated into this solvent and improved chromatography.

2.3.1.7 Validation of continuous liquid/liquid extraction technique

This was undertaken to monitor the process and to determine if the continuous liquid/liquid extraction technique was reliable. A neutral white wine was split into 2 x 300 mL volumes spiked with a known concentration of ACTPY (determined by GC/MS) and internal standard, then extracted and analysed using the method described in Section 2.2.3. Table 2.2 shows the amount and recovery of ACTPY determined by peak area integration with respect to the internal standard (4-acetylpyridine). The recovery of ACTPY was high and within an acceptable experimental range, indicating that the continuous liquid/liquid extraction technique was reliable.

Table 2.2 The amount of ACTPY added and the recovery with respect to 4-acetylpyridine of the spiked ACTPY tautomers after extraction and analysis using the continuous liquid/liquid extraction method (see Section 2.2.3.3 for extraction, Section 2.2.3.3B for analysis), determined by either integration of the area or height of the target peaks with respect to the internal standard (see Section 2.2.3.4 for quantification). The concentration of ACTPY is expressed as the sum of the 2 tautomers.

Sample	Area	
	ACTPY (µg/L)	Recovery (%)
Spike	41.9	
Wine 1	39.5	94.4
Wine 2	35.8	85.6

The comparison between the known addition of 4-acetylpyridine (53.0 µg/L) and that determined with respect to the second internal standard, 3-acetylpyridine (50.4 µg/L), after replicate analysis of the spike mix (containing ACTPY and known amount of 4 and 3-acetylpyridine) indicated a high degree of accuracy is achieved by this method for the estimation of the amount ACTPY spiked into the wines (Table 2.2). A more satisfactory method of carrying out the validation would have been to use a known amount of ACTPY determined by weight, but as described for response factor calculations (Section 2.3.1.2), accurate weight measurement of ACTPY was suspect.

2.3.2 ETPY

This compound was previously proposed as a cause of mousy taint in alcoholic cider (Tucknott, 1977). It was also detected in a mousy wine (Tucknott, 1978), although, later studies could not detect this compound in mousy wines or in model systems (Craig and

Heresztyn, 1984; Heresztyn, 1986a). A re-examination of this compound was undertaken, with the benefit of the more sensitive analytical technique.

2.3.2.1 Synthesis

By comparison with published mass spectral data (Tucknott, 1977; Craig and Heresztyn, 1984), synthesis of ETPY was confirmed. The mass spectral data of synthetic ETPY, *m/z* (%): 112 (7), 111 (67), 110 (100), 96 (25), 83 (25), 82 (48), 68 (5), 56 (40), 55 (22), 54 (14), 41 (15). Direct retention time comparison for the Carbowax 20 CAM capillary GC column could not be done as there was no published data. A sample of reference ETPY had a LRI of 1217. No attempt was made to further purify ETPY (95% purity by GC/MS). Aroma assessment of the sample by judges familiar with mousy taint confirmed the literature description of a mousy impact (Tucknott, 1977; Craig and Heresztyn, 1984), and GC–sniff by the author corroborated a previous report (Craig and Heresztyn, 1984) that ETPY itself has a mousy odour.

2.3.2.2 Identification of ETPY

To confirm the presence of ETPY in mousy sample extracts, synthetic ETPY was spiked into a fermentation extract (*B. anomalus* fermentation, with L-lysine treatment, Section 3.3.3, Table 3.8) containing ETPY tentatively identified by comparison with mass spectral data. The addition was made with enough synthetic ETPY to increase the total ion intensity by a factor of 2-times relative to the first internal standard.

The results of this coinjection analysis are described in Figure 2.4. This data clearly shows that the tentatively identified compound was indeed ETPY, with the peak symmetrically enhanced upon coinjection. No difference was observed in the mass spectrum produced from the target and reference compound or from the coinjection analysis. The importance of this compound to mousy taint, however, is unclear.

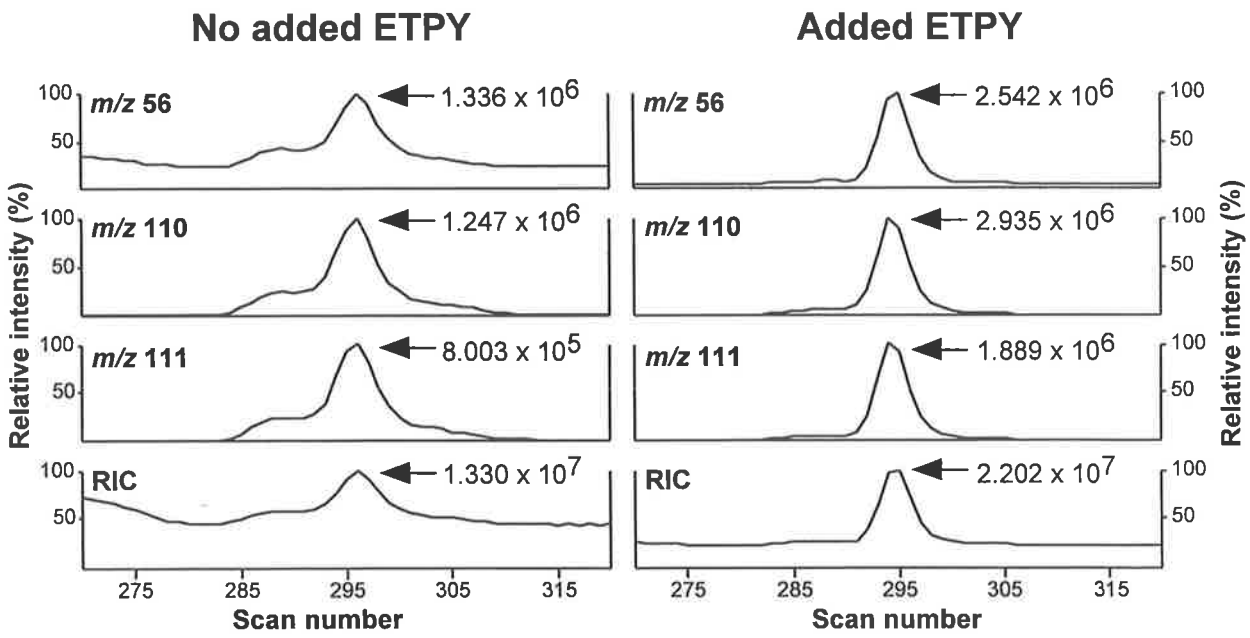


Figure 2.4. Identification of ETPY by coinjection analysis; after the addition of synthetic ETPY (**Added ETPY**) to an extract containing tentatively identified ETPY (**No added ETPY**). These mass chromatograms of selected ions (*m/z* 56, 110 & 111) and total ion abundance (RIC) show an increase in ion abundance of selected ions of approximately 2.2-times after the addition of ETPY, indicating that ETPY was identified correctly.

2.3.3 ACPY

The potential of this compound to induce mousy taint in wine was examined. ACPY was reported to be responsible for mousy taint in wetted pearl millet (Seitz *et al.*, 1993). This highly potent compound has structural and stability characteristics similar to those of ACTPY in that it degrades rapidly on exposure to air and forms brown polymeric material, and that it is best stored as a dilute solution (Buttery *et al.*, 1983; De Kimpe *et al.*, 1993). Due to its similarity in physical properties with ACTPY, the extraction, analysis and quantification was carried out in the same manner as for ACTPY. The extraction procedure was not, however, optimised for ACPY since adequate quantities of authentic reference material were not available to do this.

2.3.3.1 Analysis

Mass spectral data of reference material, *m/z* (%): 111 (M^+ 26), 83 (45), 82 (6), 69 (18), 68 (26) 55 (4) 43 (100), 42 (23), 41 (48), and a LRI 1318 was in agreement with published data (Buttery *et al.*, 1983; Schieberle and Grosch, 1987b).

2.3.3.2 GC–sniff

The reference sample had an intense mousy aroma, but some minor by-products were present in the reference sample (determined by GC/MS analysis). To confirm the mousy characteristic of ACPY and to exclude any interference from other compounds, this material was evaluated further by GC–sniff. ACPY was confirmed as having a very strong mousy character by experienced judges familiar with this off-odour. Minor impurities did not contribute to overall mousy aroma. This compound has a 30-fold lower odour threshold in air than ACTPY [odour threshold in air: ACPY, 0.02 µg/L; ACTPY, 0.6 µg/L, (Schieberle and Grosch, 1991)], and may therefore be very important to mousy taint perception. Thus, it could be detected at concentrations below that of the threshold of ACTPY, and at these low concentrations produce a mousy impact.

2.3.4 Mousy wine analysis

Previously, sensory evaluation was the only method available for the assessment of mousy wine. Several relatively recent reports have described methods for analysis of mousy beverages, although none presented quantitative data (Tucknott, 1977; Craig and Heresztyn, 1984; Strauss and Heresztyn, 1984; Heresztyn, 1986a). With the development of the extraction, analysis and quantification technique (described in Sections 2.2.3.3, 2.2.3.3B and 2.2.3.4 respectively), this type of data was available for the first time. Table 2.3 details the results of the analysis of a number of mousy wines.

Table 2.3. Concentration of mousy impact compounds in wine. These wines were all described as mousy after sensory evaluation by a panel of judges familiar with mousy taint. These compounds were not detected in sound wine. The concentration of ACTPY is expressed as the sum of the 2 tautomers.

Sample	Compounds (µg/L)		
	ETPY[a]	ACTPY[b]	ACPY[c]
Red A	ND	4.8	7.8
Red B	ND	106	7.1
Red C	1.9	39	0.2
Rosé A	4.5	14.7	ND
White A	ND	4.3	ND

ND Not detected.
[a] Taste threshold in wine; 150 µg/L (Craig and Heresztyn, 1984).
[b] Odour threshold in water; 1.6 µg/L (Teranishi *et al.*, 1975).
[c] Odour threshold in water; 0.1 µg/L (Buttery *et al.*, 1983).

The detection of ACPY in samples Red A, B and C is the first report of the association of this compound with mousy wine. ACPY, which has only been detected at very low

concentrations, may strongly influence the impact of the taint, due to its highly potent nature (low odour threshold).

The analysis of these wines also confirms the earlier report of ETPY in mousy wine (Tucknott, 1978). Subsequent work had failed to isolate this compound from mousy wines, leading to the conclusion that this compound was not responsible for mousy taint in wine (Craig and Heresztyn, 1984; Strauss and Heresztyn, 1984).

The present work has detected ETPY in mousy wines (Table 2.3), although it was at concentrations well below its reported taste threshold in wine, suggesting it would have little or no impact on mousy taint. In contrast to ACPY and ETPY, ACTPY was detected in all mousy wines analysed (Table 2.3). This suggests that ACTPY is the most important compound in mousy taint production. This is the first quantitative study of ACTPY, although previous research has identified ACTPY in mousy wines (Strauss and Heresztyn, 1984; Heresztyn, 1986a). A broad range in the concentration of this compound was found.

The concentrations of ACTPY and ACPY in the mousy wines described in Table 2.3 all exceeded reported odour thresholds in water. The sensory thresholds for these compounds in wine have not been established. However, all these wines exhibited mousy taint spoilage by sensory evaluation, indicating that the taste threshold was exceeded.

Determination of taste thresholds for mousy compounds in wine by standard sensory evaluation techniques would be problematic, due to their lability and other physio-chemical characteristics. The mousy taste in the mouth generally persists for a long time after the sample has been expectorated, even up to several minutes, which makes sensory evaluation difficult as one sample potentially could interfere with the next. Another sensory characteristic of mousy taint is the observation that individuals range from insensitive to very sensitive in their perception of the taint, and this variation between individuals will strongly influence the results of any sensory investigation.

A physio-chemical characteristic of the mousy compounds that may also influence sensory research is the fact that these compounds will interact with the wine matrix. For example, ACTPY and SO_2 forming an adduct, which could influence taste perception, similarly ETPY and tartaric acid, the most important organic acid constituent in wine can form a stable salt (Tucknott, 1977). It is unknown how these interactions would effect sensory detection. This suggests the reported taste threshold for ETPY in wine (Craig and Heresztyn, 1984) could be an overestimation (which would perhaps increase the importance of ETPY to mousy taint). To translate a taste threshold across all wine styles would be impossible, due to the variation in the composition of different wines and to produce reliable and meaningful sensory data would require a long and careful study, considering all the variables described above.

Nothing is known about the sensory interactions between mousy compounds. In all but one sample, the mousy compounds were detected in association with at least one other compound. A more detailed sensory analysis is required to clearly establish the importance of the individual compounds to mousy taint. A technique such as aroma extract dilution analysis could be used to investigate the impact of the individual compounds (see Grosch (1994), for a review of this topic). Time constraints did not allow for this type of analysis to be carried out on mousy wine.

The development of a reliable extraction and analysis technique for the detection of ACTPY in wine has enabled a more detailed investigation of the compounds related to mousy taint, with the discovery of a new wine component (ACPY), and the re-examination of another (ETPY). This procedure has also allowed for the continuation of research into the microorganisms involved in mousy taint formation and the metabolism and physiology of the production of these compounds. Further optimisation of this technique, perhaps through the use of stable isotope labelled internal standards would further enhance the

depth of understanding of the nature of mousy impact compounds. A detailed study of the sensory perception of these compounds is required to determine the level of importance of each to mousy taint.

CHAPTER 3 Mousy Taint Production by *Dekkera/Brettanomyces*

3.1 Introduction

Several studies have investigated the importance of strain or species of *Dekkera/Brettanomyces* on formation of mousy taint in a beverage or culture medium. Tucknott (1977) examined six strains, comprising two strains of *B. anomalus*, two strains of *B. bruxellensis* and one strain each of *B. claussenii* and *B. lambicus*, for the production of mousy taint by culturing them in an apple juice medium. All strains produced a taint that was detected by sensory evaluation and furthermore, a strain of *B. anomalus* produced the mousy tasting heterocyclic ETPY in a chemically defined medium. Heresztyn (1986a; b) found that five strains of *Brettanomyces*, that included three strains of *B. intermedius*, a *B. lambicus* and a *B. anomalus* strain, could form a mousy taint when these yeast were grown in wine or grape juice. The *B. intermedius* strains produced a mousy taint in a chemically defined medium. The mousy taint tautomers of ACTPY were detected by GC/MS in the culture medium extracts of these strains. An unidentified strain of *Dekkera* was also found to produce mousy taint in a grape juice and a chemically defined medium (Heresztyn, 1986b). These studies, while covering two of the *Dekkera* species currently accepted, namely *D. bruxellensis* and *D. anomala* (Smith *et al.*, 1990; Boekhout *et al.*, 1994), have not considered the three species of *Brettanomyces*, *B. custersianus*, *B. nana* and *B. naardenensis*. Of the type strains of *Dekkera/Brettanomyces* that have been described (Table 3.1) only five of thirteen strains have been surveyed for mousy taint production.

The aim of this research was to investigate the relationship between mousy taint formation and all species and type strains of *Dekkera/Brettanomyces* so far described, to determine if mousy taint production was a general characteristic of *Dekkera/Brettanomyces* or if it was

confined to the species and strains associated with alcoholic beverages. This was carried out by culturing these yeast in a variety of media and under conditions known to enhance the mousy characteristic.

As no specific growth data was provided in any of the earlier reports for the *Dekkera/Brettanomyces* strains investigated, a study was undertaken to examine the growth of the two species of *Dekkera* found or associated with wine, *D. anomala* and *D. bruxellensis* (Barnett *et al.*, 1990). Four type strains representing these species were investigated by culturing in two commercially produced wines. The wines were selected and treated so as to allow the conditions to be conducive for growth. The strains selected had been previously reported to occur or grow in wine.

Twelve type strains of *Dekkera/Brettanomyces*, representing four species were screened for mousy taint production in a grape juice medium. The method used was designed to produce rapid results without the need for complex analysis. *B. nana*, formerly *E. nanus,* was not examined as at the time of this investigation it was still considered separate from *Brettanomyces.*

The type and concentration of taint compounds produced by the five species of *Dekkera/Brettanomyces* was investigated in detail. All thirteen type strains were examined and the mousy taint compounds were analysed by the highly sensitive extraction, analysis and quantification technique developed (Chapter 2). This involved culturing the strains under defined conditions to quantify the production of the nitrogen heterocyclic mousy taint compounds ACPY, ETPY, and ACTPY, and to determine the taxonomic significance formation of these compounds. The role of L-lysine in the formation of mousy taint compounds by these selected strains in a chemically defined medium was also investigated.

3.2 Materials and Methods

3.2.1 Yeast strains

The *Dekkera/Brettanomyces* yeast used were all type strains obtained from the Centraalbureau voor Schimmelcultures (CBS), Delft, The Netherlands. The strain identification numbers and their origin are listed in Table 3.1. *S. cerevisiae* has not been reported as a mousy taint producing yeast, and therefore the strains AWRI 796 and AWRI 729 were used as negative controls. All yeast were maintained on slants of MYPG [malt and yeast extract 0.3% (w/v), bacteriological peptone 0.5% (w/v), D-glucose 1% (w/v), and bacteriological agar 2% (w/v), stored at 4°C. For the *Dekkera/Brettanomyces* yeast, calcium carbonate 5% (w/v)] was included in the MYPG formulation.

Table 3.1. Type strains of *Dekkera/Brettanomyces* yeast and their origin.

Type strain	CBS no.	AWRI no.	Origin
D. anomala	8139	953	soft drink
B. anomalus	77	1101	stout
B. claussenii	76	1128	beer
D. bruxellensis	74	952	beer
B. bruxellensis	72	1102	beer
D. intermedia	4914	1129	beer
B. intermedius	73	1104	grape must
B. lambicus	75	1127	beer
B. custersii	5512	1103	brewery
B. abstinens	6055	1130	soft drink
B. custersianus	4805	950	brewery
B. nana	1945	1201	beer
B. naardenensis	6042	951	soft drink

3.2.2 Yeast growth and mousy taint formation in wine

The growth and survival of four type strains of *Dekkera/Brettanomyces* was studied in two commercially produced wines. The wines were chosen to provide conditions conducive for yeast growth, with low (< 5 mg/L) free SO_2 and an ethanol concentration below 12% (v/v) (Table 3.2). Both wines were sourced from the Barossa Valley, South Australia, vintage 1992.

The yeast selected had all been reported in wine produced from grapes (Table 3.3), although the actual strains used were not originally isolated from wine.

Table 3.2. Analysis of the commercial wines. Free and total SO_2 and titratable acidity determined using the methods described in Iland *et al.* (1993), ethanol concentration determined as described in Section 3.3.1, glucose and fructose concentration determined as described in Section 3.2.3.2.

Analysis	White wine	Red wine
pH	3.32	3.30
Glucose + fructose (mg/L)	0.1	0.0
SO_2 free (mg/L)	3	0
SO_2 total (mg/L)	43	15
Titratable acidity (g/L)	10.0	7.3
Ethanol (% v/v)	10.6	11.2

Table 3.3. Strains used in the wine growth experiments and an example reporting the occurrence of each strain in wine.

Strain	Wine isolation
B. anomalus	Schanderl and Draczynski (1952)
B. bruxellensis	Querol and Huerta (1987)
B. intermedius	van der Walt and van Kerken (1959a)
D. intermedia	Froudiére and Larue (1990)

3.2.2.1 Culture conditions

Each strain was subcultured in a pre-starter culture and an adaptation medium before being inoculated into the wine medium. A pre-starter culture of each strain was established by inoculating a single loop-full from the respective agar slant into 50 mL of YPG [yeast extract 1% (w/v), bacteriological peptone 2% (w/v) and D-glucose 2% (w/v)] in a baffled 250 mL conical flask plugged with cotton wool, and grown at 25°C in a shaking waterbath at 200 oscillations per minute (opm). Cultures were monitored by cell counts (using a haemocytometer cell counting chamber) until the cell number reached 1 x 10^8/mL.

The cultures were then inoculated into an adaptation medium. This medium consisted of 50% white wine/50% grape juice (the white wine was described in Section 3.2.2, and the grape juice was Muscat Blanco Gordo, 1991 vintage, Riverland, South Australia). *Dekkera/Brettanomyces* have an auxoheterotrophic requirement for the vitamins biotin and thiamin (Peynaud and Domercq 1956, van der Walt and van Kerken 1959a). *Dekkera anomala* also requires *myo*-inositol for growth (Barnett *et al.*, 1990). These vitamins were therefore added to the medium (Table 3.4). A nitrogen supplement was also added in the form of diammonium phosphate (200 mg/L). The medium was adjusted to pH 3.5 and then sterilised by filtration through a 0.2 µm membrane.

Table 3.4. Essential vitamins for the growth of *Dekkera/Brettanomyces,* adapted from Barnett *et al.* (1990).

Vitamin	Amount required (per litre)
Biotin	20 µg
Thiamin HCl	400 µg
myo-Inositol	10 mg

The adaptation cultures were inoculated at a density of 5 x 10^6 cells/mL and grown to 1 x 10^8cells/mL in a volume of 150 mL. All other growth conditions and monitoring were the same as for the pre-starter cultures.

The adapted cultures were then inoculated into the wine media. The wine media were supplemented with diammonium phosphate (200 mg/L), L-lysine.HCl (50 mg/L) and vitamins (Table 3.4). The wines were adjusted to pH 3.5 and then sterile membrane filtered (0.2 µm). The wines were sparged with sterile CO_2 throughout handling to minimise oxygen pick-up.

The culture vessels used were 200 mL medical flat bottles, closed with No. 33 Suba-seals. As an airlock, a 18G x 1.5 needle was inserted into the Suba-seal to which a 20 cm length of autoclavable silicon tubing (internal diameter 0.5 cm) was attached. The fermentation equipment was then sterilised by autoclaving at 121°C and 100 kPa for 15 min. After sterilising, the tubing was then inserted into a sterile 10 mL tube containing approximately 8 mL of sterile water.

Prior to filling the bottles with the wine medium, an inoculum was added to produce a final cell density of 1 x 10^5 cells/mL. The bottles were gassed with filter sterilised CO_2 for 10 min, then filled with the wine medium (200 mL final volume) via a calibrated peristaltic

pump, and sparged for a further 10 min. Each strain was tested in triplicate for the two wine types. The inoculated wines were statically incubated at 25°C.

3.2.2.2 Sampling

Each culture was mixed and then sampled (0.2 mL) at 3 to 4 day intervals, and after sampling each bottle was gassed with filter sterilised N_2. The samples were diluted with bacteriological peptone [0.1% (w/v)] to the appropriate range of 25-250 colony forming units (cfu) per 25 µL, in sterile 96 well micro-titre plates. Samples were spot-plated (3 x 25 µL spots per replicate) onto YPG plus bacteriological agar [2% (w/v)] and YPG agar plus cycloheximide [0.05% (w/v)] to determine culture viability. Each dilution was plated out in triplicate.

3.2.2.3 Mousy taint assessment

At the end of the growth monitoring period, the wines were examined for mousy taint by sensory evaluation using the alkaline paper strip method. This technique was adapted from Heresztyn (1986a). The paper strips (4-5 mm x 50 mm) were prepared by soaking in NaOH (0.1M) and drying overnight at room temperature. The strips were dipped into cell free samples (centrifuged) and assessed for mousy taint by sniffing. This was an informal assessment carried out by a panel of individuals who were familiar with mousy taint odour.

3.2.3 Type strain screening trial for mousy taint formation

3.2.3.1 Yeast strains

All *Dekkera/Brettanomyces* type strains (except *B. nana*) (Table 3.1) and the non-mousy taint producing control, *S. cerevisiae* AWRI 796, were streaked out for single colonies onto YPG agar. Several colonies were taken from 7 day old plates and used to inoculate sterile membrane filtered (0.2 µm) grape juice medium.

3.2.3.2 Culture conditions and sampling

The grape juice medium was produced from Doradillo grape juice of neutral aroma and flavour, obtained from the Riverland, South Australia, 1992 vintage. Free SO_2 was removed from the juice by the appropriate addition of H_2O_2 [30% (v/v)] (Hoffman and Edwards, 1975; Anonymous, 1997). Further additions and modifications were then made as shown in Table 3.5.

The screening trial was carried out using 50 mL conical screw cap Kimax flasks containing the grape juice medium (30 mL) inoculated with 3 colonies and grown at 25°C in a shaking water bath at 150 opm. The flasks were sampled for taint development every 3 days by sniffing at the neck of the flask, and using the alkaline paper strip method (Section 3.2.2.3).

Table 3.5. Initial analysis of neutral Doradillo grape juice and the additions and modifications made to formulate the grape juice medium. Analysis methods as described in Table 3.2.

Analysis	Initial	Final
pH	3.26	3.80
Glucose + fructose (g/L)	186	186
SO_2 free (mg/L)	10	0
SO_2 total (mg/L)	103	9
Vitamins	ND	see Table 3.4
Diammonium phosphate (mg/L)	ND	200
L-lysine.HCl (mg/L)	ND	50

ND Not determined.

Sugar catabolism was monitored by measuring the refractive index of the culture supernatant, and glucose plus fructose concentration was determined by UV enzymatic assay (Boehringer Mannheim test combination Cat. No. 139 106).

