FORMATION OF MOUSY OFF-FLAVOUR IN WINE BY LACTIC ACID BACTERIA

by

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Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university. To the best of my knowledge and belief, no material described herein has been previously published or written by any other person except when due reference is made in the text.

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Peter James Costello

THESIS SUMMARY

Formation of mousy off-flavour in wine by lactic acid bacteria.

Mousy off-flavour is an infrequent yet serious spoilage phenomenon in wine and other fermented beverages, which is commonly associated with the growth and metabolism of certain lactic acid bacteria (LAB) and the spoilage yeast *Dekkera / Brettanomyces*. Two compounds known to cause the characteristic and offensive mousy-like off-flavours are the N-heterocyclic volatile bases 2-acetyltetrahydropyridine (ACTPY) and 2-ethyltetrahydropyridine (ETPY). Since there is no satisfactory method for the removal of mousy off-flavour, this spoilage can invoke substantial economic loss to the wine producer. The aims of this thesis were to investigate the following aspects of the formation of mousy off-flavour by wine LAB:

1. Development of a sensitive and reliable procedure for the quantification of N-heterocyclic compounds causing mousy off-flavour in wine;
2. Survey the abilities of wine LAB and other wine bacteria to produce mousy off-flavour and the causative mousy compounds;
3. Investigate the substrates and metabolism of mousy compound formation by LAB.

Difficulties were encountered in the analysis of ACTPY due to its chemical and chromatographic instability, suggesting why previous research efforts have failed to quantify mousy compounds. Of several procedures assessed for the reliable extraction and quantification of low concentrations (µg/L level) of mousy compounds, a continuous liquid - liquid extraction (CLLE) method was developed and used in association with gas chromatography - mass spectrometry (GC-MS). The CLLE / GC-MS method was validated by demonstrating efficient and artefact-free recovery of mousy compounds from spiked Riesling wine. Using this procedure, three structurally related compounds, ACTPY, ETPY and a newly discovered and highly potent N-heterocycle, 2-acetyl-1-pyrroline (ACPY), were found to be unique components of mousy wines. Of the three mousy compounds, ACTPY was the most common and occurred at the highest concentration (106 µg/L), whereas ACPY and ETPY occurred less frequently and at maximum concentrations of 7.8 and 4.5 µg/L, respectively. The mousy aroma properties of ACPY were confirmed by GC-sniff analysis.

Thirty five LAB were screened for the ability to produce mousy off-flavour by a qualitative alkaline test strip procedure. In addition to *Lactobacillus brevis* and *L. cellobiosus*, which were known to be associated with mousy off-flavour, a diversity of LAB species, particularly heterofermentative *Lactobacillus* spp. and *Oenococcus oeni,* exhibited this ability in a range of ethanolic and wine-based media. The homofermentative *Pediococcus* spp., however, were generally lacking in this ability.

Selected wine bacteria were tested for the production of mousy compounds in nutritionally complex (Carr-MEt) and chemically defined (S1) media. In Carr-MEt medium, strains of *Lactobacillus* spp., *O. oeni*, *Pediococcus* spp. and *Gluconobacter oxydans* each produced one or more of ACTPY, ACPY and ETPY generally in the concentration range of <0.1 to 30 µg/L. Exceptionally high concentrations of ACTPY (259 µg/L) were produced by the type strain *L. hilgardii* DSM 20176. In contrast, synthetic (S1) medium supported only limited production of mousy compounds, despite similar growth characteristics to those with Carr-MEt medium.

The metabolism of mousy compounds by LAB was studied utilising a high cell density incubation (HCDI) technique with a basal assay (BA) medium, the main components of which were D-fructose (50 g/L), ethanol (5% v/v), L-lysine (5g/L), L-ornithine (5 g/L), metal salts and organic acids. Essential substrates of ACPY and ACTPY formation by *L. hilgardii* DSM 20176 were the availability of a fermentable carbohydrate (e.g. D-fructose), ethanol and iron (ferrous sulfate). In addition, L-ornithine stimulated the formation of ACPY and repressed ACTPY, whereas L-lysine stimulated the formation of ACTPY and repressed ACPY. The formation of ETPY, however, was little influenced by the availability of carbohydrate, L-ornithine or L-lysine. Other nutritional factors found to affect the formation of ACPY and ACTPY by *L. hilgardii* DSM 20176 in BA medium included the presence of malic acid and acetaldehyde, and the source of carbohydrate and amino acid. Replacement of ethanol with *n*-propanol led to the formation of propionyl-tetrahydropyridine, although this reaction did not occur with *iso*-propanol. The incorporation of deuterated ethanol (d_6-ethanol) into the acetyl side chain ACTPY and ACPY, and of deuterated acetaldehyde (d_4-acetaldehyde) into the acetyl side chain of ACTPY, confirmed that ethanol and acetaldehyde were precursors of these mousy compounds. These results also suggested that the attachment of the carbonyl side chain involved prior reduction of a primary alcohol to the corresponding aldehyde.