3.2.4 Identification and quantification of mousy taint compounds

3.2.4.1 Strains and culture conditions

The type strains of *Dekkera/Brettanomyces* (Table 3.1) and the *S. cerevisiae* control, AWRI 729, were inoculated (1 x loop-full) into 50 mL of chemically defined medium (CDM) at pH 3.8 in baffled 250 mL conical flasks, plugged with cotton wool, and incubated at 25°C in a shaking waterbath at 200 opm. The formulation of the CDM is described in Table 3.6. The starter medium did not contain L-lysine. When the cell number reached 1 x 10^8 cells/mL (by haemocytometer count) the strains were inoculated in duplicate into 1 L Schott bottles containing 600 mL of CDM, pH 3.8, incubated aerobically (cotton wool plugged) at 25 °C and 150 opm, until all the glucose had been consumed or ceased to be used. There were two conditions investigated; (1) with, or (2) without the addition of 100 mg/L L-lysine.HCl. The flasks were sampled (1 mL) daily, growth was monitored by absorbance (650 nm), glucose level estimated by refractive index and reaction with Cu_2SO_4/NaOH Clinitest tablets (Ames).

At the end of the fermentation, cells were removed by centrifugation (10 min, 4500g) and membrane filtration (0.2 μm) and the filtrates stored at –20 °C prior to extraction and analysis for mousy taint compounds by the technique developed (Section 2.2.3.2).

Table 3.6. Chemically defined culture medium, adapted from Heresztyn (1986b).

Classification	Addition	Amount (per litre)
Carbon	Glucose	50 g
Nitrogen	$(NH_4)_2SO_4$	2 g
	L-lysine HCl	100 mg
Salts & Minerals	$MgSO_4$ $7H_2O$	250 mg
	KH_2PO_4	2 g
	Citric acid	200 mg
	$CaCl_2$ $2H_2O$	310 mg
	$ZnSO_4$ $7H_2O$	2 mg
	$FeSO_4$ $7H_2O$	10 mg
Vitamins	*myo*-Inositol	25 mg
	Pyridoxine HCl	1 mg
	Nicotinic acid	10 mg
	Thiamin HCl	1 mg
	Biotin	30 mg

3.3 Results

3.3.1 Yeast growth and mousy taint formation in wine

The growth of the type strains of *B. anomalus, B. bruxellensis, B. intermedius* and *D. intermedia* in two different wines was monitored over a period of 52-55 days. It was evident that growth occurred in both red and white wine, especially with *B. bruxellensis* (Figure 3.1), and to a lesser extent with *B. anomalus* (Figure 3.2). After inoculation, the viability of *B. bruxellensis* declined before entering an exponential growth phase after 14 days. This yeast achieved a similar maximum cell number in both the white (3.59×10^5 cfu/mL) and red wine medium (3.28×10^5 cfu/mL). However, stationary phase was reached in the white wine medium after 21 days, with little change in viable cell number after 28 days. In the red wine medium, the maximum cell number was not reached until 52 days, the final sample point (Figure 3.1).

Brettanomyces anomalus reached a maximum viable cell number of 5.34×10^5 cfu/mL in the red wine and 7.49×10^5 cfu/mL in the white wine media (Figure 3.2). This was achieved after 28 days in both media, and throughout the remainder of the growth monitoring period there was a slow decrease in cell viability.

The viability of the type strain *B. intermedius* and its teleomorph *D. intermedia* gradually declined over the time frame of this experiment (Figures 3.3 and 3.4). In the white wine medium the viability of *B. intermedius* dramatically reduced, a decrease of approximately 2 $\log_{10}$ units. This reduction was greater than that observed in the red wine medium, where yeast viability decreased from 3.44×10^5 cfu/mL to 3.96×10^4 cfu/mL (Figure 3.3). The larger reduction in viability in the white wine compared with the red wine medium was also observed with *D. intermedia*. For the latter yeast the change in viable cfu/mL in the white wine was greater than 1 $\log_{10}$ unit, from 5.44×10^5 cfu/mL to 1.47×10^4 cfu/mL. A slight increase in viable cells was found in the red wine with a maximum of

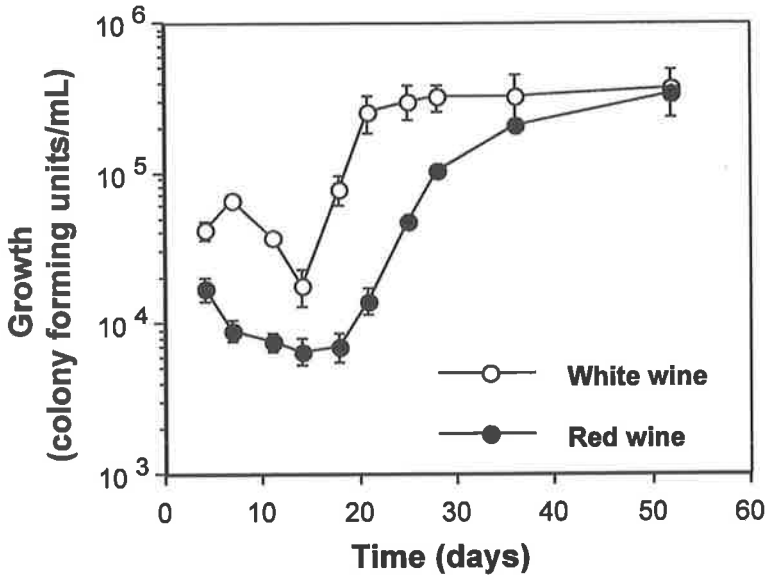


Figure 3.1. Growth of *B. bruxellensis* type strain in a red and white wine, as determined by viable colony plate count.

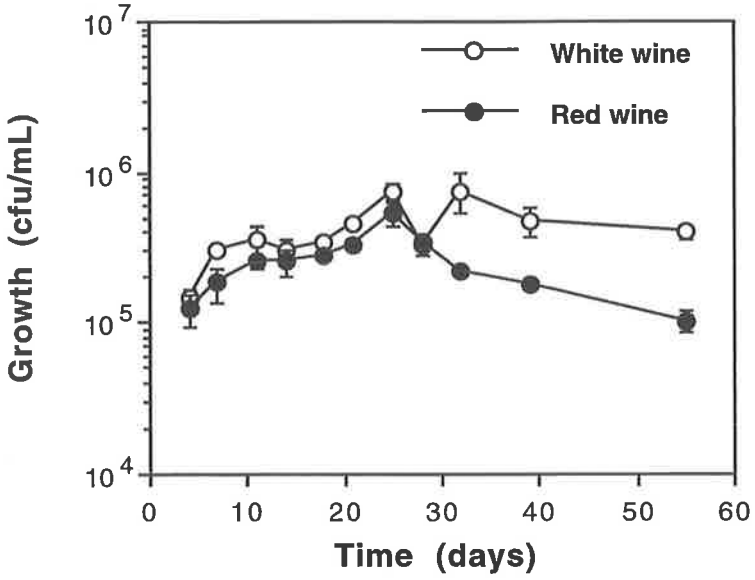


Figure 3.2. Growth of *B. anomalus* type strain in a red and white wine, as determined by viable colony plate count.

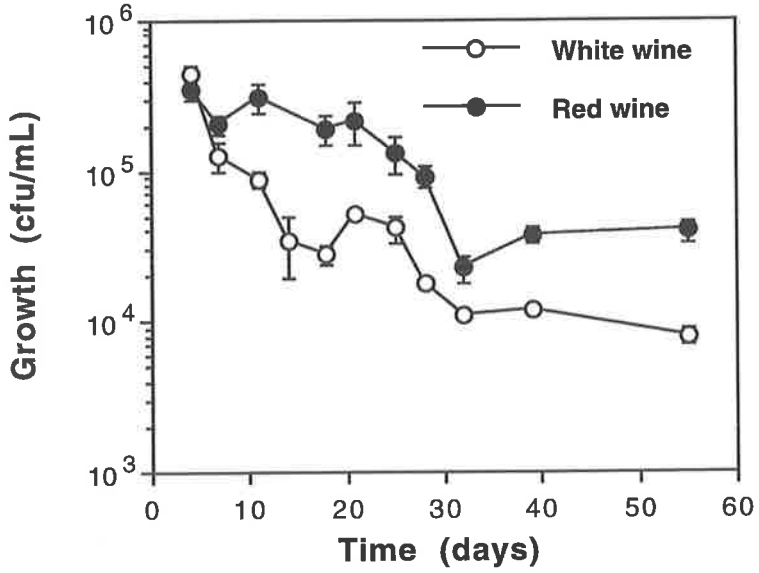


Figure 3.3. Growth of *B. intermedius* type strain in a red and white wine, as determined by viable colony plate count.

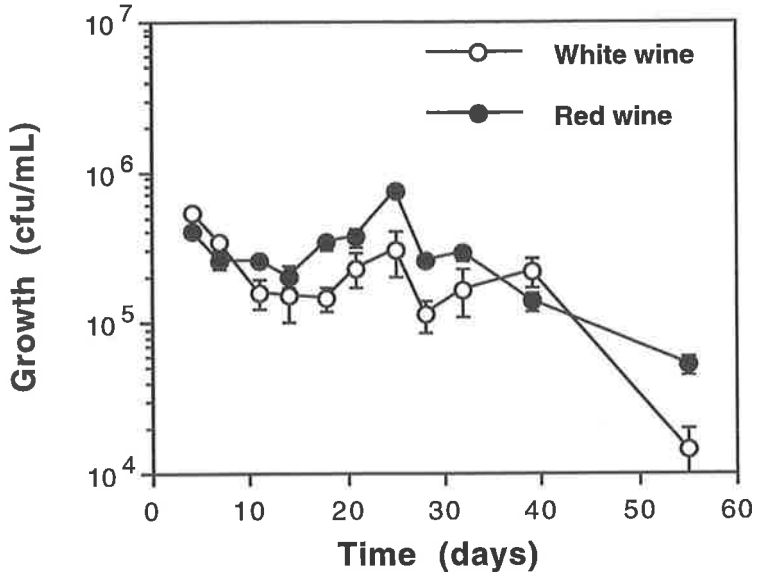


Figure 3.4. Growth of *D. intermedia* type strain in a red and white wine, as determined by viable plate colony count.

7.33 x 10^5 cfu/mL achieved after 28 days. After this point, however, the viability decreased (Figure 3.4).

No carbon source was added to these wines, but trace concentrations of residual sugars (including glucose, fructose, galactose and trehalose) may have provided sufficient energy and carbon for growth. Under the air-limited conditions used in this experiment, there is a possibility that ethanol could be utilised for energy as well. The ethanol concentration was determined at the end of the trial by near infra-red reflectance spectroscopy (Bran and Luebbe 260 Infra-alyser). The loss of ethanol from the white wines was on average 0.9% (v/v) and from the red wine, 0.74% (v/v). No significant change in pH or titratable acidity was observed.

A slight mousy taint was detectable by the alkaline paper strip method in both red and white wines in which *D. intermedia* had been inoculated. A moderate level of taint was detected for *B. anomalus* and *B. intermedius* in the red wine but only a slight taint had developed in the white wine samples. *B. bruxellensis* produced a moderate mousy taint in both the red and white wines.

3.3.2 Type strain screening trial for mousy taint formation

The *Dekkera/Brettanomyces* type strains, as well as an uninoculated control and *S. cerevisiae* (AWRI 796) were screened for mousy taint production in the grape juice medium. Figure 3.5 reports the degree of mousy taint in the culture supernatant detected by sensory evaluation using the alkaline strip method. No taint could be detected without the aid of the alkalised strip.

The metabolic activity of the yeasts, monitored by glucose and fructose catabolism, varied considerably both between and within *Dekkera/Brettanomyces* species (Figure 3.6 and 3.7). The *S. cerevisiae* control strain completely consumed the available sugar more

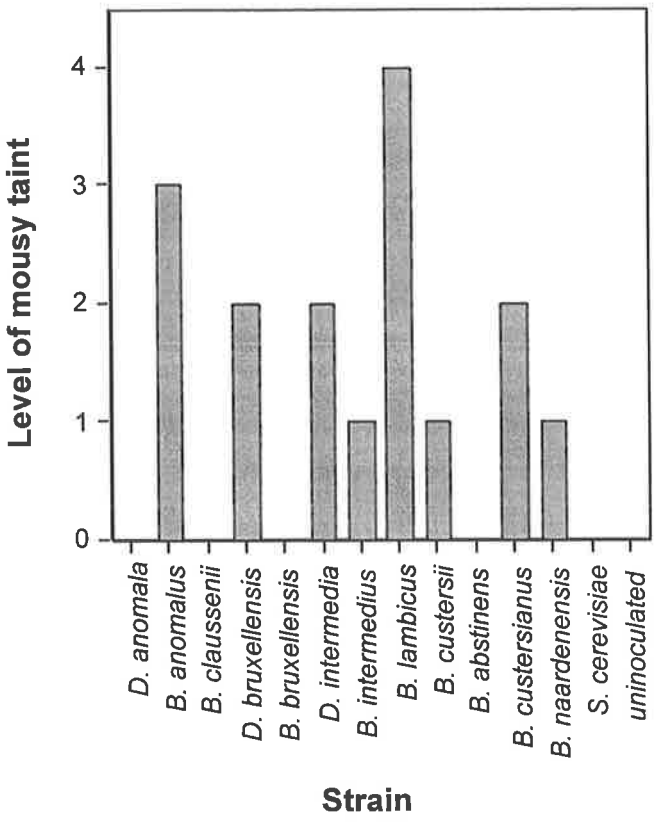


Figure 3.5. Mousy taint production by type strains of *Dekkera/Brettanomyces* grown in a grape juice medium. Mousy taint assessment was carried out on culture filtrates by sensory evaluation using the alkaline paper strip method. The intensity of the taint was ranked on a scale: (0) no taint, (1) slight taint, (2) moderate taint, (3) strong taint, (4) very strong taint.

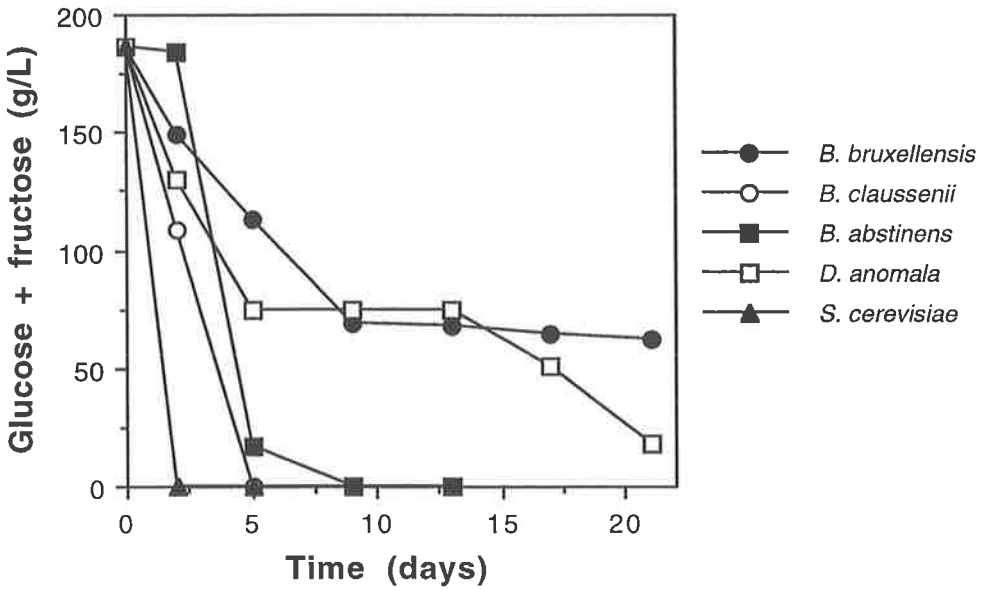


Figure 3.6. Catabolism of glucose and fructose by non-mousy taint producing type strains of *Dekkera/Brettanomyces* and a *S. cerevisiae* strain, grown aerobically in a grape juice medium, pH 3.8 at 25°C.

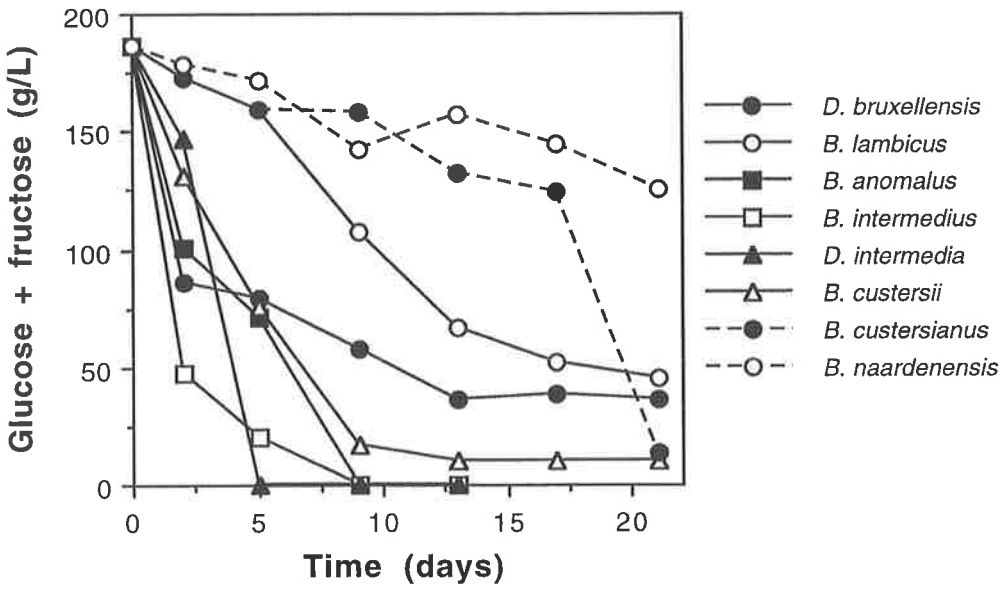


Figure 3.7. Catabolism of glucose and fructose by mousy taint producing type strains of *Dekkera/Brettanomyces,* grown aerobically in a grape juice medium, pH 3.8 at 25°C.

rapidly than any *Dekkera/Brettanomyces* type strain (Figure 3.6). *B. claussenii* and *D. intermedia* were the first *Dekkera/Brettanomyces* strains to metabolise all the sugar, while many strains did not consume all available glucose and fructose within the time frame of the experiment. These included *B. naardenensis*, *B. bruxellensis* and *B. lambicus* (Figure 3.6 and 3.7).

Metabolic activity generally coincided with mousy taint production although, there were some exceptions (Figure 3.5, 3.6 and 3.7). For example, *B. claussenii* which rapidly removed all the sugar from the medium, did not produce a detectable taint, whereas *B. naardenensis* used only approximately 30% of the available glucose and fructose and produced a mousy taint.

Not all type strains of *Dekkera/Brettanomyces* produced detectable mousy taint by the alkaline strip sensory method (Figure 3.5). Mousy taint or mousy-like aromas were not detected in the *B. bruxellensis*, *B. claussenii*, *B. abstinens,* and *D. anomala* ferments, or in the uninoculated control. The aroma of acetaldehyde was dominant in the *S. cerevisiae* ferment with no mousy taint.

Other commonly recorded aromas produced by *Dekkera/Brettanomyces* were yeast, cheese, bread, sour apple and butterscotch. The pungent odour of acetic acid was frequently detected, even using the alkaline strip method. This aroma became stronger as the fermentations progressed, and occurred with almost all *Dekkera/Brettanomyces* except *B. naardenensis*.

3.3.3 Identification and quantification of mousy taint compounds

Culture filtrates of *Dekkera/Brettanomyces* type strains, the uninoculated controls and the control strain of *S. cerevisiae* grown in CDM with and without L-lysine were analysed for the presence of the mousy taint compounds, ACPY, ETPY and ACTPY. The *S. cerevisiae*

strain AWRI 729 was used as the negative control in this case, as strain AWRI 796 did not grow in CDM.

Under these conditions no ACPY was detected in any sample. ACTPY was produced by all type strains of *Dekkera/Brettanomyces*, including the recently reclassified *B. nana* (formally *E. nanus*) (Table 3.7). This result indicates that the production of ACTPY is a characteristic of the *Dekkera/Brettanomyces* genera. A wide range in the concentration of this compound was observed, but no direct relationship to species was found. There was variation within and between the species groups. For example between *D. anomala* and *B. anomalus*, and *B. nana* and *B. naardenensis* there was at least a 10-fold variation in the amount of ACTPY produced (Table 3.7).

Exogenous L-lysine was not required for ACTPY formation, which is in contrast to a previous report that addition of L-lysine to the medium was required for the production of ACTPY (Heresztyn, 1986a). L-Lysine supplementation did, however, stimulate the production of ACTPY. For example, this was as much as 70-fold for *B. abstinens* (Table 3.7), which showed the greatest response to L-lysine.

ETPY was produced by all type strains of *Dekkera/Brettanomyces* assessed, as was the case for ACTPY (Table 3.8). The concentration of ETPY was in general much lower than that of ACTPY, with some strains, for example *D. anomala*, unable to produce a detectable amount without lysine added to the medium. As with ACTPY, there appeared to be no relationship between production of ETPY and species groups.

Table 3.7. Production of ACTPY by type strains of *Dekkera/Brettanomyces*. The yeasts were cultured in a CDM in the presence or absence of exogenous L-lysine. Strain names in bold type face represent a *Dekkera/Brettanomyces* species group described by Boekhout *et al.* (1994). The concentration of ACTPY is expressed as the sum of the two tautomers.

Strain	ACTPY (µg/L)	
	No L-lysine	**Plus L-lysine**
D. anomala	Trace	9.4
*B. anomalus**	4.1	140.3
B. claussenii	0.3	14.5
D. bruxellensis	4.4	33.8
B. bruxellensis *	0.3	88.1
D. intermedia *	Trace	83.5
B. intermedius *	1.1	38.8
B. lambicus *	Trace	45.8
B. custersii	2.1	63.1
B. abstinens	3.2	224.9
B. custersianus	7.1	73.9
B. nana	4.6	166.1
B. naardenensis	Trace	16.7
S. cerevisiae	ND	ND
CDM	ND	ND

ND Not detected.
* *Dekkera/Brettanomyces* strains previously reported to occur or grow in wine (van der Walt and van Kerken, 1959a; Heresztyn, 1986a; b; Querol and Huerta, 1987; Froudiére and Larue, 1990).

Table 3.8. Production of ETPY by type strains of *Dekkera/Brettanomyces*. The yeasts were cultured in a CDM in the presence or absence of exogenous L-lysine. Strain names in bold type face represent a *Dekkera/Brettanomyces* species group by Boekhout *et al.* (1994). The concentration of ACTPY is expressed as the sum of the two tautomers.

Strain	ETPY (µg/L)	
	No L-lysine	Plus L-lysine
D. anomala	ND	Trace
B. anomalus	Trace	2.4
B. claussenii	Trace	1.5
D. bruxellensis	Trace	2.3
B. bruxellensis	Trace	12.7
D. intermedia	0.2	0.4
B. intermedius	2.5	3.0
B. lambicus	ND	2.8
B. custersii	Trace	0.6
B. abstinens	Trace	3.7
B. custersianus	ND	1.7
B. nana	ND	5.2
B. naardenensis	Trace	2.3
S. cerevisiae	ND	ND
CDM	ND	ND

ND Not detected.

3.4 Discussion

3.4.1 Growth and mousy taint production

The growth of *B. bruxellensis* and *B. anomalus* in wine has been previously reported (Schanderl and Draczynski, 1952; Heresztyn, 1986b; Querol and Huerta, 1987), and therefore the growth of these strains in the two wine media used was expected. *Brettanomyces intermedius* and its perfect form *D. intermedia,* have also been reported to occur in wine (Froudiére and Larue, 1990). Limited growth for *D. intermedia* and no growth for *B. intermedius* under the conditions investigated in this present study were observed (Figures 3.3 and 3.4). Interestingly, *B. intermedius* is the most commonly isolated *Brettanomyces* strain from wine (Barret *et al.*, 1955; Peynaud and Domercq, 1956; van der Walt and van Kerken, 1959a; Wright and Parle, 1974; Heresztyn, 1986a; Gaia, 1987; Ciolfi *et al.*, 1988) and is generally associated not only with the production of mousy taint but also with the off-flavour ethylphenol (Chatonnet *et al.*, 1992; Chatonnet *et al.*, 1995). That no significant growth was achieved by either *B. intermedius* or its teleomorphic form *D. intermedia* suggests that the conditions were not conducive for growth or that these particular strains cannot grow in wine. The latter possibility seems the most likely since growth response was similar in the two wines used. However, both of these yeast remained viable after 55 days.

The effect of ethanol and SO_2 concentration on the growth and survival of *Dekkera/Brettanomyces* in wine has been investigated (van Zyl, 1962; Froudiére and Larue, 1990). An ethanol concentration greater than 16% (v/v) with free SO_2 present at greater than 60 mg/L are reported to be inhibitory to *Dekkera/Brettanomyces* (van Zyl, 1962). No pH data was provided, thus careful interpretation of these results must be made. Froudiére *et al.* (1990) found that *Dekkera/Brettanomyces,* including *B. intermedius* and *D. intermedia,* did not survive in wine with an ethanol concentration greater than 15% (v/v) and 11 mg/L free SO_2 at pH 3.5. Conditions used in the survival experiment described in

this thesis were well below the concentrations of ethanol and free SO_2 reported for the growth limits of *Dekkera/Brettanomyces*.

Unlike the present study, the growth and survival studies of van Zyl (1962) and Froudiére and Larue (1990) did not investigate mousy taint production by *Dekkera/Brettanomyces*. Mousy taint was detected with all strains in the yeast growth experiment, but only at a low level with *D. intermedia*. Strains of *B. intermedius* have been isolated previously from mousy wines (Peynaud and Domercq 1956; van Zyl 1962; Heresztyn 1986a). It was also shown that *B. intermedius* could produce a taint when inoculated into a sound wine (Heresztyn, 1986a; b). Although *B. bruxellensis* and *B. anomalus* have been detected in wine, there have been no reports of their isolation from mousy wines. Both these yeast have been reported as producing mousy taint in an apple or blackcurrant juice medium and *B. anomalus* in a chemically defined culture medium (Tucknott, 1977). Further, it has been found that *B. anomalus* could produce a mousy taint when inoculated and grown in a grape juice or a wine (Heresztyn, 1986b).

The results from the yeast growth trial verifies previous work linking *B. intermedius* and *B. anomalus* with mousy taint production. These data also confirm that the two species of *Dekkera/Brettanomyces* associated with wine, *D. anomala* (synonym *B. anomalus*) and *D. bruxellensis* (synonym *B. bruxellensis*, *B. intermedius* and *D. intermedia*), contain strains that can produce mousy taint in wine.

The eight taint producing strains identified from the screening experiment are representative of the four species, *D. anomala*, *D. bruxellensis* and *B. naardenensis* and *B. custersianus* that were included in the classification of the *Dekkera/Brettanomyces* genera (Smith *et al.*, 1990). This was the first observation of production of mousy taint by *B. naardenensis* and *B. custersianus,* neither of which have previously been associated with wine or any alcoholic beverage. The strains that produced a strong and consistent mousy taint, such as *B. lambicus, B. anomalus* and *D. intermedia,* have been related to wine

(Schanderl and Draczynski, 1952; Heresztyn, 1986a; Froudiére and Larue, 1990). Mousy taint was consistently observed throughout the type strain screening trial with *D. intermedia,* which only produced slight taint when grown in wine. *Brettanomyces bruxellensis,* which produced taint when grown in wine, did not produce taint in the grape juice medium. Mousy taint, however, was detected with *D. bruxellensis,* the teleomorph of *B. bruxellensis*. These variations in mousy taint detection between the yeast growth and screening trial experiments may simply be differences in media composition, growth conditions, or a reflection of the limitation of the sensory evaluation technique employed, that is, the alkaline paper strip assessment. Any sensory evaluation technique relies on a subjective judgement by individuals, and this may account for the variation observed, although other factors such as masking effects, may also influence the results.

One possible masking compound was acetic acid. Production of this compound is a characteristic of these genera (Barnett *et al.*, 1990; Boekhout *et al.*, 1994). The aroma of acetate tended to increase as the screening progressed and was perceived on the alkaline strip. Acetic acid is produced by *Dekkera/Brettanomyces* via the oxidation of ethanol (Henrici, 1941; Skinner, 1947). By examining the growth of these yeasts under anaerobic conditions, the production of acetic acid would be limited. However, growth rate of the yeasts under these conditions would also be reduced.

Clearly, chemical analysis and quantification of the compounds responsible for mousy taint would remove any ambiguity. The development of the reliable extraction and analysis method for the compounds responsible for mousy taint, enabled an investigation of the typed strains of *Dekkera/Brettanomyces* for the production of mousy taint compounds without the need for subjective sensory assessment. There appeared to be no species or strain link to the formation of mousy taint compounds, as all *Dekkera/Brettanomyces* tested produced these compounds when L-lysine was added to the medium. There was also considerable variation in the range of concentrations of taint compounds produced within the species groups. This was especially true with ACTPY,

with all the strains previously reported to occur and grow in wine producing this compound.

The production of the mousy taint compounds by all the *Dekkera/Brettanomyces* strains examined indicates the formation of these compounds is a characteristic of these genera. That the recently classified *Brettanomyces* species *B. nana,* previously known as *E. nanus* (Hoeben and Clark-Walker, 1986; Clark-Walker *et al.*, 1987; Boekhout *et al.*, 1994), also produced both these compounds provides further evidence for the incorporation of this yeast into the *Brettanomyces* genus.

In all of the different growth media used, the control strains of *S. cerevisiae* (AWRI 796 and 729) did not produce any detectable mousy taint by sensory evaluation or by chemical analysis, nor was any taint detected in the uninoculated controls. That no mousy taint compounds were detected in the uninoculated controls indicates that these compounds are not produced as artefacts of the extraction and analysis procedure.

This systematic approach to investigating mousy taint across *Dekkera/Brettanomyces* yeast, over species as well as strains, has established that mousy taint production is a generic character not just confined to particular strains. This would suggest that any *Dekkera/Brettanomyces* yeast has the potential to cause mousy taint spoilage, further strengthening the position that adequate winery hygiene must be maintained to limit growth of any *Dekkera/Brettanomyces* yeast. Previous investigations of mousy taint production were limited by the analytical technique and instrumentation available. The development of the reliable and sensitive extraction and analysis technique has allowed a more detailed investigation of the compounds responsible for mousy taint.

3.4.2 Culture conditions

The ability of *B. bruxellensis* and *B. anomalus* strains to grow and produce taint in wine which is considered to be 'dry', shows that wines with a very low concentration of residual sugars are not protected from the growth of these spoilage yeast. This characteristic has been previously described for *Dekkera/Brettanomyces* (Peynaud and Domercq, 1956; van der Walt and van Kerken, 1958; van Zyl, 1962). Chatonnet *et al.* (1995) investigated the role of *Dekkera/Brettanomyces* yeast in the production of ethylphenols and determined that 275 mg/L of residual sugars (glucose + fructose + galactose + trehalose) was a sufficient amount of carbon to produce the threshold level concentration of ethylphenols. Only the glucose and fructose concentrations for the wines used in this experiment was determined, but it can be speculated that adequate amounts of residual sugars were present to support growth and produce taint.

The wine medium was held under air limiting conditions, but some oxygen may have been available to stimulate fermentation via the Custers effect. That the ethanol concentration was slightly reduced in all wine samples at the end of the growth experiment, even in those where growth did not occur, is not conclusive evidence for utilisation of ethanol as a carbon source. No significant difference in loss of ethanol was observed between wine where yeast growth occurred and wine that had no growth. Fermentation of ethanol by *B. bruxellensis* under aerobic conditions has been reported previously by Sanfaçon *et al.* (1976). They inoculated a *B. bruxellensis* strain into a radioactively labelled (1-^{14}C) ethanol medium, and were able to detect $^{14}CO_2$ generation. If glucose, however, was also present in the medium, no radioactive CO_2 was produced, indicating that glucose was the preferred carbon source. This suggests that residual sugars would be used before ethanol was metabolised and the change in ethanol concentration observed can probably be attributed to loss due to sampling and sparging of the wines throughout the trial.

The results of this yeast growth experiment suggest that while carbon source(s) concentration may have some influence, it is not necessarily a critical factor in taint production, as a viable non-growing strain (slowly diminishing in number) was sufficient for taint production. The minimum yeast population level required to achieve this detectable taint is unknown. There appears to be some effect due to strain as there were differences in taint production observed between the strains examined.

Metabolic activity of the strains screened in the grape juice medium was monitored by sugar utilisation. The rate of utilisation of the available sugar did not always relate to taint production. For example, *B. lambicus* was the strongest taint producer (Figure 3.5), but the fermentation proceeded at a slower rate than *B. intermedius* (Figure 3.7). Non-taint producing strains were also able to use glucose and fructose indicating that no obvious differences in metabolism of these carbohydrates exists that could account for the difference in taint and non-taint producing strains. Previous investigations examining growth and taint production by *Dekkera/Brettanomyces* in grape juice did not report any carbohydrate utilisation data (Heresztyn, 1984; 1986a; b).

The chemically defined culture media used by both Tucknott (1977) and Heresztyn (1986a; b) was initially based on the medium employed by Wikén *et al.* (1961) to investigate the Custers effect. Heresztyn (1986b) reported mousy taint production by *B. intermedius, B. anomalus* and a *Dekkera* strain, this representing at least 2 species groups, in a slightly different medium to Heresztyn (1986a). As described previously (Section 3.2.2.1) *D. anomala* requires *myo*-inositol for growth, and therefore this vitamin was incorporated into the CDM (Heresztyn, 1986b). This was the CDM used in the present study.

A stimulation of taint compound production was observed upon the addition of L-lysine to the CDM. This supports the data of previous investigations of mousy taint in relation to the role of this amino acid (Tucknott, 1977; Heresztyn, 1986a). The observation that L-

lysine supplementation is not required for the production of taint compounds is in contrast to previous work, which used several of the same strains and similar culture conditions as this trial. ACTPY was not detected when L-lysine was omitted from the growth medium (Heresztyn, 1986a). The concentration of the taint compounds in this present investigation without L-lysine added were not high but may still be important. That low levels of ETPY and ACTPY were produced without exogenous L-lysine is not unexpected, as L-lysine can be synthesised by these yeast and therefore would be present in the intracellular amino acid pool occurring naturally in the cell (Bode *et al.*, 1990). This endogenous L-lysine present in the cell may be acting as the precursor to the mousy taint compounds.

3.4.3 Mousy taint compounds

All strains of *Dekkera/Brettanomyces* examined in the CDM produced the mousy taint compound ACTPY, in the presence or absence of L-lysine. As described above a stimulatory effect on the production of taint compounds upon the addition of L-lysine was observed. This was most apparent for ACTPY. The increase of ACTPY production on the addition of L-lysine has been previously reported (Heresztyn, 1986a), but this the first report of the quantification of ACTPY. That significant amounts of ACTPY can be produced in the absence of L-lysine has implications for mousy taint production in wine; even the low levels of this amino acid found in wine could provide the stimulation for ACTPY formation. Further to this, it also indicates that a wine with no L-lysine is not protected from ACTPY production by *Dekkera/Brettanomyces.*

This study has confirmed that *B. intermedius* can produce ACTPY, and also that *B. anomalus* can produce ETPY, when these yeast were cultured in chemically defined media (Tucknott, 1977; Heresztyn, 1986a). The strain producing the lowest concentration of ACTPY, *D. anomala,* and the highest producing strain, *B. abstinens,* were both isolated from soft drink (Kolfschten and Yarrow (1970), Yarrow and Ahearn (1971) respectively). Mousy taint spoilage has not been reported in soft drink.

In the presence of L-lysine, all the *Dekkera/Brettanomyces* strains could produce ETPY, but to a much smaller extent than that of ACTPY. ETPY was also produced in the absence of L-lysine, but not for all strains examined. This further highlights the importance of ACTPY and confirms the assumption that it is the most critical compound to mousy taint. However, ACTPY and ETPY are in general always detected in association.

No clear relationship between species groups and the formation of ACTPY or ETPY was established. Why previous studies did not detect ETPY is unclear; it may be due the sensitivity of the analysis employed or simply that it was not looked for (Heresztyn, 1986a; b). Other studies did not detect this compound in mousy wine and therefore considered it unimportant (Craig and Heresztyn, 1984; Strauss and Heresztyn, 1984).

ACPY was not detected in any of the culture medium extracts of the *Dekkera/Brettanomyces* strains examined in the presence or absence of L-lysine. This result suggests that either the appropriate precursor(s) was not present in the chemically defined culture medium (L-ornithine not L-lysine is the likely precursor) or that *Dekkera/Brettanomyces* yeast are unable to produce ACPY. If this was the case the detection of ACPY in mousy wine could possibly be interpreted as an indicator of spoilage by bacteria. The role of these yeast in the production of ACPY was investigated further in Chapter 5.

CHAPTER 4 Physiological Aspects of Mousy Taint Production

4.1 Introduction

Recent studies investigating mousy taint production by *Dekkera/Brettanomyces* have focused on determining the compounds responsible for the taint and establishing what the precursors were in the formation of these compounds. It was the work of Tucknott (1977) and Heresztyn (1986a) that showed L-lysine and ethanol are important to the production of ETPY and ACTPY. Both used similar culture media with aerobic incubation. Neither of these studies reported any relationship to growth phase, or attempted to relate the physiology of *Dekkera/Brettanomyces* to taint production. Apart from helping in the understanding of the mechanism of mousy taint formation, identifying physiological aspects could lead to better preventative strategies for controlling mousy taint in the winery.

Mousy taint has long been associated with wines subjected to exposure to air. Wines exhibiting very strong mousy taints are often oxidatively spoiled as well (Henschke and Leske, unpublished observations). Some earlier work has investigated the role of oxygen in taint production. However, these studies examined mousy taint as purely the result of a chemical process. It was proposed that mousy taint was the result of a chemical reaction at high redox potential, although it was conceded that microorganisms, in this case bacteria, may be accelerating the oxidative process [(Drboglav, 1940) cited by Tucknott, 1977]. Osterwalder (1948) suggested that the physico-chemical taint was not a true mousy taint and this resulted in the debate over its origin. The evidence for a chemical reaction responsible for mousy taint was shown to be incomplete and confused (Tucknott, 1977).

In a review of bacterial spoilage of wine, bacteria were classified according to the types of spoilage reactions undertaken; under all categories, in both aerobic and anaerobic reactions, mousy taint was listed (Vaughn, 1955). This suggested that bacteria can

produce taint under varying physiological influences, but does not clarify the role of air or oxygen. Furthermore, Tucknott (1977) suggested that oxidation is an important factor in the production of mousy taint by LAB and *Dekkera/Brettanomyces* yeast, although it was not investigated.

There is therefore a limited understanding of the physiology of taint production in yeast and bacteria. Factors affecting taint development, such as role of growth, need to be investigated. An aim of this research was to examine when in the growth cycle production of mousy taint compounds by *Dekkera/Brettanomyces* occurs.

The influence of aeration on the growth and fermentation of *Dekkera/Brettanomyces* yeast with respect to production of mousy taint was investigated. Physiological investigation of *Dekkera/Brettanomyces* have previously employed whole cell incubation (WCI) studies. For example, the accumulation of acetate and succinate and the utilisation of ethanol as a carbon source in *B. bruxellensis* has been examined using this type of methodology (Sanfaçon *et al.*, 1976). Other studies have used whole cell incubations or suspensions to investigate the Custers effect (Scheffers, 1966; Gaunt, 1988). Development of a model system to investigate mousy taint production by *Dekkera/Brettanomyces* yeast using a high cell density WCI was undertaken. This system was then adapted to examine the effect of air on taint production. Using the WCI as a rapid assay system for investigating mousy taint formation, both anaerobic and aerobic treatments could be undertaken within the same experimental conditions. Through these investigations a better knowledge of the critical factors in mousy taint formation in wine was developed.

4.2 Materials and methods

4.2.1 Mousy taint production and stage of growth

The production of mousy taint was monitored throughout the growth cycle by a 'kill-type' or sequential sacrifice experiment to avoid problems of removing large volumes of sample from the fermentation vessel. Using this protocol, an inoculated culture was aliquoted into separate fermentation vessels. Over the period of the experiment, the fermentations in these individual vessels was stopped sequentially throughout the growth stages at 24 hr intervals and the resultant culture filtrate was then analysed for the accumulation of mousy taint compounds.

4.2.1.1 Yeast strain and culture conditions

Brettanomyces anomalus (AWRI 1101) was inoculated into a capped culture tube containing 10 mL of CDM, at pH 3.8 and incubated at 25°C. This medium did not contain L-lysine. For the formulation of the CDM see Section 3.2.4.1. After incubating for 7 days, 200 mL of the CDM (without L-lysine) was inoculated and cultured aerobically (200 opm) at 25°C for 4 days. This culture was then added to 1.5 L of CDM (without L-lysine) and incubated aerobically (200 opm) at 25 °C until the cell number reached 1 x 10^8 cells/mL (by haemocytometer count). The culture was then used to inoculate 12 L of CDM containing L-lysine (100 mg/L) to a cell density of 5 x 10^6 cells/mL. Five litres of CDM without L-lysine was also inoculated at the same cell density. The inoculated CDM supplemented with L-lysine was then aliquoted (500 mL) into 24, 1 L Schott bottles (cotton wool plugged) and the inoculated CDM without L-lysine into 10, of the same 1 L Schott bottles. These flasks were then incubated aerobically (150 opm) at 25 °C. Sampling (1 mL) was undertaken every 24 hr for the CDM plus L-lysine treatment, with cell number determined by haemocytometer count and glucose catabolism monitored by refractive index and Clinitest. Growth was stopped in two flasks daily by centrifugation and membrane filtration (0.2 μm). The filtrate was then

stored at –20 °C prior to further analysis. This process was carried out for 7 days and then final duplicate samples were taken after a further 9 days. The procedure was the same for the CDM without L-lysine treatment, with the exception that sampling was carried out every 48 hr. The final samples were taken at the same time as for L-lysine supplemented treatment.

Other analyses carried out on these samples included glucose and ammonia determination by UV enzymatic assay (Boehringer Mannheim test combination Cat. No. 139 106 and 542 946 respectively), L-lysine by HPLC [according to the method of Haynes *et al.* (1991)], and the concentration of mousy taint compounds in the samples by the continuous liquid/liquid extraction and analysis technique (Section 2.2.3.2).

4.2.2 Influence of air on mousy taint production

4.2.2.1 Fermentation

A. Yeast strain and culture conditions. *B. lambicus* (AWRI 1127) the strain identified from the 'Type strain screening trial for mousy taint formation' (Figure 3.5) as producing the strongest mousy taint was used in this experiment. A starter culture was prepared by inoculation (1 x loop-full) into YPG (120 mL) in a cotton wool plugged, baffled, 250 mL conical flask. The culture was incubated aerobically (200 opm) at 25 °C until a cell number of 1 x 10^8 cells/mL was reached (determined by a haemocytometer count). The fermentations were then inoculated to produce a cell density of 5 x 10^6 cells/mL in 1.2 L of medium.

B. Treatments. Two conditions were investigated; (1) air-limited, the medium was nitrogen gas sparged prior to inoculation and the headspace was sparged throughout the fermentation, (2) air-saturated, the medium was not protected from exposure to air by nitrogen gas sparging at any stage. The growth medium used was based on the grape juice

medium of the 'Type strain screening trial for mousy taint formation' (Section 3.2.2) (Table 4.1 for the analytical details and modifications made to prepare the medium, the grape juice used was Doradillo, obtained from the Riverland, South Australia, 1993 vintage). To reduce the amount of SO_2 present in the grape juice to the level indicated in Table 4.1, an appropriate addition of H_2O_2 [30% (v/v)] was made (Hoffman and Edwards, 1975; Anonymous, 1997).

Table 4.1. Initial analysis of neutral Doradillo grape juice and the additions and modifications made to formulate the grape juice medium. Analysis methods as described in Table 3.2.

Analysis/Addition†	Initial	Final
pH	3.08	3.5
Glucose + fructose (g/L)	204	204
SO_2 free (mg/L)	16.7	0
SO_2 total (mg/L)	122	6.7
Vitamins†		see Table 3.4
Diammonium phosphate (mg/L)†		200
L-lysine HCl (mg/L)†		50

Special conditions were required to establish the air-limited treatment. After additions and modifications were made to formulate the grape juice medium, oxygen was removed by sparging with sterile filtered (0.2 µm) N_2 for 2 hr (Figure 4.1). The grape juice medium was then transferred anaerobically using a peristaltic pump to a 2 L fermentation vessel which had been pre-gassed with sterile N_2 for 15 min to remove air (Figure 4.2). The grape juice medium was treated in the same way as for the air-saturated condition, except that no N_2 sparging was used and the fermentations were carried out in cotton wool plugged 2 L Schott bottles.

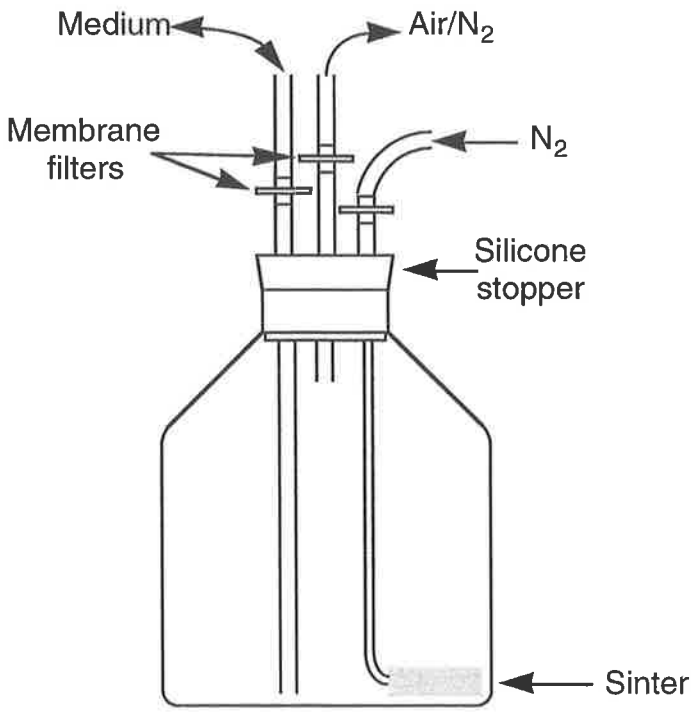


Figure 4.1. Apparatus for preparation of the air-limited grape juice medium.

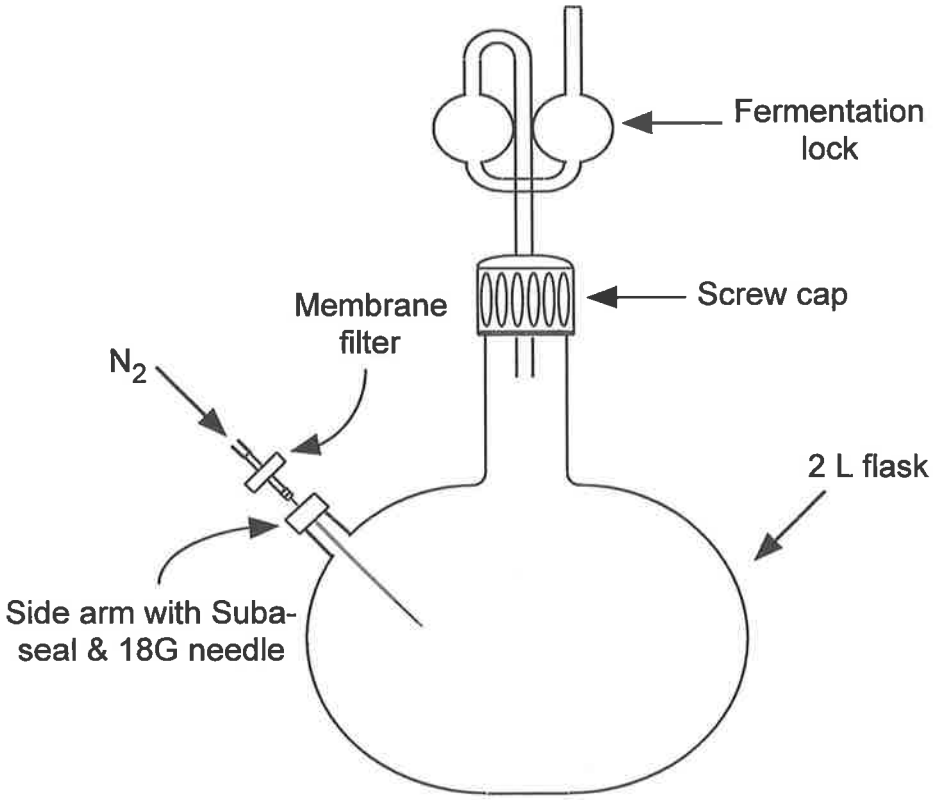


Figure 4.2. Vessel used for fermentation of grape juice medium by *B. lambicus* under air-limited conditions.

All treatments, in duplicate, were placed in an orbital shaking water bath, and incubated at 100 opm and 25 °C. The fermentations were monitored for growth by determining culture absorbance (650 nm) and fermentation activity estimated using refractive index measurement and Clinitest analysis, which were used to measure the removal of sugar from the medium. When Clinitest analysis indicated that all glucose and fructose was consumed, yeast cells were removed by centrifugation and membrane filtration (0.2 µm). The culture filtrates were then stored at –20 °C prior to further analysis. Other analyses carried out included glucose and fructose (UV enzymatic method), and the extraction and analysis procedure for mousy taint compounds as described in Section 2.2.3.2.

4.2.2.2 Whole cell incubation studies

A. Whole cell incubation method development. Using the WCI methodology of Sanfaçon *et al.* (1976) as a basis, a WCI assay as a model system for mousy taint production by *B. anomalus* (AWRI 1101) was established. Two test media were initially investigated; a Yeast nitrogen base (Difco) medium (Sanfaçon *et al.*, 1976), and the CDM used in the investigations described in this thesis (Section 3.2.4.1).