A pathway for the formation of ACPY and ACTPY by heterofermentative LAB is proposed. In this scheme, the co-metabolism of exogenous carbohydrate and ethanol force the accumulation of C-2 intermediates of the heterolactic fermentation of sugars (e.g. acetyl-coenzyme A). These C-2 compounds may then concurrently acetylate N-heterocyclic intermediates of L-ornithine and L-lysine metabolism, thus leading to the production of ACPY and ACTPY.

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Part of the work described in this thesis of Peter James Costello has been published:

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2. Herderich, M.; Costello, P.J.; Grbin P.R.; Henschke, P.A. Occurrence of 2-acetyl-1-pyrroline in mousy wines. Nat. Prod. Lett. 7; **1995**: 129-132.

3. Grbin, P.R.; Costello, P.J.; Herderich, M.; Markides, A.J.; Henschke, T.H.; Lee, T.H. Developments in the sensory, chemical and microbiological basis of mousy taint in wine. Stockley, C.S.; Sas, A.N.; Johnstone, R.S.; Lee, T.H., eds. Proceedings of the ninth Australian wine industry technical conference; 16-19 July 1995; Adelaide, SA: Adelaide SA: Winetitles; **1996**: 57-61.

TABLE OF CONTENTS

1. INTRODUCTION AND AIMS

Throughout the winemaking process, wine is continuously exposed to the risk of contamination by bacteria and other microorganisms which may grow and produce a diverse range of metabolic end-products. The final concentrations and sensory properties of these metabolites may yield desirable or undesirable effects on wine quality. Moreover, the microbiological formation of undesirable flavours can have serious economic consequences for the wine producer.

Lactic acid bacteria (LAB) are ubiquitous microorganisms which form part of the natural microflora of red and white table wines. The LAB of importance to winemaking comprise the genera *Lactobacillus*, *Pediococcus* , *Leuconostoc* and also *Oenococcus*. These bacteria, particularly *Oenococcus oeni* [formerly *Leuconostoc oenos* (Dicks *et al.* 1995)], are recognized as important bacteria for carrying out the malolactic fermentation (MLF) (Davis *et al.* 1985, Wibowo *et al.* 1985, Kunkee 1991, Henick-Kling 1993). Further to MLF, the growth and metabolism of certain LAB can also induce a range of wine spoilage reactions including acidification, mannitol taint, ropiness, formation of diacetyl and the formation of acrolein and bitterness (Vaughn 1955, Sponholz 1993). Wine conditions which are conducive to these spoilage reactions are generally those which encourage the growth of the causal bacteria, including high pH (> 3.5) and lack of sulfite. Moreover, the type and extent of the spoilage is also dependent upon the nature of the substrate attacked and upon the bacterial species involved. Moreover, while LAB favour conditions of reduced oxygen tension, the acetic acid bacteria are another group of spoilage organism which can cause acetification in grapes, must and wine under aerobic conditions.

Another potentially serious wine spoilage is the formation of mousy off-flavour (Heresztyn 1986, Rapp and Pretorius 1989, Sponholz 1993). This has long been recognized as an infrequent spoilage phenomenon in wines and other alcoholic beverages and is characterized by the development of an offensive 'mousy-like' off-flavour. In severe cases, this spoilage can render wine totally unpalatable and, since there is no known satisfactory method for its removal, its occurrence can cause major economic loss to the wine producer (Costello *et al.* 1993, Grbin *et al.* 1996). The persistent, albeit irregular occurrence of mousy off-flavour warrants further investigation of the factors which control this reaction.