Starter cultures were prepared by inoculating 50 mL of each media: (I) Yeast nitrogen base (6.7 g/L), D-glucose (30 g/L), in potassium phosphate buffer (0.2 M), pH 6.6; (II) CDM standard formulation at pH 6.6. These starters were cultured in 500 mL cotton wool plugged conical flasks with 3 x loop-full of *B. anomalus*, aerobically (200 opm) at 27 °C for 72 hr. Pre-cultures were then prepared from the starter cultures. The pre-cultures used the same media as the starter culture, 400 mL in 2L cotton wool plugged conical flasks, which were then inoculated at 5 % (v/v) (*ca.* 1 x 10^7 cell/mL) and cultured under the same conditions as the starter culture for 72 hr. Cells from the pre-culture were then washed (2 x 10 min, 4500g, with 2 x 200 mL sterile Milli-Q water) and resuspended in the incubation media to give a final absorbance (650 nm) of 10 units. The incubation media contained: (a) Yeast nitrogen base (6.7 g/L), D-glucose (15 g/L), ethanol [1.5 % (v/v) redistilled 96 %

(v/v)] and L-lysine.HCl (100 mg/L), in potassium phosphate buffer (0.2 M) at pH 6.6; (b) CDM standard formulation with the following exceptions, D-glucose (15 g/L), ethanol (1.5% v/v) and L-lysine.HCl (100 mg/L) at pH 6.6. The incubation mix [medium + cells from the pre-culture at 10 absorbance units (650 nm), total volume *ca.* 100 mL] was then incubated for 20 hr in cotton wool plugged 500 mL conical flasks at 200 opm and 27 °C. All pre-cultures and incubation cultures were prepared in duplicate.

Sensory evaluation of the incubation cultures was then carried out by alkaline strip assessment (see Section 3.2.2.3), after first adjusting the sample to approximately pH 8 (using NaOH (0.1M) with phenol red as an indicator). The evaluation was carried out by a panel of individuals familiar with mousy taint aroma. This data indicated that while a slight taint was detected in the Yeast nitrogen base incubation culture sample, the CDM incubation culture produced a much stronger mousy taint odour. On this basis, the CDM medium was investigated further.

Optimisation 1. To standardise conditions, the same concentration of glucose (50 g/L) was used in the starter culture, pre-culture and incubation media. Several incubation media were initially trialed, all containing ethanol [5 % (v/v)] and L-lysine.HCl (1 g/L); (c) no glucose, (d) acetate (1 g/L, as sodium acetate), (e) pH 3.8, and (f) pH 6.6. These incubation cultures were prepared, incubated and subjected to sensory assessment in the same manner as described above. The incubation culture volume used was approximately 50 mL, and the incubations were conducted in 250 mL conical flasks.

A dramatic loss in taint intensity was observed when glucose was excluded, while pH 3.8 and the acetate treatments produced a moderate mousy taint, stronger than the initial CDM treatment. This indicated that ethanol, L-lysine, and glucose were beneficial to taint development.

Optimisation 2. Several other conditions were examined on the basis of the results of Optimisation 1. All incubation media were at pH 3.8 and with a glucose concentration of 50 g/L but varying acetate concentrations; (g) acetate and L-lysine (both at 1 g/L), (h) acetate and L-lysine (both at 5 g/L), and (i) as for (g) with acetaldehyde (150 mg/L. These incubation cultures were prepared, incubated and subjected to sensory assessment in the same manner as Optimisation 1. The shaking platform oscillation speed was reduced to 100 opm to minimise any loss of volatile compounds.

Alkaline strip assessment indicated stronger mousy taint production in the 5 g/L acetate and L-lysine treatment (h) than the 1 g/L treatment (g). The addition of acetaldehyde did not enhance taint production. The mousy taint odour produced in any of the above treatments, however, had only been moderate to strong, and so further optimisation was required.

Optimisation 3. Two other treatments were examined; (h) as a reference, (j) as for (h) but not aerated, incubated in a capped bottle with minimal headspace, (k) as for (h) with double the cell density, 20 absorbance units (650 nm). These incubation cultures were prepared, incubated and subjected to sensory assessment in the same manner as described in Optimisation 1.

The double cell density samples (k) produced a very strong mousy taint. The importance of air or oxygen in mousy taint was indicated by treatment (j), as only a slight taint was detected under this non-aerated condition. These data demonstrated that treatment (k) would be suitable as a model system for investigating mousy taint production. At this stage it was necessary to re-examine some of these treatments via the analytical procedure developed for ACTPY, to confirm that mousy taint compounds were being produced in the WCI.

Mousy taint compound analysis. The strongest taint producing treatments from each optimisation step were analysed for mousy taint compounds (Table 4.2). A scaled down version of the continuous liquid/liquid extraction was required, as the sample volumes were only approximately 50 mL. Quantification was required to compare the relative concentration of the mousy taint compounds within a particular experiment.

A mini continuous liquid/liquid extractor with a sample volume of approximately 35 mL was used, while all other conditions in the extraction, analysis and quantification were the same as the full scale method (Section 2.2.3.2). A sample from a fermentation experiment that was known to contain ACTPY (Section 5.3.3; *B. anomalus*, in CDM with L-lysine) was extracted using the scaled down procedure. ACTPY, ETPY and both internal standards were recovered successfully. A further refinement of this technique was investigated, whereby the initial step, removal of the acid and neutral volatiles, was omitted. This was again carried out on the fermentation sample. The mousy taint compounds and internal standards were detected using the no acid/neutral extraction procedure, indicating the scaled down extraction technique would be suitable for the analysis of small volume samples.

Analysis of the WCI optimisation samples by this technique indicated that ACTPY was being produced in the WCI (Table 4.2). The double cell density condition [*i.e.* treatment (k)] was adopted as the standard condition for the WCI model system.

Table 4.2. Whole cell incubation optimisation; analysis of strongest taint producing treatments from WCI Optimisation 1, 2 and 3. The concentration of ACTPY is expressed as the sum of both tautomers.

Incubation culture conditions	ACTPY (µg\L)
(d) CDM with, lysine (1 g/L), ethanol (5% v/v), pH 6.6, cell density 10 absorbance units (650 nm)	38.4
(e) CDM with lysine (1 g/L), acetate (1 g/L), ethanol [5% (v/v)], pH 3.8, cell density 10 absorbance units (650 nm)	15.9
(h) CDM with lysine (5 g/L), acetate (5 g/L), ethanol [5% (v/v)], pH 3.8, cell density 10 absorbance units (650 nm)	47.7
(k)* CDM with lysine (5 g/L), acetate (5 g/L), ethanol [5% (v/v)], pH 3.8, cell density 20 absorbance units (650 nm)	311.1

*adopted as WCI standard condition

B. Effect of air. The WCI assay was used to investigate the influence of air or oxygen on taint production as determined by GC/MS analysis. The major advantage the of the WCI assay over the fermentation system described in Section 4.2.2.1 is the reduction in time taken to complete the experiment, as air-limiting or anaerobic fermentation by *Dekkera/Brettanomyces* are slow. Using the WCI, aerobic and anaerobic treatments were investigated within the same time frame, although working culture preparation times varied. This methodology also allowed any variation due to differences in culture biomass to be eliminated.

The conditions investigated in relation to the role of air in taint production using the WCI assay were: (A) aerobic pre-culture followed by aerobic or anaerobic incubation, (B) anaerobic pre-culture then aerobic or anaerobic incubation. All experiments were carried

out in duplicate and repeated with freshly prepared cells. The incubation medium formulation did not change from the standard condition described in Section 4.2.2.2A, with the exception of the aerobiosis/anaerobiosis conditions.

To prepare strictly anaerobic media required the use of an anaerobic hood (Levett, 1991). This apparatus allowed the absolute removal of oxygen from the media. The anaerobic pre-culture medium (500 mL) was sparged for 2 hr with oxygen stripped N_2. The medium was then transferred to the anaerobic hood and sterile membrane filtered (0.2 μm) into 1 L Schott bottles (4 x 100 mL). The bottles were then loosely capped and left for 24 hr in the anaerobic hood.

The starter culture, which was grown aerobically, was centrifuged (4500 g, 10 min) and the supernatant discarded. The cells were then transferred to the anaerobic hood, and resuspended in sterile oxygen free water (prepared by degassing for 2 days in the anaerobic hood). The washing was repeated, then the cells resuspended in 5 mL of pre-culture medium and inoculated at 5% (v/v) into the Schott bottles. The lids were then sealed and the pre-cultures removed from the anaerobic hood and incubated at 200 opm for 14 days at 27°C.

The incubation medium was prepared (200 mL) and placed in the anaerobic hood, sterile membrane filtered (0.2 μm) and allowed to degas for 24 hr prior to use. Anaerobic working culture cells were harvested and treated as per the standard condition, except all manipulations were carried out in the anaerobic hood. Oxygen contamination via the centrifugation process was considered to be negligible after centrifuging oxygen free water containing an oxygen indicator [resazurin 0.1% (w/v), Levett (1991)] showed no oxygen pick-up throughout the handling process.

To reduce variability during sample processing both anaerobic and aerobic test incubation cultures and pre-cultures were prepared in the anaerobic hood. The anaerobic incubations

were carried out in 100 mL Schott bottles and the aerobic incubations were undertaken in 100 mL conical flasks that were cotton wool plugged), and treated using the standard test conditions.

4.3 Results

4.3.1 Mousy taint production and stage of growth

The production of mousy taint compounds was monitored throughout the growth cycle of a *B. anomalus* (AWRI 1101) fermentation in the presence or absence of exogenous L-lysine. Without the L-lysine addition to the fermentation medium the ACTPY concentration did not increase beyond a low baseline level (*ca.* 0.2 µg/L) throughout the growth cycle (Figure 4.3). ETPY production was also limited under these conditions. In the L-lysine supplemented cultures, amino acid analysis of the fermentation samples indicated that lysine was taken up from the growth medium within the first 24 hours. The uptake of L-lysine corresponded to the initiation of production of ACTPY (Figure 4.4). During exponential growth the formation of this compound increased until the onset of stationary phase when the maximum concentration was measured. A decrease in ACTPY was found during the mid stationary phase which then stabilised during the later stationary phase. ETPY formation was delayed with respect to ACTPY production, with maximum production occurring well into the stationary phase (Figure 4.4). As expected, no ACPY was produced in either the presence or absence of L-lysine.

No difference was observed between the treatments (presence or absence of L-lysine) in the rate of glucose consumption and demand for nitrogen (determined as ammonia). Glucose was completely consumed by day 6 and residual ammonia remained at approximately 50 mg/L from day 5 until day 16, the final time point.

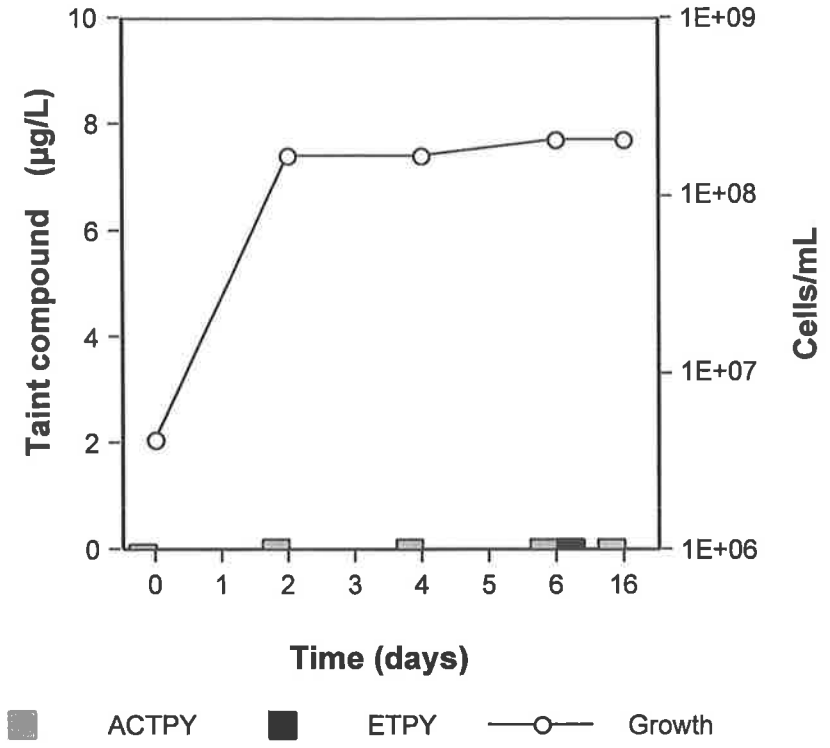


Figure 4.3. Production of taint compounds by *B. anomalus* (AWRI 1101) during growth cycle with no L-lysine added to the medium.

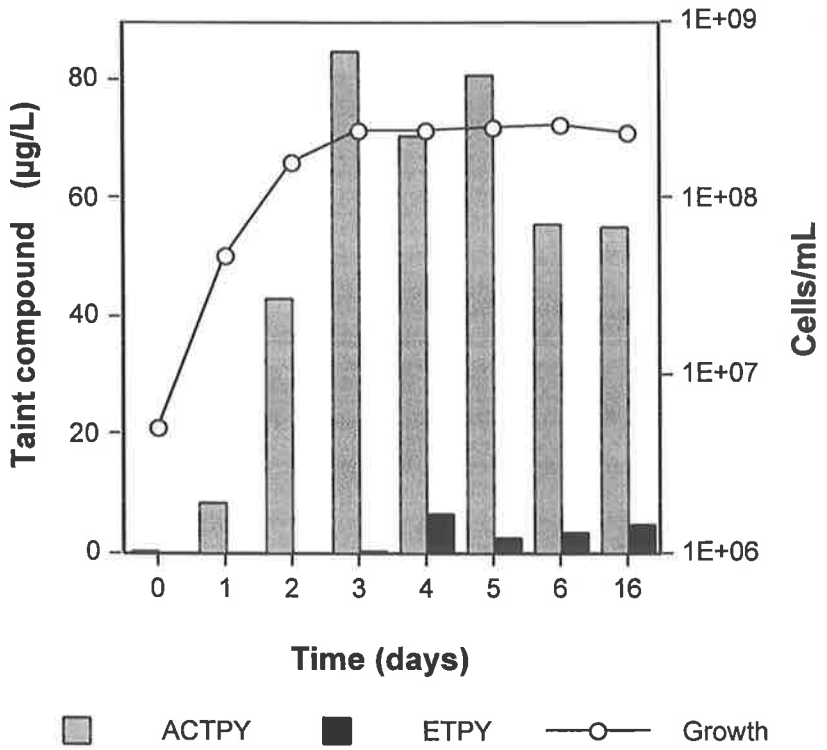


Figure 4.4. Production of taint compounds by *B. anomalus* (AWRI 1101) during growth cycle with L-lysine added to the medium.

4.3.2 Influence of air on taint production

4.3.2.1 Fermentation

The effect of air on mousy taint production by *B. lambicus* (AWRI 1127) grown in a grape juice medium was investigated. The time taken to completely metabolise all glucose and fructose varied between the air-saturated and the air-limited treatments (*ca.* 2-fold respectively) (Figure 4.5 and 4.6). This difference was expected, as air or oxygen stimulates fermentation in *Dekkera/Brettanomyces* via the Custers effect. A higher biomass (determined by absorbance at 650 nm) was produced under the air-saturated treatment (35% higher) when compared with the air-limited condition (Figures 4.5 and 4.6). The production of mousy taint compounds also differed between treatments (Table 4.3). Under these conditions no ACPY was detected. However, both ETPY and ACTPY were produced at the highest concentrations under the air-saturated treatment (Table 4.3).

Table 4.3. Mousy taint compounds produced by *B. lambicus* (AWRI 1127) grown in grape juice medium under air-limited and air-saturated conditions. The concentration of ACTPY is expressed as the sum of both tautomers.

Treatment	Compound (µg/L)		
	ACPY	ETPY	ACTPY
Air-limited	ND	9.0	6.6
Air-saturated	ND	40.4	42.0

ND indicates not detected

4.4.3.2 Whole cell incubation

When pre-cultures were aerobically grown (Condition A), only a slight variation was observed between aerobic and anaerobic incubations; a reduction in ACTPY of 15.8% under the anaerobic incubation. When anaerobically treated pre-cultures were employed

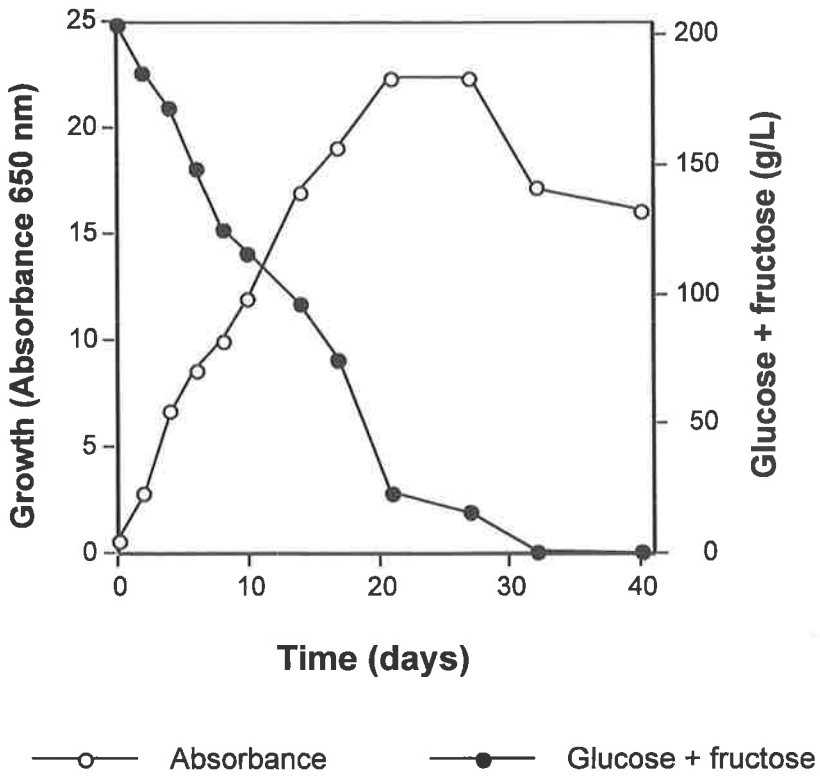


Figure 4.5. Growth of *B. lambicus* (AWRI 1127) and sugar consumption in a grape juice medium under air-saturated conditions.

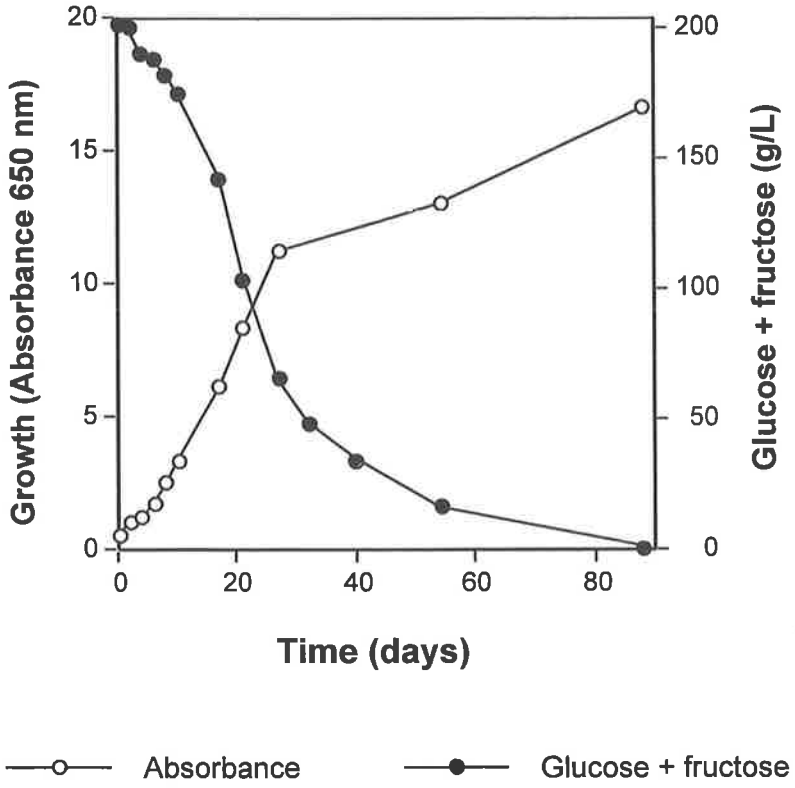


Figure 4.6. Growth of *B. lambicus* (AWRI 1127) and sugar consumption in a grape juice medium under air-limited conditions.

(Condition B), the aerobic/anaerobic incubation had a large effect on ACTPY production. The amount of ACTPY formed was reduced by 83.6% when the anaerobically grown pre-culture treatment was incubated anaerobically when compared to the anaerobically grown pre-culture treatment that was incubated aerobically. This data highlights the importance of oxygen or air, and together with the fermentation data (Section 4.3.2.1), is the first report of the influence of air on mousy taint production by yeast.

4.4 Discussion

Prior to discussing the implications of the experiments described above, it is important to clarify some of the choices made in strain and media selections. The fermentation experiment (Section 4.3.2.1) to examine the influence of oxygen on mousy taint production was conducted before the detailed investigation of mousy taint production by all type strains of *Dekkera/Brettanomyces* ('Identification and quantification of mousy taint compounds' Section 3.3.3), and during the final validation of the extraction and analysis procedure. This is why the grape juice medium was used, as at that time it was the only culture medium tested that consistently allowed the production of mousy taint. *Brettanomyces lambicus* was selected for the fermentation experiment as it was identified through the 'Type strain screening trial for mousy taint formation' (Section 3.3.2) as the strongest mousy taint producing strain. After the survey of all *Dekkera/Brettanomyces* species grown in CDM was conducted (Section 3.3.3), this culture medium and the strain *B. anomalus* (AWRI 1101) was selected for further investigation. This strain was chosen for several reasons; it was previously reported to grow in wine (Schanderl and Draczynski, 1952; Heresztyn 1986b), it produced mousy taint in the 'Yeast growth and mousy taint formation in wine' experiment (Section 3.3.1), it was a consistent taint producer in the 'Type strain screening trial for mousy taint formation' (Section 3.3.2), it produced the highest ACTPY concentration of all the strains surveyed that were associated with wine (Table 3.7), it was the strain found to produce mousy taint in chemically defined, apple and

blackcurrant juice culture media (Tucknott, 1977), and it was reported to produced mousy taint in grape juice, wine and a chemically defined culture medium (Heresztyn, 1986b).

The relationship between growth phase and mousy taint formation was not investigated in previous studies. In the present study, using the CDM and the sequential sacrifice protocol, an increasing concentration of ACTPY was detected as *B. anomalus* progressed through the growth cycle from early exponential to stationary phase. This suggests a link between mousy taint production and associated growth metabolism. Stimulated ACTPY production coincided with the uptake of L-lysine from the growth medium, while without the addition of this amino acid, only a background level of ACTPY was produced. As *Dekkera/Brettanomyces* can synthesise L-lysine (Bode *et al.*, 1990), this intracellular or endogenous lysine is probably acting as the precursor to ACTPY when no exogenous L-lysine is available. It is therefore likely that exogenous L-lysine increased the intracellular lysine pool thereby stimulating taint formation.

Cell number was not affected by the presence or absence of L-lysine (2.3×10^8 cells/mL and 2.1×10^8 cells/mL respectively) and therefore does not account for the difference in ACTPY produced. The slightly higher nitrogen content in the medium containing L-lysine also does not explain the ACTPY production, as the residual ammonia detected at the end of the trial in both treatments was at approximately the same concentration. This provides further evidence that the catabolism of exogenous L-lysine is central to significant taint production.

While the growth experiment showed the relationship between growth and mousy taint formation, taint was detected in another experimental system in which growth was not observed. A slight mousy taint was detected in the 'Yeast growth and mousy taint formation in wine' experiment (Section 3.3.1) that was produced by non-growing viable cells, which suggests that growth is not necessary for taint production. Thus, the limited metabolic activity associated with these viable cells was sufficient for taint production. It

should be noted that the non-growing cells were in contact with the wine-based medium for 55 days, suggesting that the rate of mousy taint formation was very slow under the metabolically limited conditions. The strongest taints, however, were detected in the wines in which yeast growth had occurred.

A conclusion which could be made from these observations is that the intracellular concentration of lysine may influence the rate of taint formation. This could be determined by examining the intracellular concentration of lysine and relating this to the rate of taint accumulation in the medium.

Two experimental systems were used to investigate the effect of oxygen or air on mousy taint production. Firstly, a batch growth experiment, and secondly, a whole cell incubation protocol. The WCI assay allowed the effect of oxygen on the pre-culturing of the cells to be examined independently of the incubation process, during which taint compounds biosynthesis could take place.

A large difference in the concentration of mousy taint compounds was observed between the air-saturated and air-limited fermentations carried out by *B. lambicus* in the grape juice medium. Air stimulated the production of these compounds. However, the difference in growth rate of at least half the time taken to completely use all glucose and fructose, and 35% more biomass produced under the air-saturated treatment, may also account for the increase in taint production. This indicated that oxygen was important to taint production, either directly, or by providing a stimulus to the growth of *Dekkera/Brettanomyces* yeast via the Custers effect.

To overcome the problem of difference in growth rate and biomass formation, the WCI was developed. This technique enabled direct comparison of taint production under aerobic and strict anaerobic conditions where biomass was constant. The incubations were

conducted at a very high cell density where no growth or limited growth occurred (determined by monitoring cell density before and after incubation).

A strong influence of the pre-culturing conditions was observed, with the anaerobic pre-culture condition strongly affecting ACTPY production in the WCI. The anaerobic incubation of the anaerobic pre-culture cells repressed ACTPY formation. However, if the anaerobic pre-cultured cells were transferred to the aerobic incubation condition ACTPY production was stimulated. This data suggests that oxygen may be influencing taint production directly, not just by stimulating growth and biomass formation. In this way oxygen may be directly involved in the biosynthesis of taint compounds via an oxygen dependent enzyme system or an oxidative chemical mechanism. Elucidation of the biochemical pathway(s) of mousy taint formation would clarify the role of oxygen; this is discussed in Chapter 6. The mechanism of oxygen stimulation was not further investigated here, as the aim was to examine the importance of oxygen to mousy taint production.

Why taint production was not strongly repressed under the aerobic pre-culturing followed by anaerobic WCI, is unclear. Aerobic pre-culturing could be influencing cell physiology by predisposing or adapting the yeast for taint production, which then occurred in the presence of the appropriate precursors (during the WCI) irrespective of the subsequent anaerobic incubation condition. Therefore, if the yeast was pre-cultured anaerobically this adaption for taint production in the presence of oxygen during the WCI stage would not have the opportunity to take place. Presumably growth is required for this adaptation to occur which is essentially inhibited by the WCI. The WCI data serves to highlight that the initial growth and physiological state of the yeast is important.

The results pertaining to the anaerobic pre-culture/aerobic incubation could serve as a model for mousy taint development in the winery situation, with oxygen as the switch for mousy taint biosynthesis. Wine, harbouring a population of viable or growing

Dekkera/Brettanomyces yeast, in the absence of oxygen will not become mousy, despite the presence of the other necessary precursors (for example, lysine). However, when this wine is exposed to oxygen via a processing or handling procedure, oxygen could act as a trigger for mousy taint production. A practical implication of the induction of taint formation by oxygen is that wine, which potentially contains all the necessary precursors for mousy taint production (yeast, lysine and ethanol) must be carefully protected from oxygen. If this cannot be achieved alternative steps must be undertaken to maintain strict winery hygiene, to limit the growth and occurrence of these yeast.

The detection of ETPY in early to middle stationary phase of growth indicates the biosynthesis is delayed with respect to ACTPY production. Thus, an alternative pathway of ETPY formation is likely. This could also suggest that a critical concentration of ACTPY is required to stimulate the formation of ETPY. The maximum concentration of ACTPY was achieved before significant amounts of ETPY were found, with the production of ETPY coinciding with a decrease in the overall concentration of ACTPY. Some loss in ACTPY concentration could be expected due to the volatile and highly reactive physico-chemical properties of this compound. The fact that there was a delay in the production of ETPY suggests that it is the product of further catabolism of ACTPY. If ETPY formation was purely due to the chemical degradation of ACTPY, it would be detected in a solution of synthetic ACTPY. From the results presented in Section 2.3.1.1 this was not the case. There have been no other reports of any chemical or metabolic association between ACTPY and ETPY. The only reported breakdown product of ACTPY, 2ACP (Buttery *et al.*, 1971), was not detected in any culture medium extracts.

Further evidence for a relationship between ACTPY and ETPY was found in the influence of air fermentation experiment in which *B. lambicus* was cultured in a grape juice medium. The ratio of the concentration of ETPY to ACTPY was considerably higher than found under other conditions presented in this thesis. In general, ACTPY was always detected at a higher concentration than ETPY. For example, *B. lambicus* cultured in CDM produced

16.4-fold more ACTPY than ETPY (see Tables 3.7 and 3.8). However, under air-saturated treatment the ACTPY concentration was only 1.04-times more than ETPY, and with the air-limited treatment, more ETPY than ACTPY was detected (Table 4.3). This experiment was also performed over a long time period, 40 days for the air-saturated and 88 days for the air-limited whereas all other fermentation trials lasted between 6 to 20 days. This increase in time could be sufficient to allow the slow metabolic transformation of ACTPY to ETPY. A further difference was the high sugar concentration of 204 g/L in the grape juice medium compared with 50 g/L in CDM. It is possible that cellular metabolism in the presence of a high sugar concentration could favour ETPY formation. This could be investigated by examining the ratio of ACTPY to ETPY production over a range of sugar concentrations.

The results of the experiments described in this Chapter show that cell physiology influenced the production of mousy taint by *Dekkera/Brettanomyces*. The biosynthesis of mousy taint compounds, in particular ACTPY, was stimulated in the presence of oxygen. The WCI analysis suggested that this stimulation was independent of growth. However, further work is required to determine the actual biosynthetic involvement of oxygen. The production of ACTPY increased throughout the growth cycle until the middle of the stationary phase, when an increase in the accumulation of ETPY was also observed. The detection of ACTPY coincided with the uptake of L-lysine from the culture medium.

CHAPTER 5 Metabolism of Mousy Taint Compounds: the Role of Amino Nitrogen

5.1 Introduction

Previous studies have described the importance of L-lysine to the production of mousy taint by *Dekkera/Brettanomyces*. Tucknott (1977) demonstrated that the L-lysine addition to a chemically defined culture medium stimulated the development of mousy taint by *B. anomalus*. This observation inferred a role for L-lysine in the formation of ETPY. L-Lysine was shown to be essential for the accumulation of ACTPY by several strains of *B. intermedius* in a chemically defined medium (Heresztyn, 1986a). Further to this, experiments described in this thesis (Section 3.3.3 and 4.3.1) have substantiated the central role of L-lysine in the production of ACTPY and ETPY by all *Dekkera/Brettanomyces* strains and species investigated.

The aim of this research was to obtain a more detailed understanding of the metabolic process by which mousy taint compounds are derived from L-lysine. The catabolism of L-lysine by *Dekkera/Brettanomyces* was investigated by several means. Firstly, growth of two species of *Dekkera* in CDM with L-lysine as a sole source of nitrogen was examined. This study was undertaken to determine if forcing nitrogen metabolism to utilise only L-lysine would enhance mousy taint compound formation. An investigation was carried out to determine the dose response in ACTPY and ETPY production relative to L-lysine concentration by *B. anomalus*. This was carried out to ascertain if exogenous L-lysine provided the stimulatory effect over a wide concentration range, as taint production relative to L-lysine concentration is of interest from a practical winemaking viewpoint. Finally, studies were undertaken to identify the nitrogen precursor to ACPY.

A variety of forms of L-lysine naturally occur, including the enantiomer D-lysine and various acetylated and methylated derivatives. Substitution of L-lysine with these

compounds in CDM was used to obtain evidence for the biochemical mechanism of mousy taint compound formation.

The use of mass labelling, or more commonly described as stable isotope labelling, is an elegant technique routinely employed for the elucidation of biochemical transformations in a variety of biological systems. This technique relies upon the naturally occurring non-radioactive isotopes of, for example, nitrogen (^{15}N), carbon (^{13}C) and hydrogen (deuterium, D). The stable isotope of nitrogen, ^{15}N, has been used extensively in lysine catabolism research (Weissman and Schoenheimer, 1941; Clark and Rittenburg, 1951; Rothstein and Miller, 1954b; Tanenbaum and Kaneko, 1964; Grove *et al.*, 1969; Miller and Rodwell, 1971; Read *et al.*, 1971; Mizon *et al.*, 1972; Guenegerich *et al.*, 1973; Leistner and Spenser, 1973; Guenegerich and Broquist, 1976; Fangmeier and Leistner, 1980; Irving *et al.*, 1983; Wickwire *et al.*, 1990). Recently the biosynthesis of ACPY by *Bac. cereus* was investigated via the incorporation of stable isotope labelled amino acids (Romanczyk Jr *et al.*, 1995).

Uniformly labelled L-lysine with ^{15}N and ^{13}C was used to provide a definitive answer as to how this amino acid was involved in the biosynthesis of ACTPY and ETPY by *Dekkera/Brettanomyces*. To establish which nitrogen of L-lysine was incorporated into these mousy taint compounds, individually labelled α-^{15}N and ε-^{15}N L-lysine were used as ACTPY/ETPY precursors in supplementation experiments.

5.2 Materials and methods

5.2.1 Sole nitrogen source

The nitrogen precursors for the three mousy taint compounds ACPY, ETPY, and ACTPY was investigated by fermentation experiments. Three strains of two species of *Dekkera, D. anomala* and *D. bruxellensis,* were assessed for growth and production of mousy taint

compounds in CDM with various sole sources of nitrogen; (1) ammonia ($(NH_4)_2SO_4$) as the standard nitrogen source of CDM, (2) the substitution of ammonia with L-lysine.HCl, and (3) the substitution of ammonia with L-ornithine.HCl. The formulation of the CDM is given in Section 3.2.4.1. The strain of *D. anomala* used was *B. anomalus* (AWRI 1101), and *B. intermedius* (AWRI 1104), and *B. lambicus* (AWRI 1127) were the *D. bruxellensis* strains investigated.

Each strain was inoculated (1 x loop-full) into a culture tube containing MYPG (5 mL) and incubated for 7 days at 25°C. A 1 mL aliquot of this culture was used to inoculate CDM (200 mL) at pH 3.8. The fermentations were conducted in cotton wool plugged 500 mL conical flasks, which were incubated at 150 opm and 25°C until the cell number reached 1 x 10^8 cells/mL, as determined by haemocytometer count. The strains were then inoculated into duplicate cotton wool plugged 1 L Schott bottles containing CDM (500 mL), that contained the appropriate nitrogen source (Table 5.1). The culture media were at pH 3.8 and incubated aerobically at 150 opm and 25°C. The fermentations were continued until glucose consumption ceased or all the glucose had been metabolised. Uninoculated controls of all media were also included. The nitrogen content of all treatments was standardised (Table 5.1). The standardised nitrogen content was based on CDM (that contained 2 g/L $(NH_4)_2SO_4$, see Table 3.6) supplemented with 100 mg/L L-lysine.HCl, a total of 439.4 mg/L N.

Table 5.1. Standardised nitrogen content of media used in sole nitrogen source experiment.

Medium	Nitrogen source	Amount (mg/L)
(1)	$(NH_4)_2SO_4$	2072
(2)	L-lysine.HCl	2864
(3)	L-ornithine.HCl	2644

The flasks were sampled every 24 hr and growth was monitored by measuring absorbance at 650 nm and glucose estimated by refractive index measurement and Clinitest tablets. At the end of the fermentation, cells were removed by centrifugation for 10 min at 4500g followed by membrane filtration (0.2 µm). The filtrates were stored at –20°C prior to extraction and analysis for mousy taint compounds by the procedure described in Section 2.2.3.2.

5.2.2 Dose response to L-lysine

The production of ACTPY relative to the concentration of L-lysine in the growth medium was investigated. The strain *B. anomalus* AWRI 1101 was inoculated using 3 x loop-full into CDM (60 mL) at pH 4.5 in a cotton wool plugged 250 mL conical flask and incubated aerobically at 180 opm and 27°C. The starter culture medium did not contain L-lysine. The starter culture was monitored by cell counts using a haemocytometer counting chamber, and used when the cell number reached 1 x 10^8 cells/mL.

This starter culture was then used to inoculate to a cell density of 5 x 10^6 cells/mL, duplicate cotton wool plugged 250 mL conical flasks containing CDM (100 mL) at pH 4.5, supplemented with L-lysine.HCl at four concentrations as described in Table 5.2. An additional treatment of CDM supplemented with L-ornithine.HCl (100 mg/L) was also included. All other conditions were the same as for the L-lysine treatments. These cultures were then incubated at 180 opm and 27°C. Daily samples of 1 mL were taken to monitor growth (by absorbance at 650 nm) until stationary phase was reached. The cells were then removed by centrifugation for 10 min at 4500g. The supernatant was then membrane filtered (0.2 µm) and stored at –20°C prior to extraction and analysis. The scaled down extraction procedure described in Section 4.2.2.2.A was employed.

Table 5.2. The additions of L-lysine.HCl used to examine the dose response in ACTPY and ETPY formation.

L-Lysine addition (mg/L)
10
50
100
1000

5.2.3 L-Lysine derivatives

Several derivatives of L-lysine were assessed for their ability to act as precursors for the formation of ACTPY by *B. anomalus* (AWRI 1101) cultured in CDM. The addition of each derivative was standardised for nitrogen content, based upon 100 mg/L of L-lysine.HCl giving 15.34 N mg/L. The treatments examined are described in Table 5.3.

Table 5.3. The concentration of L-lysine and L-lysine derivatives used to provide 15.34 mg/L nitrogen.

Supplement	Addition (mg/L)
L-lysine.HCl	100
α-acetyl-L-lysine	103
ε-acetyl-L-lysine	103
ε-methyl-L-lysine.HCl	108

The culture conditions and growth monitoring are as described in Section 5.2.1, with the following exceptions; only *B. anomalus* was examined, and a final fermentation volume of 200 mL in duplicate cotton wool plugged 500 mL Schott bottles was used. Extraction and

analysis of ACTPY was carried out using the scaled down extraction procedure described in Section 4.2.2.2.A.

5.2.4 D-lysine

The potential of D-lysine to act as a precursor to mousy taint compounds was investigated. This was carried out under two conditions using CDM supplemented with D-lysine.HCl (100 mg/L), and with D-lysine.HCl as the sole source of nitrogen.

The fermentation and extraction protocol used for the 100 mg/L treatment was as described in Section 5.2.2. Uninoculated controls were included as well the standard controls of L-lysine.HCl (100 mg/L) and no lysine added treatments. All treatments were conducted in triplicate. Uptake of D-lysine from the growth medium was monitored by HPLC analysis, as described for L-lysine in Section 4.2.1.1.

For the sole nitrogen source treatment, the amount of D-lysine added to the CDM was 2864 mg/L. This was the same concentration as for L-lysine when used as a sole nitrogen source, as described in Section 5.2.1. A control using L-lysine.HCl as the sole nitrogen source was included, and the fermentations and extractions carried out as described in Section 5.2.2. The strain used in all the treatments was *B. anomalus* (AWRI 1101).

5.2.5 Stable isotopes of L-lysine

The addition of stable isotope or mass labelled L-lysine to culture media was used to monitor the production of ACTPY and ETPY by *B. anomalus* (AWRI 1101).

5.2.5.1 Chemicals

Initially, L-lysine uniformly labelled with the stable isotope of carbon (^{13}C) and the stable isotope of nitrogen (^{15}N) was used in a feeding experiment. The mass labelling increased

the molecular weight of L-lysine.HCl from 182.6 to 190.6. In further experiments the stable isotopes α-^{15}N L-lysine.2HCl and ε-^{15}N L-lysine.2HCl, both with a molecular weight of 220.1, were also used to determine which nitrogen of L-lysine was incorporated into the mousy taint compounds. The purity of all stable isotope labelled L-lysine compounds was greater than 98%, and the uniformly labelled L-lysine had an isotopic enrichment of greater than 98% for ^{13}C, and between 96-99% for ^{15}N. The isotopic enrichment for the single nitrogen labelled compound α-^{15}N L-lysine was between 95-99% and greater than 98% for ε-^{15}N L-lysine. The three compounds were purchased from Cambridge Isotope Laboratories, Andover, MA, USA.

5.2.5.2 Uniformly labelled L-lysine (U-$^{13}C_6$ + $^{15}N_2$ L-lysine)

Following the fermentation protocol described in Section 5.2.2, a *B. anomalus* fermentation supplemented with U-$^{13}C_6$ + $^{15}N_2$ L-lysine.HCl (100 mg/L) was conducted. Two controls were also used, an addition of unlabelled L-lysine.HCl (100 mg/L) and no addition of L-lysine. All treatments were carried out in triplicate.

5.2.5.3 Single nitrogen labelled L-lysine

This experiment followed the same procedure as described in Section 5.2.5.2. All treatments, carried out in triplicate, are described in Table 5.4, with all L-lysine additions at 100 mg/L. Two controls were used in this trial; no L-lysine addition, and unlabelled L-lysine (Table 5.4).

Table 5.4. The supplementation made to CDM to determine which nitrogen of L-lysine was incorporated into mousy taint compounds.

Supplement
no L-lysine
L-lysine.2HCl
α-^{15}N L-lysine.2HCl
ε-^{15}N L-lysine.2HCl
U-$^{13}C_6$ + $^{15}N_2$ L-lysine.HCl

5.2.5.4 Analysis of labelled mousy compounds

Extraction, analysis and quantification of the fermentation samples were carried out as described in Section 5.2.2. Identification of labelled ACTPY and ETPY was determined by comparison of retention times with that of reference samples of unlabelled mousy taint compounds. Predictions of the molecular ion and fragmentation pattern of the labelled compounds were also used to aid the identification of the target compounds. The predicted molecular ion for the addition of U-$^{13}C_6$ + $^{15}N_2$ L-lysine as a precursor was *m/z* 131, and with the single labelled precursor, a molecular ion of *m/z* 126 was proposed.

5.3 Results

5.3.1 Sole nitrogen source

The effect of nitrogen source on the production of the mousy taint compounds, ACPY, ETPY and ACTPY, by three strains of two species of *Dekkera* grown in CDM was investigated. Marked differences in the pattern of production of these taint compounds were observed with respect to the source of nitrogen (Table 5.5). Each strain gave qualitatively similar but quantitatively different results.

Table 5.5. Production of mousy taint compounds ACPY, ETPY and ACTPY by three *Dekkera/Brettanomyces* yeasts grown in CDM with three different sole sources of nitrogen. The concentration of ACTPY is expressed as the sum of both tautomers.

Sole nitrogen source	Strain	Compound (µg/L)		
		ACPY	ETPY	ACTPY
$(NH_4)_2SO_4$	*B. anomalus*	ND	ND	0.8
	B. intermedius	ND	ND	0.4
	B. lambicus	ND	ND	1.3
L-lysine	*B. anomalus*	ND	ND	Trace
	B. intermedius	ND	1.0	232.4
	B. lambicus	ND	ND	98.8
L-ornithine	*B. anomalus*	6.5	ND	13.7
	B. intermedius	1.4	ND	7.9
	B. lambicus	Trace	ND	2.3

ND Not detected.

Low concentrations of ACTPY were detected when $(NH_4)_2SO_4$ was the nitrogen source, with no ACPY or ETPY detected under this condition. Formation of ACPY only occurred when L-ornithine was used as the sole nitrogen source.

When L-lysine was used as the sole nitrogen source, ACTPY production was stimulated in two strains of *D. bruxellensis*; *B. lambicus* and *B. intermedius*. However, this was not the case for the *D. anomala* strain, *B. anomalus*, which only produced a trace amount of ACTPY. Both species of *Dekkera* (including all strains tested) produced ACPY when L-ornithine was the nitrogen source (Table 5.5).

With respect to growth rate and biomass production, ammonia or L-ornithine provided the best sole sources of nitrogen (Figures 5.1–5.3), although this was slightly reduced for *B. intermedius* with L-ornithine as the nitrogen source (Figure 5.2). When L-lysine was the sole source of nitrogen, growth rate and biomass production was dramatically reduced indicating that L-lysine is a relatively poor sole source of nitrogen. Glucose was completely consumed by all strains when ammonia or L-ornithine was the nitrogen source. However, the glucose was not completely catabolised when L-lysine was the nitrogen source for all three strains. Glucose concentrations of approximately 1.5–2% remained in all the sole L-lysine treatments.

5.3.2 Dose response to L-lysine

The production by *B. anomalus* of two mousy taint compounds relative to the initial concentration of L-lysine was determined and the results described in Table 5.6.

Table 5.6. The dose response of ACTPY and ETPY synthesis relative to L-lysine concentration. The concentration of ACTPY is expressed as the sum of both tautomers.

L-Lysine addition (mg/L)	Compound (µg/L)	
	ETPY	ACTPY
10	Trace	37.0
50	Trace	66.1
100	ND	72.3
1000	Trace	82.0

ND Not detected.

These data indicate that the production of ACTPY appears to be stimulated by increasing concentrations of L-lysine. However, the relationship was not directly proportional, as was

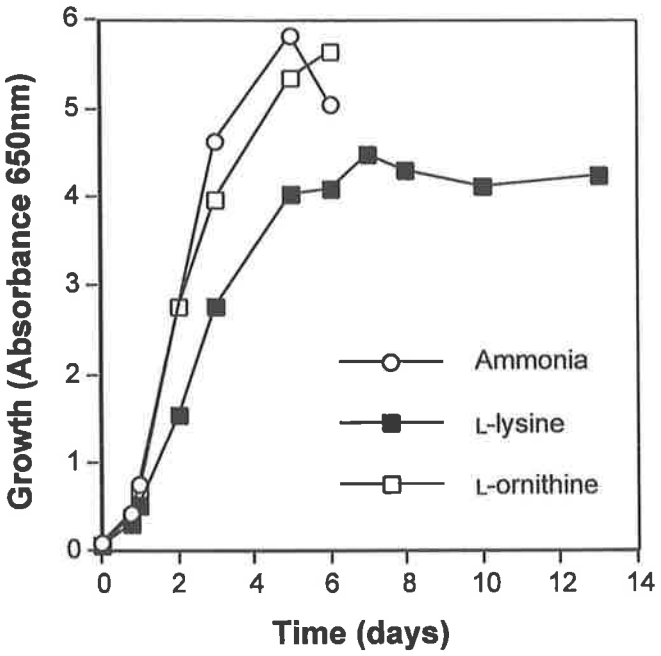


Figure 5.1. The growth of *B. anomalus* (AWRI 1101) in CDM with various sole nitrogen sources.

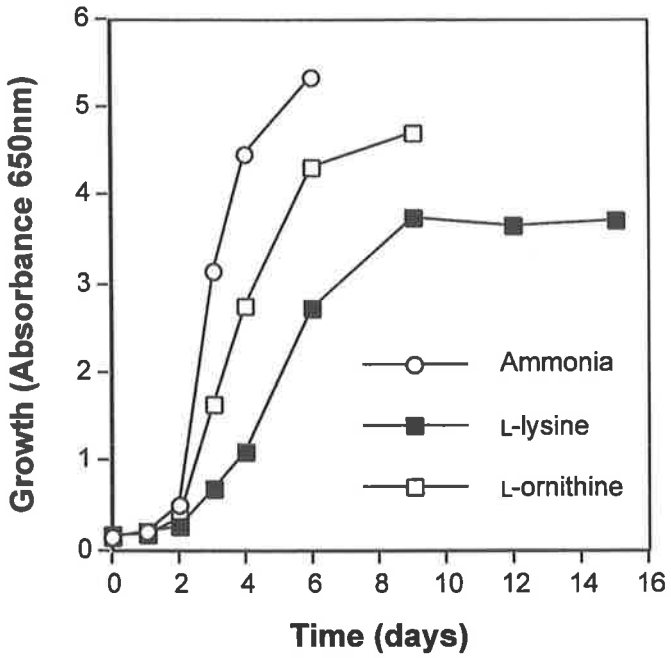


Figure 5.2. The growth of *B. intermedius* (AWRI 1104) in CDM with various sole nitrogen sources.

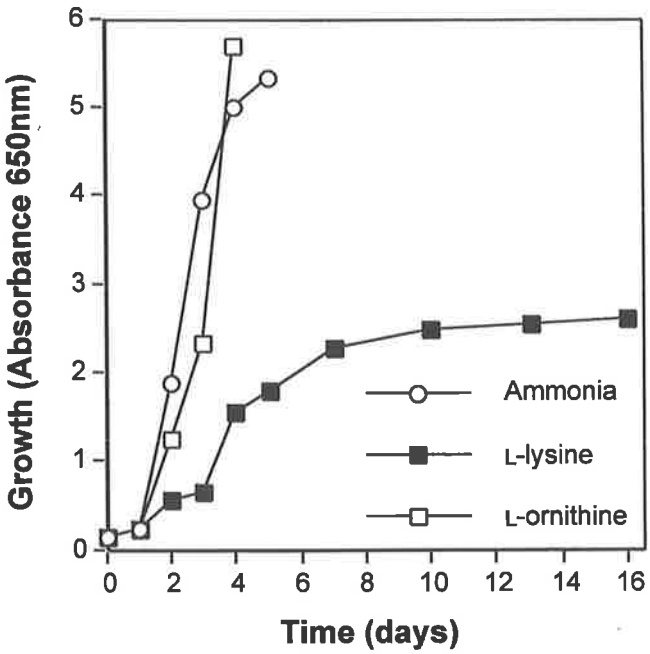


Figure 5.3. The growth of *B. lambicus* (AWRI 1127) in CDM with various sole nitrogen sources.

highlighted when the concentration of L-lysine added was 1000 mg/L producing only approximately 10 µg/L more than when 100 mg/L was the initial L-lysine concentration. No influence on the amount of ETPY produced was found relative to L-lysine concentration. The treatment of 100 mg/L L-ornithine indicated that ACPY could be produced by *B. anomalus*, although only a low concentration was detected (0.5 µg/L). No ETPY and 2.0 µg/L ACTPY were detected under this condition.

5.3.3 L-Lysine derivatives

The L-lysine derivatives, α-acetyl N, ε-acetyl N and ε-methyl N did not act as strong stimulants for the production of ACTPY relative to L-lysine. No differences in growth between L-lysine derivative ferments were observed. Both ε-acetyl and ε-methyl analogues produced only a small amount of ACTPY, while a higher concentration of ACTPY was detected when α-acetyl-L-lysine was supplemented into the growth medium (Table 5.7). Proposed higher analogues of ACTPY, *i.e.* ACTPY with additional acetyl or methyl groups, were not detected by ion trace monitoring.

Table 5.7 The production of mousy taint compounds by *B. anomalus* (AWRI 1101) grown in CDM supplemented with different L-lysine analogues. The concentration of ACTPY is expressed as the sum of both tautomers.

Supplement	Compound (µg/L)		
	ACPY	ETPY	ACTPY
L-lysine	ND	Trace	85.0
α-acetyl-L-lysine	ND	ND	9.8
ε-acetyl-L-lysine	ND	ND	0.7
ε-methyl-L-lysine	ND	ND	1.7

ND Not detected.

5.3.4 D-lysine

Brettanomyces anomalus produced ACTPY and ETPY when growing in CDM supplemented with D-lysine (Table 5.8). HPLC analysis revealed that like L-lysine, D-lysine was removed from the growth medium within the first 24 hr. Growth in the treatments with no lysine, and 100 mg/L D- or L-lysine occurred at a similar rate and all achieved almost equivalent biomass (Figure 5.4).

Table 5.8. The effect of the enantiomer of lysine on the production of mousy taint compounds by *B. anomalus* (AWRI 1101) in CDM. Lysine was used as either a 100 mg/L supplement, a sole source of nitrogen or no lysine added. The concentration of ACTPY is expressed as the sum of both tautomers.

Treatment	Compound (µg/L)		
	ACPY	ETPY	ACTPY
no lysine	ND	ND	Trace
D-lysine	ND	ND	83.8
L-lysine	ND	1.2	82.3
D-lysine (sole N source)	ND	ND	2.4
L-lysine (sole N source)	ND	0.6	38.5

ND Not detected.

Some ACTPY was detected when *B. anomalus* was cultured in CDM with D-lysine as the sole source of nitrogen (Table 5.8), although, as shown in Figure 5.4, growth in this medium was limited.

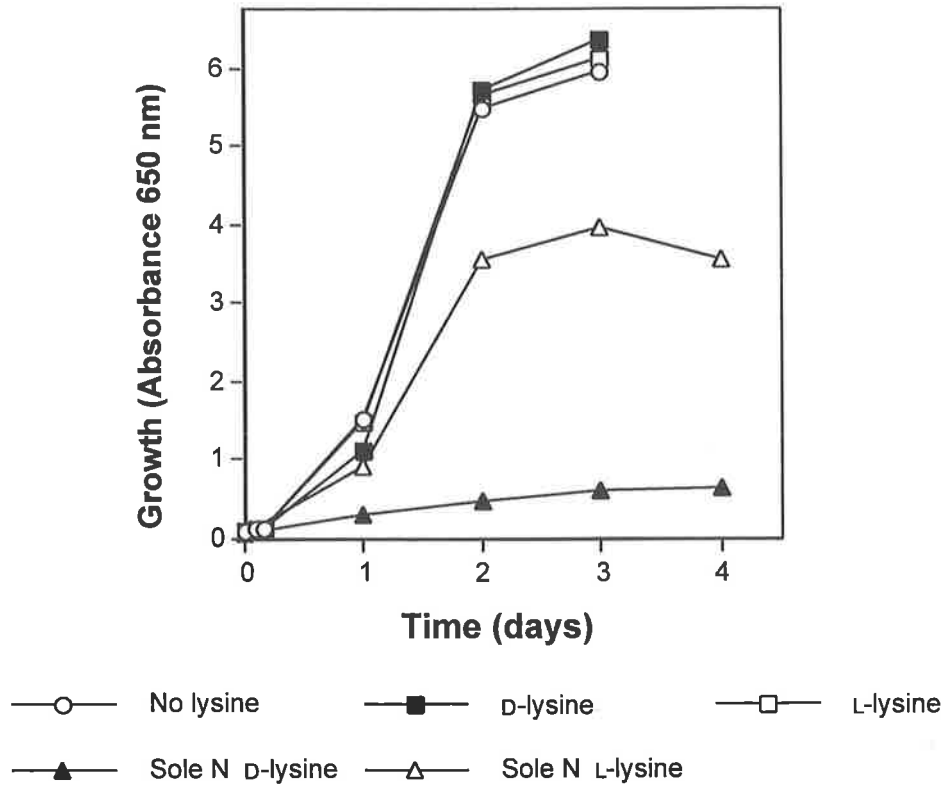


Figure 5.4. The growth of *B. anomalus* (AWRI 1101) in CDM: without lysine, in the presence of lysine enantiomers supplemented into CDM at 100mg/L, or lysine enantiomers as sole nitrogen sources.

5.3.5 Stable isotopes of L-lysine

5.3.5.1 U-$^{13}C_6$ + $^{15}N_2$ L-lysine

Growth of *B. anomalus* in CDM in the presence of U-$^{13}C_6$ + $^{15}N_2$ L-lysine confirmed that L-lysine was the direct precursor to ACTPY. ACTPY incorporating five ^{13}C and one ^{15}N, increasing the molecular mass to 131, was detected in the fermentation extracts. The mass spectra of the two 'heavy' tautomers of ACTPY and unlabelled ACTPY are shown in Figure 5.5 and 5.6. The identity of the labelled ACTPY was confirmed by comparison of retention time data with unlabelled ACTPY, and from the predicted fragmentation pattern for ACTPY. The odour of extracts containing labelled ACTPY had the same intense mousy aroma as unlabelled ACTPY. The concentration of ACTPY detected in all treatments is shown in Table 5.9.

Table 5.9. The concentration of unlabelled and labelled ACTPY detected when *B. anomalus* (AWRI 1101) was grown in CDM supplemented with one of the following: no addition, L-lysine, and U-$^{13}C_6$ + $^{15}N_2$ L-lysine. The concentration of ACTPY is expressed as the sum of both tautomers.

Supplement	Unlabelled ACTPY (µg/L)	Labelled ACTPY (µg/L)
no L-lysine	Trace	ND
L-lysine	85.6	ND
U-$^{13}C_6$ + $^{15}N_2$ L-lysine	3.3	87.3

ND Not detected.

Labelled ACTPY could only be detected when the CDM was supplemented with U-$^{13}C_6$ + $^{15}N_2$ L-lysine, although a low concentration of unlabelled ACTPY was also detected in these extracts (Table 5.9).

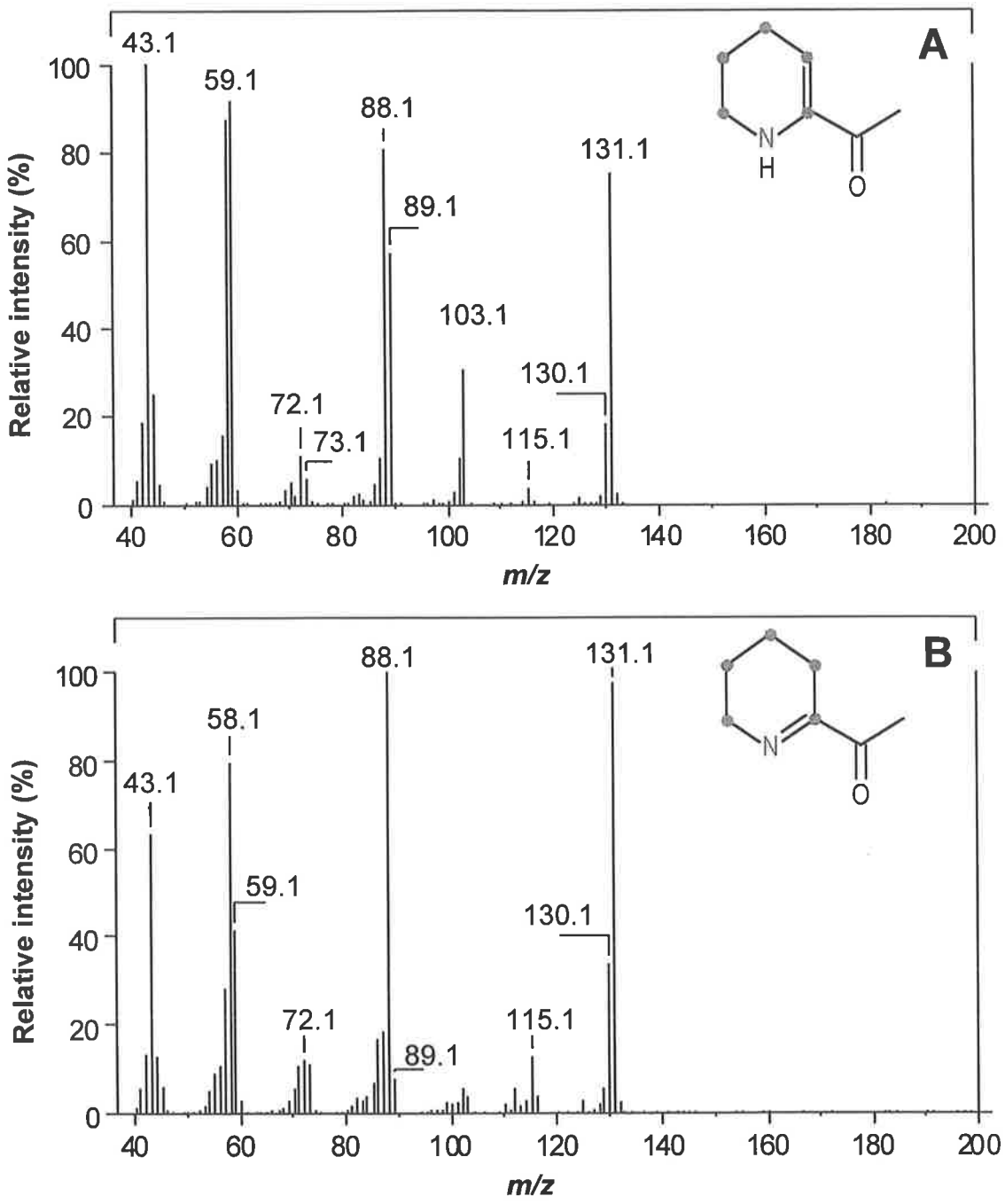


Figure 5.5. ^{15}N and ^{13}C labelled ACTPY; (A) tautomer I, (B) tautomer II. Grey in chemical structure indicates the position of incorporation of the label.

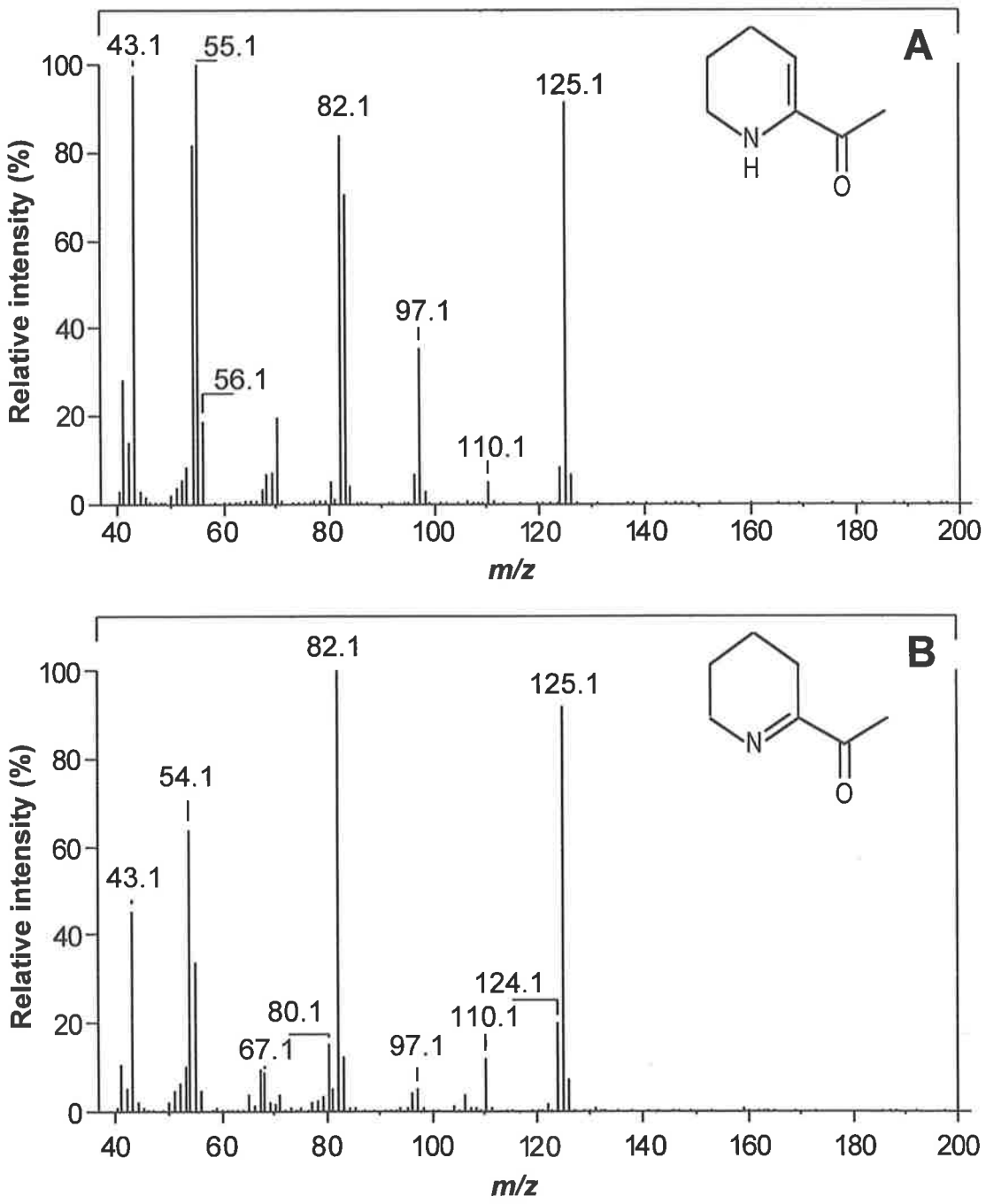


Figure 5.6. Unlabelled ACTPY; (A) tautomer I, (B) tautomer II.

5.3.5.2 ^{15}N labelled L-lysine

Analysis of extracts of *B. anomalus* fermentations conducted with supplementation of single ^{15}N labelled L-lysine, revealed that the ε-nitrogen of L-lysine was incorporated into ACTPY. The mass spectra of this labelled ACTPY, with the molecular weight increased by 1, are shown in Figure 5.7. No incorporation of the α-labelled nitrogen occurred, as shown by Figure 5.8. Quantification of the ^{15}N ACTPY revealed a slightly lower concentration than expected, when compared to the addition of unlabelled L-lysine or U-$^{13}C_6$ + $^{15}N_2$ L-lysine. This reduced concentration of ACTPY was also found in the α-^{15}N L-lysine treatment (Table 5.10).

Table 5.10. The concentration of unlabelled and labelled ACTPY detected when *B. anomalus* (AWRI 1101) was grown in CDM supplemented with one of the following: no addition, L-lysine, α-^{15}N L-lysine, ε-^{15}N L-lysine, and U-$^{13}C_6$ + $^{15}N_2$ L-lysine. The concentration of ACTPY is expressed as the sum of both tautomers.

Supplement	Unlabelled ACTPY (µg/L)	Labelled ACTPY (µg/L)
no L-lysine	2.8	ND
L-lysine	84.9	ND
α-^{15}N L-lysine	74.8	ND
ε-^{15}N L-lysine	15.2	62.4
U-$^{13}C_6$ + $^{15}N_2$ L-lysine	2.0	87.0

ND Not detected.

Labelled or 'heavy' ETPY was detected in the ε-^{15}N L-lysine and U-$^{13}C_6$ + $^{15}N_2$ L-lysine treatments (Figures 5.9 and 5.10). The identity of these labelled products was confirmed by the same method used for ACTPY. The concentration of labelled ETPY in both treatments was less than 2.0 µg/L. The mass spectrum of the labelled ETPY produced in

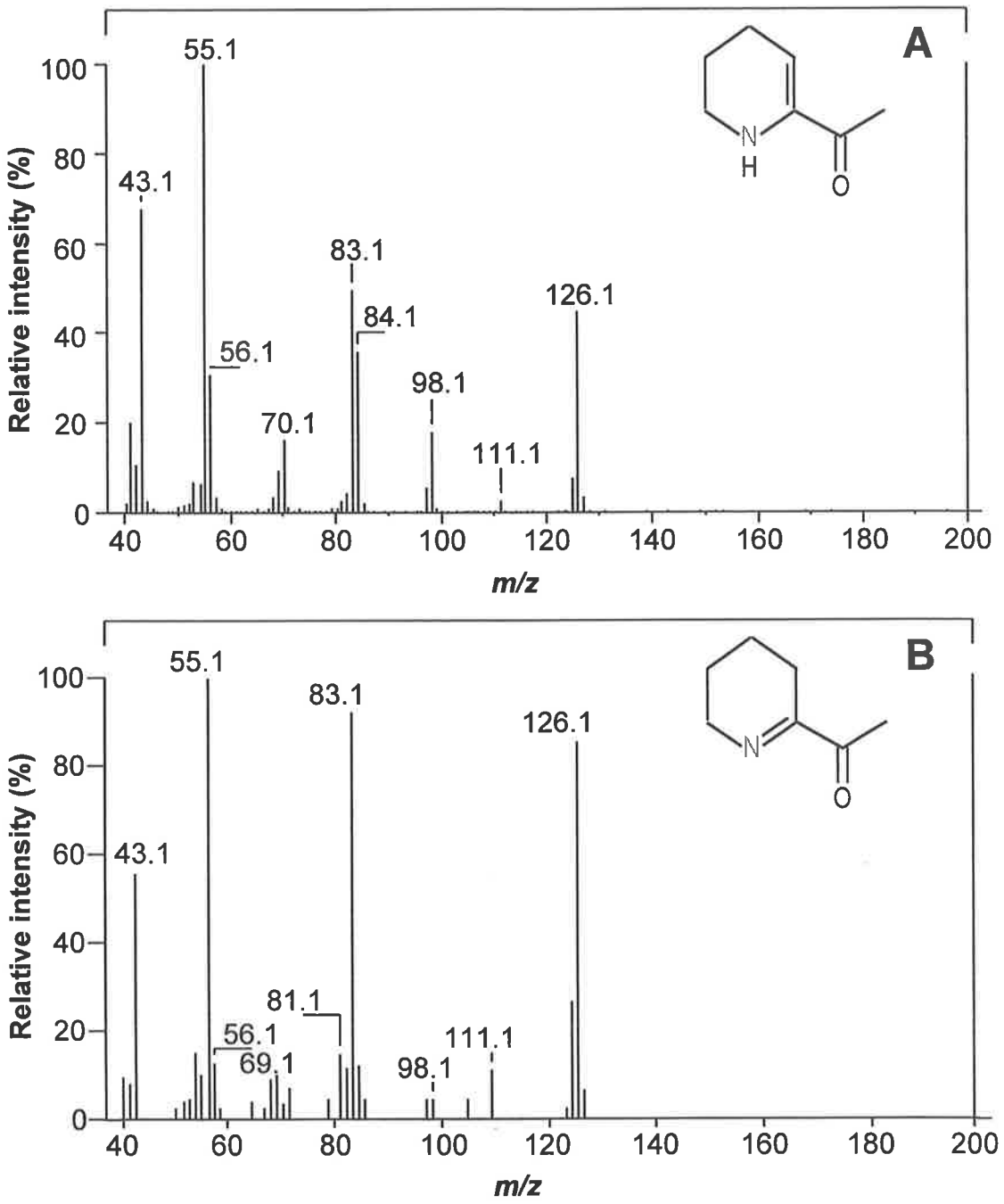


Figure 5.7. ^{15}N labelled ACTPY; (A) tautomer I, (B) tautomer II. Grey in chemical structure indicates the position of incorporation of the label.

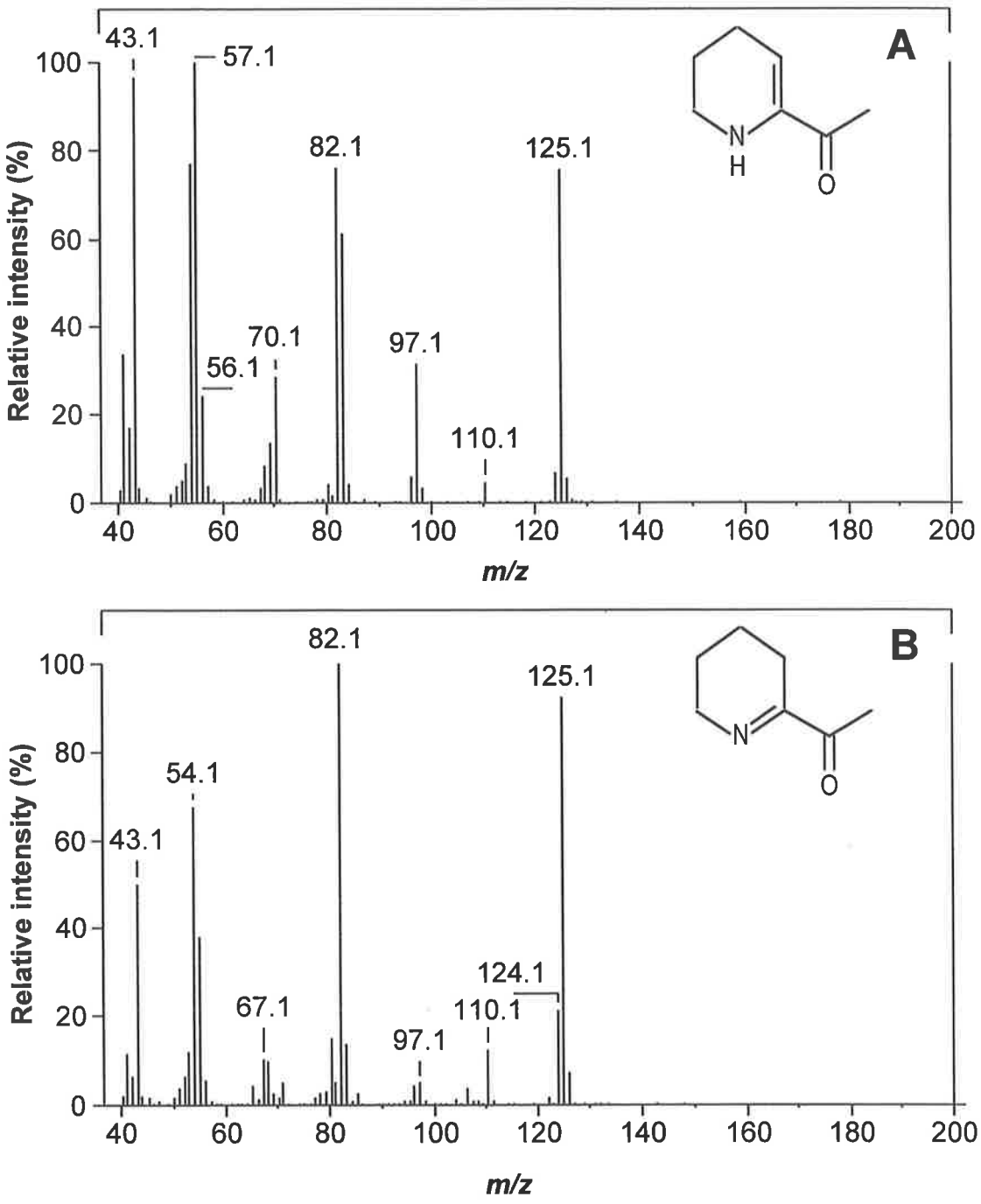


Figure 5.8. Unlabelled ACTPY; (A) tautomer I, (B) tautomer II.

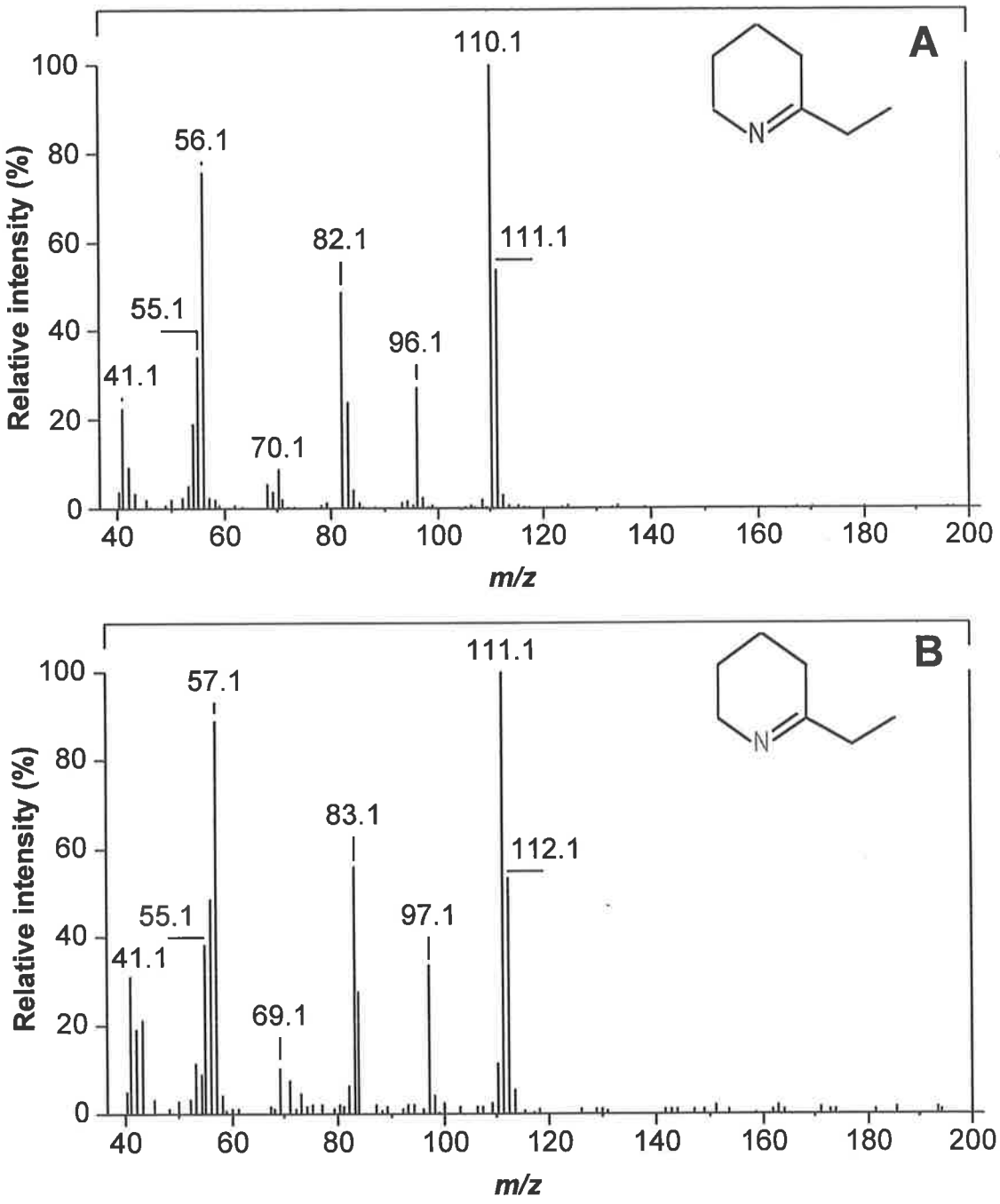


Figure 5.9. ETPY; (A) unlabelled (B) ^{15}N labelled. Grey in chemical structure indicates the position of incorporation of the label.

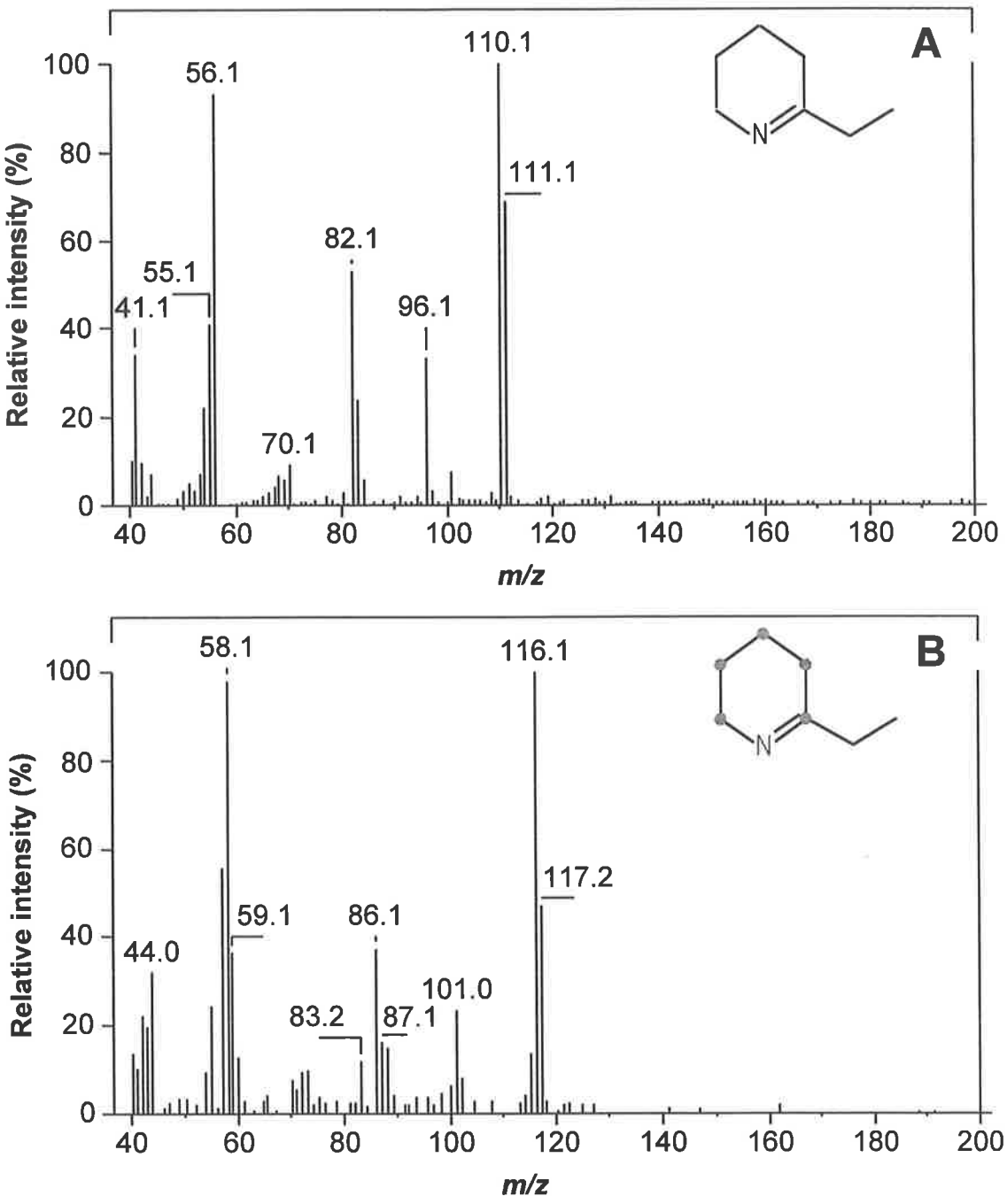


Figure 5.10. ETPY; (A) unlabelled, (B) ^{15}N and ^{13}C labelled. Grey in chemical structure indicates the position of incorporation of the label.

the U-$^{13}C_6$ + $^{15}N_2$ L-lysine treatment clearly shows the addition of 6 mass units, as found with ACTPY produced under these conditions (Figure 5.10). The addition of 1 mass unit was shown in the mass spectrum of ETPY formed when ε-^{15}N L-lysine was the precursor (Figure 5.9). As expected, in any of the treatments using labelled or non-labelled L-lysine, no ACPY was detected.

5.4 Discussion

As shown by the work of Tucknott (1977), and Heresztyn (1986a), and in the current study, L-lysine plays a central role in the production of ACTPY and ETPY, although the mechanism by which this occurs had not been investigated. The aim of this research was to define the amino nitrogen precursors for the mousy taint compounds, and through this, investigate the mechanism of their biosynthesis.

The dose response of ACTPY production at a low concentration range of L-lysine, indicates a metabolic relationship between the catabolism of L-lysine and ACTPY production (Table 5.5). However, when the L-lysine concentration was increased from 100 to 1000 mg/L, there was not a concomitant 10-fold increase in ACTPY. This result supports the conclusion that under the conditions examined the formation of ACTPY was not a major metabolic outcome. The low efficiency of conversion of L-lysine to ACTPY was also shown by the fact that on average 100 mg/L L-lysine produced only 82.0 µg/L ACTPY (Sections 5.3.2–5.3.6).

No dose response between L-lysine and ETPY concentration was found, despite the fact that ETPY was detected only when L-lysine was present in the growth medium (Sections 3.3.3 and 4.3.1). This may be explained by several factors. Firstly, there is a possibility that the extraction of ETPY using the scaled down extractor was not optimal, although this compound was detectable using the scaled down technique. Secondly, the conditions used may not have been conducive to ETPY production. The formation of ETPY described in

Sections 3.3.3 and 4.3.1 occurred in fermentations that were conducted over a longer time frame. The 'Mousy taint production and stage of growth' experiment (Section 4.3.1) indicated that a relatively high ETPY concentration was detected after only 4 days. The dose response fermentations were terminated on day 3. Time may have also affected the concentration of ETPY detected in 'Influence of air on mousy taint production' fermentations (Section 4.3.2.1) which were conducted over a long time period (up to 88 days). The data suggest that the biosynthesis of ETPY may be related to a time dependent biotransformation of ACTPY.

The results of the trial investigating taint production when L-lysine was used as the sole nitrogen source indicated for at least the two strains of D. *bruxellensis*, that by forcing nitrogen metabolism via this amino acid, ACTPY production increased. This is probably the result of a dose response effect. The difference observed in the concentration of ACTPY produced by *B. anomalus* (AWRI 1101) with L-lysine as the sole nitrogen source (Section 5.3.1 and 5.3.4) is most likely explained by the difference in pre-culturing and fermentation conditions, such as pH (Section 5.3.1 pH 3.8 and Section 5.3.4 pH 4.5). A direct comparison between these experiments is problematic due to these variations in culture conditions.

Under the conditions examined (Section 5.2.1) L-lysine was a poor source of nitrogen relative to ammonia and L-ornithine. This is the first report that *Dekkera/Brettanomyces* can grow on L-ornithine as the sole nitrogen source. Limited growth of *B. anomalus* on D-lysine as the sole nitrogen source has not been previously described.

The experiment examining the role of L-ornithine in mousy taint compound biosynthesis indicated that a supplementation of 100 mg/L of L-ornithine to a *B. anomalus* CDM fermentation could stimulate the production of ACPY. This was confirmed by the detection of ACPY in fermentations by strains of *D. anomala* and *D. bruxellensis* that were conducted with L-ornithine as the sole nitrogen source. Both species could produce

ACPY, with variation in the concentration of ACPY detected between and within the species. These types of variations have also been described for the production of ACTPY and ETPY (Section 3.3.3). This is the first report of the formation of ACPY by yeast and confirms our proposal that L-ornithine is the probable precursor to this compound in yeast. Previously the only microorganisms found to produce ACPY were bacteria (Romanczyk Jr *et al.*, 1995; Grbin *et al.*, 1996). Romanczyk *et al.* (1995) showed that the compounds, L-ornithine, L-proline and L-glutamic acid, could stimulate ACPY biosynthesis by *Bac. cereus*. Incorporation of ^{15}N into ACPY was also described when ^{15}N L-proline or ^{15}N L-glutamate were added to plate count agar inoculated with *Bac. cereus*. Labelled L-ornithine was not tested. Under the conditions examined by Romanczyk *et al.* (1995) ACTPY was not detected, however, in the *Dekkera/Brettanomyces* fermentations conducted with L-ornithine as a sole nitrogen source, ACTPY was produced. This may indicate stimulation in background level synthesis of ACTPY by induction of biosynthetic pathway(s) by L-ornithine, a homologue of L-lysine.

Confirmation that L-lysine was directly converted into ACTPY by *Dekkera/Brettanomyces* came from the stable isotope feeding study, where five of the six carbon units and one of the two nitrogens of L-lysine were shown to be incorporated into the ring structure of ACTPY. Incorporation of exogenously supplied labelled U-$^{13}C_6$ + $^{15}N_2$ L-lysine into ACTPY proves that this amino acid was the direct precursor, and did not act by stimulating a biosynthetic pathway that utilised other substrates in taint compound formation. The data also showed that the L-lysine aliphatic side chain was symmetrically incorporated into ACTPY, with five of the six labelled carbons detected in labelled ACTPY. A small amount of unlabelled ACTPY was detected in the labelled L-lysine treatment. This was expected, as there would be a background level of ACTPY produced from the native or endogenous L-lysine present in the amino acid pool of the cell, as well as possible contaminating unlabelled L-lysine present in the labelled precursor material.

That only five of the ^{13}C of the uniformly labelled L-lysine precursor were detected in labelled ACTPY demonstrated that the acetyl group of ACTPY was not provided by L-lysine. Furthermore, the detection of the fragment of mass *m/z* 43, *i.e.* the acetyl group, occurred in all treatments (Figures 5.5–5.8). If ^{13}C was incorporated into the acetyl group, an increase in its mass would have been detected. This supports the earlier work that demonstrated the importance of ethanol in the formation of ACTPY by *Dekkera/Brettanomyces* and LAB (Heresztyn, 1986a). In this study, formation of ACTPY by bacteria did not occur without ethanol present in the growth medium, and by substituting ethanol with *n*-propanol, propionyl analogues of ACTPY were produced, by both *Dekkera/Brettanomyces* and bacteria. This observation provided evidence for an acetylation step in the biosynthesis of ACTPY.

The U-$^{13}C_6$ + $^{15}N_2$ L-lysine feeding experiment could not be used to show which amino nitrogen of L-lysine was incorporated into ACTPY, as both nitrogens were ^{15}N labelled. Evidence for which amino nitrogen was retained to produce ACTPY was suggested by the L-lysine derivatives study. ε-Nitrogen protected L-lysine derivatives did not act as precursors to ACTPY. The amount of ACTPY detected was consistent with results obtained when no L-lysine was added (Table 5.7). However, when the growth medium was supplemented with α-acetyl L-lysine, the concentration of ACTPY detected increased. This suggested that *B. anomalus* catabolism was proceeding via removal of the α-amino group of L-lysine, producing ACTPY and therefore incorporating the ε-amino nitrogen.

Although ACTPY was detected when α-acetyl L-lysine was added to the growth medium, it was at a concentration 8.7-fold less than when L-lysine was the supplement. This may reflect a low rate of uptake of the α-acetyl derivative from the medium. Attempts to confirm transport of the L-lysine derivatives from the medium using the same HPLC methodology as used for L- and D-lysine analysis was unsuccessful.

Supplementation studies using single ^{15}N labelled L-lysine enabled a conclusive demonstration that the ε-amino group was incorporated into ACTPY. This was determined by demonstrating an increase in the molecular weight of ACTPY by 1 mass unit when ε-^{15}N L-lysine was added to a *B. anomalus* CDM fermentation. When α-^{15}N L-lysine was the supplement under the same conditions, the incorporation of ^{15}N was not observed, which indicated that the α-amino group was removed. Production of ACTPY from the single ^{15}N labelled L-lysine precursors was lower than expected (Table 5.10). This could indicate that the purity of the precursor material was not high as reported.

That the ε-nitrogen was retained shows that a deamination of the α-amino group of L-lysine occurs in the biosynthesis of ACTPY/ETPY. While investigating L-lysine catabolism in yeast Hammer *et al.* (1991) found the principle pathway of L-lysine catabolism in *B. anomalus* was via ε-aminotransferase, however, the stable isotope study above indicated that this enzyme was not involved in ACTPY synthesis.

The removal of the α-nitrogen of L-lysine has been reported in other biological systems. A deaminating α-oxidase has been described in the fungi *Trichoderma viride* (Kusakabe *et al.*, 1980) and in chicken liver microsomes (Struck and Sizer, 1960). There have been several reports of the incorporation of the ε-^{15}N amino group of lysine in the production of the lysine catabolic intermediate L-pipecolic acid in *Pseudomonas* (Miller and Rodwell, 1971), *Neurospora crassa* (Fangmeier and Leistner, 1980), plants (Gupta and Spenser, 1969) and the rat (*in vivo*) (Rothstein and Miller, 1954a; Rothstein and Miller, 1954b; Grove *et al.*, 1969). The incorporation of ε-^{15}N amino group into pipecolate by the fungi *Rhizoctonia leguminicola* was reported by Guenegerich and Broquist (1976), however, this finding was recently disputed (Wickwire *et al.*, 1990). This investigation showed that it was the α-nitrogen of L-lysine that was retained in this fungus. There have not been any reports of α-amino deamination of lysine in yeast, except after ε-N acetylation (Rothstein, 1965; Gaillardin *et al.*, 1976; Guenegerich and Broquist, 1976; Large, 1986; Schmidt *et al.*, 1988b; Hammer *et al.*, 1991b; Bode *et al.*, 1993) (see Figure 1.3). However, no

activity of the enzyme responsible for ε-N acetylation of L-lysine in *B. anomalus* was detected (Hammer *et al.*, 1991b). If ACTPY/ETPY biosynthesis occurred via N-acetylation, it would be expected that the supplementation with the N-acetyl derivatives, as described in Section 5.3.3, would result in the production of typical concentrations of ACTPY/ETPY (*i.e.* ACTPY/ETPY concentrations produced from 100 mg/L L-lysine). As only low concentrations of ACTPY and no ETPY was produced from the N-acetyl L-lysine derivatives, this suggests that an N-acetylation pathway for mousy taint biosynthesis does not exist.

Deamination of L-lysine via an α-aminotransferase has not been reported in nature, although arginine racemase of *Pseudomonas graveolens* was found to transaminate L-lysine (Yorifuji *et al.*, 1971a; b) and a D-lysine α-aminotransferase has been described from *Bacillus. sphaericus* (Yonaha *et al.*, 1975).

Brettanomyces anomalus was able to produce ACTPY and ETPY when grown in chemically defined medium supplemented with D-lysine, but only a limited amount of ACTPY was detected when this amino acid was the sole source of nitrogen. Lysine catabolism via a DL racemase has been described in bacteria (Ichihara *et al.*, 1960; Chang and Adams, 1974; Fothergill and Guest, 1977) and fungi (Guenegerich and Broquist, 1973), although no lysine racemase activity has been previously reported for yeast. An alternative to a DL racemase in mousy taint biosynthesis would be the involvement of a non-stereospecific enzyme(s) in the biotransformation of lysine. An enzyme that catalyses the transamination of D- and L-ornithine (as well as L-lysine) has been described in *Pseudomonas graveolens* (Yorifuji *et al.*, 1971b). The activity of this enzyme on ornithine as well as lysine is of interest when considered with the data relating to the biosynthesis of both ACPY and ACTPY from L-ornithine.

The results of the investigations of the catabolism of the amino acids, L-lysine, D-lysine and L-ornithine has provided confirmation that a biological transformation is required for

mousy taint production, rather than a non-enzymatic chemical synthesis. Although previous studies have provided strong evidence for the biological nature of mousy taint spoilage this study has provided conclusive evidence of an enzyme mediated synthetic pathway in *Dekkera/Brettanomyces* yeast. A proposed biochemical pathway is discussed in Chapter 6.

CHAPTER 6 General Discussion and Proposed Pathway of Mousy Taint Biosynthesis

6.1 General discussion

This study on the physiology and metabolism of *Dekkera/Brettanomyces* has provided a more thorough understanding of mousy taint production. The development of a sensitive and reliable extraction, separation, analysis and quantification procedure has enabled a detailed investigation of the individual mousy taint compounds and the factors that influence their biosynthesis. The application of this analytical technique has allowed the identification of ACPY for the first time in wine, and the rediscovery of ETPY, which was previously considered irrelevant to mousy taint. The ability to obtain quantitative data on the compounds associated with mousy taint has permitted reliable comparison of treatments both within and between experiments, making it possible to draw satisfactory conclusions from the work.

That all species and strains therein of *Dekkera/Brettanomyces* surveyed could produce ACTPY/ETPY, indicates that mousy taint production is a characteristic of these genera. This has implications for industrial microbiology, as any *Dekkera/Brettanomyces* present has the potential to produce mousy taint compounds, given the appropriate conditions and availability of precursors. Therefore to prevent the biosynthesis of these compounds, elimination or strict control of these yeast must be maintained, particularly in the wine environment where potentially all the precursors are available. When the growth of these yeast is being encouraged to provide additional complexity to a wine style, careful strain selection and preliminary trialing would be required before full scale production commences. This would be necessary to minimise the risk of mousy taint development.

Air or oxygen was shown to be a key factor in the formation of mousy taint compounds. This is likely to be due to the stimulation of growth of the yeast by oxygen, via the Custers effect. This unique characteristic of *Dekkera/Brettanomyces* may simply be coincidental

with the unique ability (in yeast) to synthesise mousy taint compounds. However, a relationship between these metabolic processes of taint production and Custers effect could be speculated upon. The association between ACTPY accumulation and the aerobic growth cycle of *Dekkera* provides some evidence for a link between taint production and the Custers effect. Further work on the influence of oxygen is required to elucidate the relationship. Oxidative metabolism may play a role in providing a metabolic process that facilitates the biosynthesis of taint compounds.

This study has clearly established that a biotransformation of L-lysine is required for production of mousy taint, which supports earlier observations (Tucknott, 1977; Heresztyn, 1986a). It has also been shown that another basic amino acid L-ornithine is important to the biosynthesis of sensorily potent ACPY. Both these amino acids occur naturally in grape juice and wine. The concentrations of these two amino acids are quite low [reported to range from 0–64 mg/L in grape juice and wine (Ough and Amerine, 1988; Henschke and Jiranek, 1993; Lehtonen, 1996)], although this low level may still be significant, especially for L-lysine. The dose response of ACTPY production relative to L-lysine concentration revealed that as little as 10 mg/L of L-lysine was enough to produce a substantial concentration of ACTPY. In addition, sensorily detectable (with respect to odour thresholds reported in water) concentrations of ACTPY were produced without any supplementation of L-lysine (Section 3.3.3), that is, from endogenous L-lysine. However, that a dose response was identified, as well as production of a high concentration of ACTPY by a *D. bruxellensis* strain cultured on L-lysine as the sole nitrogen source, suggests that the presence of extracellular L-lysine resulted in the enlargement of the intracellular L-lysine pool with a concomitant increase in the flux of ACTPY biosynthesis. The ratio of conversion of L-lysine to ACTPY/ETPY indicated that this biotransformation was not efficient, suggesting that biosynthesis of these taint compounds was not the principal pathway of L-lysine catabolism at least under these conditions. It also indicates that this metabolic process was unfavourable and secondary.

With respect to L-ornithine and ACPY production, that only high concentrations of L-ornithine relative to those reported in wine stimulated ACPY biosynthesis may indicate that *Dekkera/Brettanomyces* yeast are not responsible for the production of this compound in wine. Detection of ACPY in a mousy beverage may therefore be an indicator of bacterial spoilage.

6.2 Proposed pathway(s) for the biosynthesis of mousy taint compounds

The stable isotope feeding experiments unequivocally identified L-lysine as the key precursor for ACTPY/ETPY biosynthesis. Using U-$^{13}C_6$ + $^{15}N_2$ L-lysine as a supplement showed that the C_5N tetrahydropyridine ring structure of ACTPY/ETPY was provided by L-lysine. Further, the εN of L-lysine was characterised as the nitrogen incorporated into these taint compounds, after supplementing the *B. anomalus* fermentation medium with ε-^{15}N L-lysine. The findings of these two critical experiments provide the necessary data to propose a biosynthetic pathway(s) for the formation of mousy taint compounds. The fact that D-lysine can also act as a precursor for ACTPY/ETPY biosynthesis must also be considered.

Provision of the C_5N backbone structure of ACTPY/ETPY by L-lysine suggests that biosynthesis would proceed via the synthesis of the piperideine or tetrahydropyridine ring. This nucleus could be compounds such as Δ^1-piperideine or Δ^1-piperideine-2-carboxylic acid (Figure 6.1). These highly reactive compounds both exist in equilibrium with their hydrated acyclic analogues, 5-aminopentanal and ε-amino-α-ketocaproic acid, respectively (Meister, 1954; Chang *et al.*, 1982; Bock and Dammel, 1987; Mann, 1987; Chang and Charles, 1995). Δ^1-Piperideine can be formed by the deamination of cadaverine which is produced by an initial decarboxylation of L-lysine [Figure 6.2; Reaction (5)], while Δ^1-piperideine-2-carboxylic acid can be produced directly from the α-N deamination of lysine [Figure 6.2; Reaction (1)]. Therefore, in nature, two distinct initial steps of lysine catabolism have been utilised for the biosynthesis of the tetrahydropyridine ring. In the

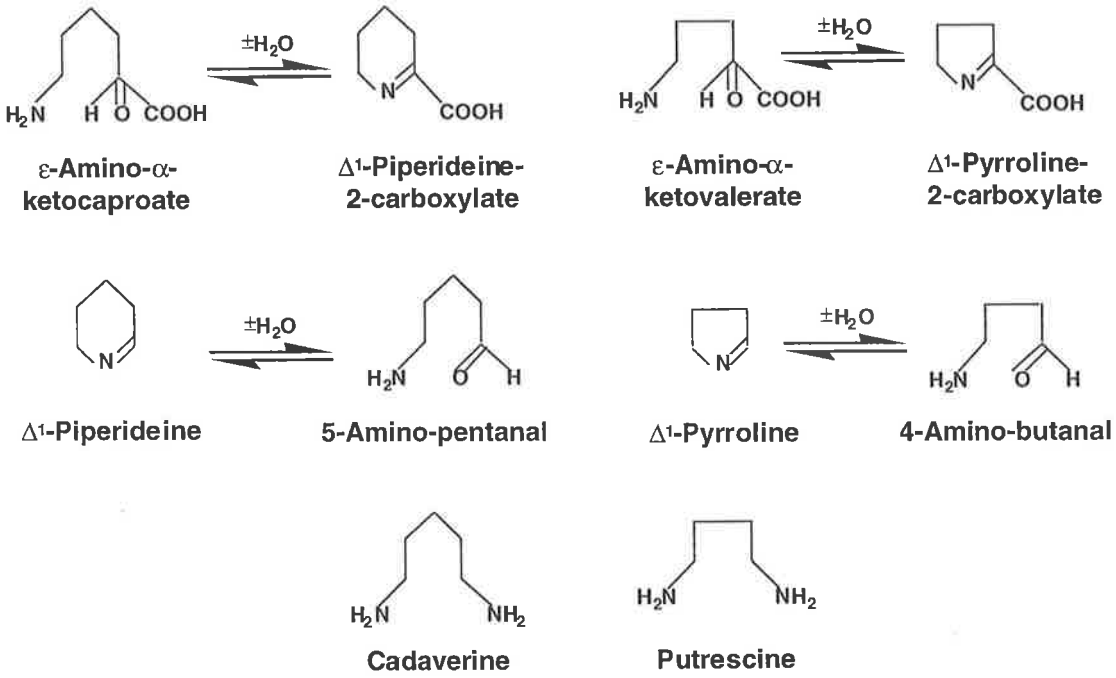


Figure 6.1. Possible biosynthetic intermediates of ACTPY/ETPY and ACPY. C_5N compounds are derived from L-lysine and C_4N from L-ornithine.

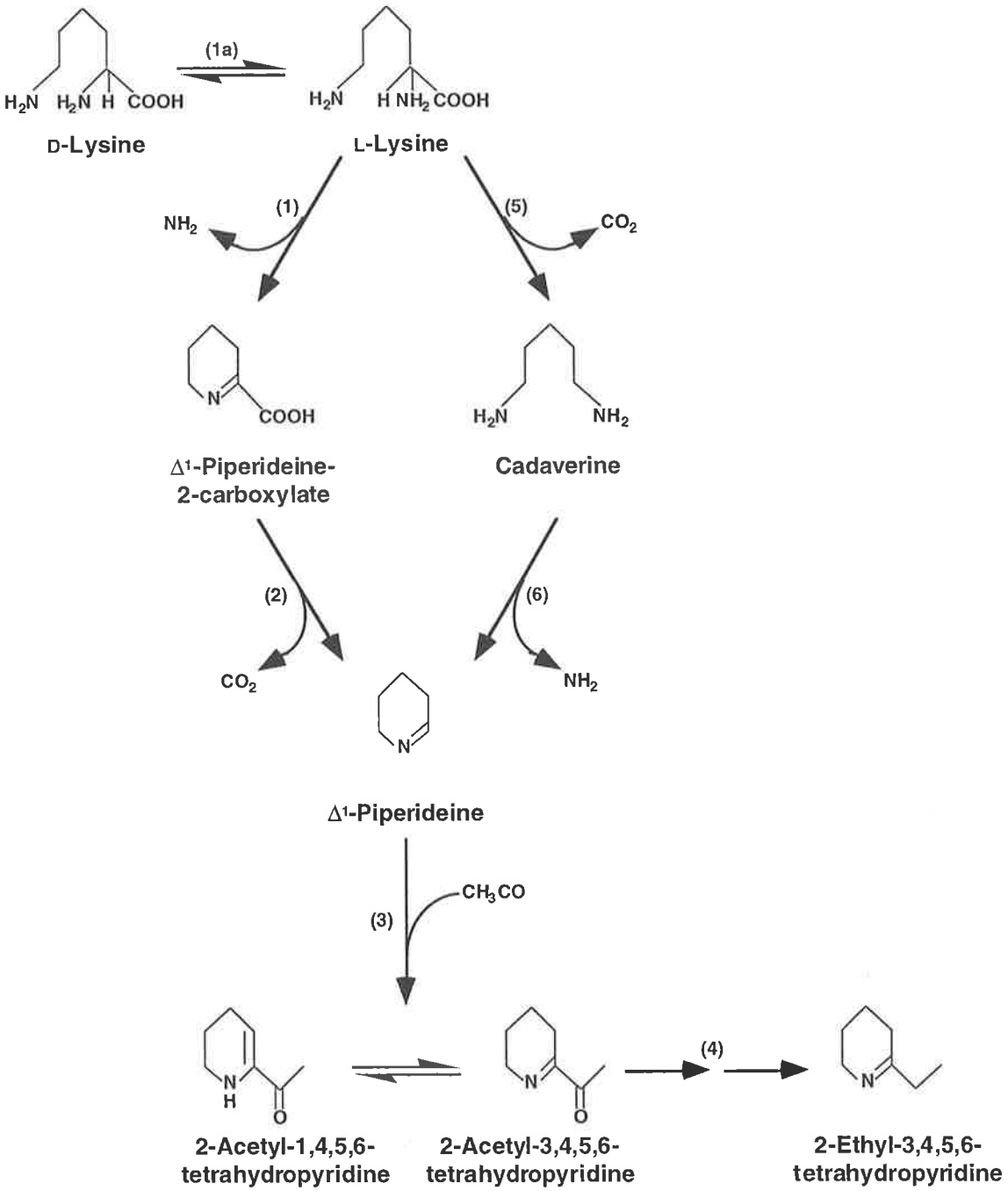


Figure 6.2. Proposed pathway(s) of biosynthesis of 2-acetyl-1,4,5,6-tetrahydropyridine/2-acetyl-3,4,5,6-tetrahydropyridine (ACTPY) and 2-ethyl-3,4,5,6-tetrahydropyridine (ETPY). Pathway A: Reactions (1)→(2)→(3)→(4). Pathway B: (5)→(6)→(3)→(4). L-ornithine can be substituted for L-lysine to produce the C_4N intermediate required for the biosynthesis of 2-acetyl-1-pyrroline (ACPY). Proposed enzyme mediated reactions; (1) L-lysine α-aminotransferase or α-oxidase (deaminating), (1a) lysine racemase, (2) Δ^1-piperideine-2-carboxylic acid decarboxylase, (3) acylase via acetyl-CoA or other condensation reaction, (4) dehydrogenase, (5) L-lysine decarboxylase or L-ornithine decarboxylase, (6) diamine aminotransferase or putrescine oxidase (deaminating).

same way, ornithine can be transformed to produce the proposed ACPY biosynthetic intermediate, the pyrroline ring, as either Δ^1-pyrroline or Δ^1-pyrroline-2-carboxylic acid (Figure 6.1).

6.2.1 Pathway A

The enzymatic deamination of lysine can occur via either an aminotransferase or an oxidase. As described above, the α-amino group of lysine is removed in the formation of ACTPY/ETPY. The loss of this amino group of lysine has not been reported in yeast previously, although acetylation of the ε-amino group of lysine prior to the deamination of the α-amino group of lysine has been described. This enzymatic transformation was not detected in *Dekkera/Brettanomyces* (Hammer *et al.*, 1991b). An L-lysine ε-aminotransferase enzyme has been characterised in yeast (see Figure 1.4), and activity of this enzyme detected in *B. anomalus* (Hammer *et al.*, 1991b). However, the product of this reaction, α-aminoadipic-δ-semialdehyde which is spontaneously dehydrated intramolecularly into Δ^1-piperideine-6-carboxylic acid, incorporates the α-N, and not the ε-N that is incorporated into ACTPY/ETPY.

A pathway has been characterised in *Pseudomonas* for the biosynthesis of Δ^1-piperideine-2-carboxylic acid via the deamination of D-lysine (Chang and Adams, 1971; Miller and Rodwell, 1971; Chang and Adams, 1974; Payton and Chang, 1982; Cao *et al.*, 1993). D-Lysine catabolism via Δ^1-piperideine-2-carboxylic acid has been described in *Bac. sphaericus* and *Neurospora crassa* (Yonaha *et al.*, 1975; Fangmeier and Leistner, 1980). Catabolism of L-lysine with Δ^1-piperideine-2-carboxylic acid as an intermediate has also been proposed in a variety of other organisms including rats [both *in vivo* (Rothstein and Miller, 1954a; Rothstein and Miller, 1954b; Grove *et al.*, 1969) and *in vitro* (Meister *et al.*, 1957; Chang, 1976; Chang, 1978)], chicken liver microsomes (Struck and Sizer, 1960), *T. viride* (Kusakabe *et al.*, 1980), and plants (Meister *et al.*, 1957; Gupta and Spenser, 1969b).

In general, separate pathways exist for the catabolism of L- and D-lysine (Chang and Adams, 1971; Miller and Rodwell, 1971; Chang and Adams, 1974; Fothergill and Guest, 1977). However, there have been reports of degradation of both enantiomers of basic amino acids by the same enzyme. Grove *et al.* (1969) showed that L- and D-lysine were catabolised to L-pipecolic acid via Δ^1-piperideine-2-carboxylic acid in the rat, although D-lysine served as a better precursor. An investigation of the enzyme arginine racemase of *P. graveolens* found L-lysine, and L- and D-ornithine α-aminotransferase activity (D-lysine was not examined) (Yorifuji *et al.*, 1971a; b). The product of the transamination of L- and D-ornithine was Δ^1-pyrroline-2-carboxylic acid, which is a proposed intermediate of ACPY biosynthesis. It could be predicted from these data, due to the structural similarity between ornithine and lysine, that Δ^1-piperideine-2-carboxylic acid would be produced from either L- or D-lysine. This type of enzyme could therefore allow the biosynthesis of ACTPY/ETPY from both L- and D-lysine.

The alternative reaction resulting in Δ^1-piperideine-2-carboxylic acid is by oxidative deamination with an α-amino acid oxidase. The enzyme L-lysine α-oxidase has been described in *Trichoderma viride* (Kusakabe *et al.*, 1980). This α-oxidase was highly specific for L-lysine and had no activity on D-lysine, but some degradation of L-ornithine. Δ^1-Piperideine-2-carboxylic acid was not produced from L-lysine in the absence of catalase. Catalase was required to prevent further oxidation of Δ^1-piperideine-2-carboxylic acid by the H_2O_2 produced by the oxidase reaction.

Amino acid oxidases are common throughout nature although many have little or no activity on lysine (Meister, 1965; Kusakabe *et al.*, 1980; Curti *et al.*, 1992). D- and L-amino acid oxidase activity has been reported in yeast, with some activity with lysine and ornithine, although no enzymes have been characterised (Sentheshanmuganathan and Nickerson, 1962; Simonetta *et al.*, 1982; Simonetta *et al.*, 1987). Catalase has been characterised in a number of yeasts (Large, 1992).

To produce ACTPY/ETPY from Δ^1-piperideine-2-carboxylic acid, further metabolic steps are required. The stable isotope study determined that one carbon of L-lysine was removed, and that only carbon from the L-lysine aliphatic side chain was incorporated into the piperideine ring structure. A decarboxylation to remove the carboxyl group of Δ^1-piperideine-2-carboxylic acid would result in the formation of Δ^1-piperideine [Figure 6.2; Reaction (2)]. This compound is proposed as a crucial intermediate in both pathways for mousy taint compound biosynthesis from L-lysine as described in Figure 6.2. Decarboxylation of Δ^1-piperideine-2-carboxylic acid has not been characterised in any biological system, although it has been proposed in the biosynthesis of Δ^1-piperideine (Gupta and Spenser, 1969a; Leete, 1969; Keogh and O'Donovan, 1970).

α-Amino deamination of ornithine would result in the formation of the ε-amino-α-ketovaleric acid that is intramolecularly dehydrated into Δ^1-pyrroline-2-carboxylic acid (Figure 6.1) (Meister, 1954; Carbello *et al.*, 1964). Decarboxylation of this compound would produce Δ^1-pyrroline. Δ^1-Pyrroline, like Δ^1-piperideine, would be an important intermediate key biosynthetic intermediate in any proposed pathway, in this case for ACPY.

The final step in the biosynthesis of ACTPY is proposed to be the addition of an acetyl group to Δ^1-piperideine [Reaction (3)]. As described previously (Section 1.4.2), earlier investigations indicated that ethanol was important to mousy taint production by *Dekkera/Brettanomyces* and LAB. Propionyl analogues of ACTPY were produced by *B. intermedius* when *n*-propanol was added to the culture medium (Heresztyn, 1986a). This was a strong indication that the acyl group was attached to the tetrahydropyridine ring. Further evidence for this was obtained from the stable isotope trial, were it was determined that only the C_5N ring of ACTPY was derived from L-lysine, and therefore the acetyl group was provided by another precursor.

Further reactions of Δ^1-piperideine or Δ^1-pyrroline are predicted to proceed by simple chemical processes (enzyme mediated), through Schiff-base formation then condensation with acyl-CoA or by other condensation type reactions (Mann, 1987). The universal acetyl group carrier, acetyl CoA is a highly reactive cofactor that is required in many enzyme catalysed acetylations, and could provide the acetyl group for the mousy taint compounds.

Acyloin condensation is a biotransformation allowing the formation of a carbon-carbon bond. An enzyme commonly associated with acyloin condensations is pyruvate decarboxylase from *S. cerevisiae* (Kren *et al.*, 1993). The decarboxylation of pyruvate produces an enzyme bound reactive C_2 donor that can react further to produce a new carbon-carbon bond (Kren *et al.*, 1993; Häring *et al.*, 1997). This active C_2 could also act as a precursor in mousy taint biosynthesis.

Several experiments outlined in this thesis suggest that ETPY production is a result of a biotransformation of ACTPY [Reaction (4)]. The delayed detection of ETPY relative to ACTPY biosynthesis in the 'Mousy taint production and stage of growth' experiment (Section 4.3.1) provides strong support for this. High concentrations of ETPY were detected in long term growth experiments investigating the effect of oxygen on taint production (Section 4.3.2.1), with limited ETPY production in the short term fermentation trials investigating lysine catabolism (Sections 5.3.2–5.3.5). This suggests that time was critical in ETPY biosynthesis, allowing for a slow biotransformation of ACTPY.

ETPY was not produced as a result of chemical degradation of ACTPY stored for 29 months in dilute solution (Section 2.3.1.1). This shows that ETPY was not derived by a simple chemical transformation from ACTPY. The biosynthesis of ETPY could not occur directly from the condensation of a tetrahydropyridine ring with a aliphatic substituent, as this would be biochemically unfavourable, and thus a more reactive intermediate is required. This also indicates that ACTPY is a likely precursor to ETPY. However, the removal of the relatively stable carbonyl group from ACTPY is probably not a simple

transformation, as the reaction appears to be slow. The reduction of the carbonyl group via a dehydrogenase would produce an allylic alcohol intermediate that could be then reduced further to form ETPY. This alcohol, however, was not detected using the mousy taint extraction and analysis procedure developed as part of this study.

6.2.2 Pathway B

Direct decarboxylation of L-lysine followed by deamination would produce the required tetrahydropyridine nucleus of ACTPY/ETPY [Figure 6.2; Reactions (5)→(6)]. The decarboxylation of L-lysine to form the symmetrical biogenic amine, cadaverine, has been widely described in nature (Leistner and Spenser, 1973; Fothergill and Guest, 1977; Mann, 1987; Madduri *et al.*, 1989). L-Ornithine decarboxylation results in the biosynthesis of putrescine (Figure 6.1) (Jakoby and Fredericks, 1959; Hölttä and Pohjanpelto, 1983; Tabor and Tabor, 1984; Tabor and Tabor, 1985; Walters, 1995).

In plants and bacteria the enzyme responsible for cadaverine biosynthesis is L-lysine decarboxylase (Soda and Moriguchi, 1969; Sabo, 1974; Fothergill and Guest, 1977; Walters, 1995). It has been demonstrated that L-lysine decarboxylase catalyses the formation of cadaverine in a stereospecific reaction (Leistner and Spenser, 1973; Sabo, 1974; Mann, 1987). No activity of this enzyme was detected with L-ornithine (Sabo, 1974). The enzyme L-ornithine decarboxylase catalyses the formation of putrescine, and in some cases, it inefficiently converts L-lysine to cadaverine. This phenomenon has been reported in bacteria, fungi and animal cells (Pegg and McGill, 1979; Paulus *et al.*, 1982; Hölttä and Pohjanpelto, 1983; Igarashi *et al.*, 1986). L-Ornithine decarboxylase has been characterised in *S. cerevisiae* (Tyagi *et al.*, 1981; Tabor and Tabor, 1985). The action of this enzyme was suggested as responsible for the biosynthesis of cadaverine by this yeast, and not by L-lysine decarboxylase (Walters and Cowley, 1996).

In the context of the proposed mousy taint compound biosynthetic pathway [Figure 6.2 Reactions (5) →(6)], that a single enzyme could be responsible for the conversion of L-lysine and L-ornithine to cadaverine and putrescine respectively is a distinct possibility. Some evidence to support this proposal was provided by data in which L-ornithine was the sole nitrogen source (Section 5.3.1), where ACPY along with ACTPY was detected in the fermentation extracts (above the typical background concentration detected when no L-lysine is added). This may indicate that an induction of the mousy taint biosynthetic pathway occurred at the high L-ornithine concentration used, allowing a greater proportion of intracellular L-lysine to be converted to ACTPY than when ammonia was the sole nitrogen source. No ACPY was detected when L-lysine was the sole nitrogen source as might be expected if an induction of the pathway occurred. However, only background ACTPY/ETPY was detected, and ACPY was never detected, without amino acid supplementation. Much lower concentrations of ACPY, by at least 10-times in most cases, were produced with any L-ornithine supplements when compared to lysine additions and ACTPY concentrations. This suggests a much lower efficiency in the production of ACPY, which could account for the lack of ACPY detected in experiments where L-lysine was the sole nitrogen source.

Further catabolism of cadaverine and putrescine occurs via deamination by aminotransferase or oxidase to produce Δ^1-piperideine and Δ^1-pyrroline respectively (Large, 1992). For cadaverine, this transformation by an oxidase has been demonstrated to be stereospecific for L-lysine in bacteria and plants (Leistner and Spenser, 1973; Mann, 1987). Putrescine oxidase (deaminating) and diamine aminotransferase have been characterised in bacteria (Yamada, 1971; Fothergill and Guest, 1977; Okada *et al.*, 1979; Large, 1992). Yeast can breakdown cadaverine and putrescine, although most reports indicate the pathway proceeds via acetylated intermediates that initially prevent the formation of the cyclic imines Δ^1-piperideine and Δ^1-pyrroline (Large, 1986; Gillyon *et al.*, 1987; Large and Robertson, 1988). Some yeast, however, can catabolise these biogenic amines via an α-amino group oxidation (Large, 1992).

There is some evidence to support Pathway B, Reaction (6) (Figure 6.2) in relation to the incorporation of ε-N into ACTPY/ETPY as described by the stable isotope study. Work investigating the biosynthesis of the piperidine nucleus of some plant alkaloids from L-lysine determined that ε-N was retained (Leete, 1969; Gupta and Spenser, 1969a; b; 1970; Keogh and O'Donovan, 1970). Further, it was found that production of these alkaloids occurs via deamination of cadaverine (Leistner and Spenser, 1973; Mann, 1987).

The reported stereospecificity of cadaverine and putrescine metabolism indicates that for D-lysine to act as a precursor to ACTPY/ETPY, an enantiomeric transformation to L-lysine would be required [Figure 6.2 Reaction (1a)]. As described previously, lysine racemase activity has been characterised in nature, therefore it could be speculated that this type of enzyme may occur in *Dekkera/Brettanomyces*. This would account for the biosynthesis of ACTPY/ETPY via Pathway B from D-lysine.

Pathway B at this point reaches the critical intermediate of Δ^1-piperideine (Δ^1-pyrroline from L-ornithine) where it converges with Pathway A, and Reactions (3)→(4) (Figure 6.2) have been described above.

6.2.3 Which pathway?

Evidence for each individual step of either Pathway A or Pathway B is not complete. However, strong proof of the existence of the critical intermediate Δ^1-piperideine, in either pathway was obtained from the stable isotope study, that also determined which amino nitrogen of L-lysine was incorporated into ACTPY/ETPY. This data may serve to eliminate other possible routes for tetrahydropyridine biosynthesis. That D-lysine can also act as a precursor suggests that Pathway B is the least likely biosynthetic route, due the stereospecificity of biogenic amine metabolism. Although cadaverine/putrescine analysis was not undertaken, the distinctive and recognisable aroma of these compounds was not detected in any fermentation, whole cell incubation or extract. This indicates further that

Pathway B is the doubtful option, and analysis for cadaverine/putrescine would help facilitate this conclusion. The possibility of an enantiomeric conversion of D-lysine via a racemase [Reaction (1a)] must be eliminated before this pathway can be completely discounted.

The initial reaction of Pathway A is proposed to occur via an α-aminotransferase or by an α-oxidase. L-Lysine α-aminotransferase has not been widely described, and the unlikeliness of this transformation well recognised (Weissman and Schoenheimer, 1941; Paik and Kim, 1964). α-Aminotransferase activity is a common process in the catabolism of a wide variety of amino acids, and that at least nine alternatives to this ubiquitous reaction for L-lysine catabolism have been described in nature (Section 1.4.1) suggests that this is the unlikely alternative. However, as indicated above, an enzyme with some L-lysine α-aminotransferase activity (with L- and D-ornithine as well) has been described (Yorifuji *et al.*, 1971a; b). This could suggest an approach for mousy taint biosynthesis. An enzyme with functionality not related to L-lysine catabolism, however, could react with lysine and ornithine (both enantiomers?) to produce the appropriate mousy taint compound precursors. If this type of system was responsible for mousy taint biosynthesis, it would also account for why the process is inefficient, as specific enzymes are not utilised.

The alternative, via an α-oxidase, may be more likely as these enzymes require oxygen, and it was determined that air or oxygen was important to mousy taint biosynthesis. Again, specific lysine or ornithine enzymes are probably not involved, otherwise the production of mousy taint compounds would be more metabolically favourable. A question remains regarding the stereospecificity of α-oxidase enzymes. In general, oxidases are stereospecific (Meister, 1965), however, the induction of L-amino acid oxidase activity by D-amino acids has been reported, and could explain ACTPY biosynthesis from D-lysine. Alternatively, as postulated in Pathway B, a lysine racemase may function.

Therefore Pathway A which utilises an α-amino acid oxidase would appear the most likely approach for the biosynthesis of mousy taint compounds. This choice best fits the available evidence, although the other options of an α-aminotransferase or via Pathway B cannot be completely eliminated.

6.3 Model for mousy taint production by *Dekkera/Brettanomyces*

A practical consequence of the information derived from this study and from previous work, is that a model for mousy taint production in wine can be proposed (Figure 6.3). The presence in wine of any viable strain or species of *Dekkera/Brettanomyces* yeast, given the conditions and precursors are available, can produce mousy taint. The ideal precursor is the basic amino acid L-lysine, although D-lysine can be substituted for the L-enantiomer in the biosynthesis of ACTPY and consequently ETPY. The amino acid L-ornithine can act as a precursor for ACPY. However, this process is probably questionable in wine as the concentration of L-ornithine required to produce a sensorily significant concentration of ACPY is not found in grape juice or wine. ACPY is therefore an indicator of bacterial spoilage. The other important precursor is oxygen, which probably serves a dual function, stimulating the growth of the yeast, as well as contributing directly to the biosynthesis of taint compounds. The active and growing yeast population provides the metabolic machinery for both the carbon and nitrogen biotransformation required.

This model can explain mousy taint production in the real winery situation. A small, viable *Dekkera/Brettanomyces* population in a wine (that contains all necessary carbon and nitrogen) with limited air contact, then exposed to air via a processing or handling procedure produces a sufficient stimulus to induce mousy taint production. This model can also demonstrate an empirical observation of a link between wine exposed to air and a high level of mousy taint detected by sensory evaluation.

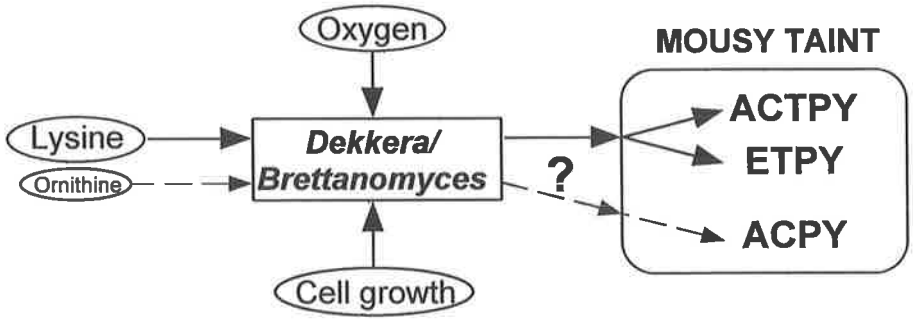


Figure 6.3. Proposed model for mousy taint biosynthesis by *Dekkera/Brettanomyces* in wine.

Mousy taint does occur in other alcoholic beverages such as beer and cider, and the model can also be used to account for taint production in these environments. Prevention in all cases is via the elimination of conditions and precursors, described in the model, which are conducive to the biosynthesis of mousy taint compounds by *Dekkera/Brettanomyces*.

6.4 Future research

Some suggestions of further research have already been made throughout the body of this thesis, therefore they do not need to be discussed further. Key future investigations of mousy taint production should focus on complete elucidation of the biochemical pathway of ACTPY synthesis. By definitively establishing the initial step in the catabolism of L-lysine, the role of oxygen in the process would be clarified. D-Lysine must be rationalised within the pathway as well.

A detailed sensory analysis of all mousy taint compounds so far described with respect to wine, would help obtain greater perspective and enable further interpretation of the data outlined in this thesis. However, only an extremely fastidious investigation would have any relevance due to the complexity and variation in the sensory response evoked by mousy taint.

The interrelationship between mousy taint and other flavour characteristics produced by *Dekkera/Brettanomyces*, both positive and negative, is also of interest. Clearly a more complete understanding of the potential of the *Dekkera/Brettanomyces* genera is worth pursuing.

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