Much controversy arose from early investigations of the causes and nature of this spoilage, which considered that either bacteria (including acetic acid bacteria), physico-chemical (oxidative) processes, or a combination of these, could cause mousy off-flavour. Furthermore, acetamide was erroneously suggested as a causative mousy compound (Erckmann 1898, Rodopulo 1952, Dymchisin 1953; cited by Tucknott 1977).

Greater understanding of this spoilage has come about in recent years. It is now known that the spoilage yeast *Brettanomyces* (*Dekkera*) spp. and also certain *Lactobacillus* spp. (*L. cellobiosus* and *L. brevis*) can cause mousy off-flavour (Tucknott 1977, Heresztyn 1986). Furthermore, with the availability of more sensitive analytical techniques and equipment, the two volatile N-heterocyclic compounds 2-ethyltetrahydropyridine and 2-acetyltetrahydropyridine have been associated with its formation. While 2-ethyltetrahydropyridine was the first compound to be attributed as the cause of mousy off-flavour (in fermentation media) (Tucknott 1977), subsequent studies (Craig and Heresztyn (1984) could not detect it in mousy wines, and Heresztyn (1986) later demonstrated that 2-acetyltetrahydropyridine was the major causal compound. 2-acetyltetrahydropyridine belongs to the potent 'cracker-like' group of aroma compounds (Teranishi *et al.* 1975, Buttery *et al.* 1982), which suggests that other structurally related N-heterocycles may also potentially contribute to mousy off-flavour in wine. Further investigations using sensitive analytical procedures are therefore warranted to determine the existence of any other mousy aroma compounds.

Apart from the recent advances concerning the nature and origin of mousy off-flavour, there is little knowledge of the factors which govern its formation by microorganisms, particularly by LAB. Ethanol is one known essential substrate of mousy compound formation, while the amino acid L-lysine has been demonstrated as another substrate for ACTPY formation by *Brettanomyces.* Reasons for the deficiency of knowledge in this area could relate to the lack of an appropriate chemically defined medium for the study of mousy off-flavour by LAB, and also the need for a sufficiently reliable procedure for the quantitative analysis of low concentrations (µg/L level) of mousy compounds.

In order to gain greater understanding and control over the formation of mousy off-flavour, further research is required to elucidate more fully the role of wine bacteria, and of the respective substrates involved in the formation of mousy compounds.

The aims of this thesis are to:

1. Survey representative wine strains of LAB including *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *O. oeni* for the ability to produce mousy off-flavour, as well as compounds causing mousy off-flavour in chemically undefined and chemically defined media;

2. Develop a reliable and sensitive procedure for the extraction and quantification of low concentrations of N-heterocyclic mousy compounds from wine and fermentation media;

3. Identify and quantify compounds in wines which exhibit mousy off-flavour;

5. Develop a high cell density incubation procedure to investigate the substrates and precursors of mousy compound formation by heterofermentative LAB.

2. LITERATURE REVIEW

2.1 Wine spoilage caused by bacteria

From an historical perspective Pasteur (1866; cited by Vaughn 1955), in his treatise *'Etude sur le vin'*, was the first to establish a causal relationship between wine spoilage and microorganisms and introduced the first classification of wine disorders caused by bacteria. This classification described particular wine 'diseases', eg. *tourne*, *pousse*, *amertume* and was embellished by later authors (Gayon and Dubourg 1894, Semichon 1905, Kayser 1913, Pacottet 1926, Dugast 1930; cited by Douglas and Cruess 1936, Vaughn 1955). Pasteur's nomenclature is still in use (Lafon-Lafourcade 1983, Peynaud1984) and has been referred to as the modern French classification of table wine spoilage (Rankine and Bridson 1971) (Table 2.1). The latter French classification, however, is based upon the constituents which are attacked by the bacteria, and at one time oenologists considered that each form of spoilage was induced by a specific organism, ie. *tourne* bacteria, *mannite* bacteria, etc. (Amerine *et al.* 1980). Later recognition that a spoilage defect can be induced by several bacterial species led to much confusion regarding spoilage nomenclature, and some authors strongly suggested the older terminologies be avoided (Müller-Thurgau and Osterwalder 1919, Arena 1936; cited by Vaughn 1955, Amerine *et al.* 1980). More rigorous differentiation of spoilage defects in terms of the causal bacteria was initiated by Müller-Thurgau and Osterwalder (1919), and from which Vaughn (1955) derived a classification dividing abnormalities into two groups of spoilage reactions, ie. aerobic (oxidative) or anaerobic (fermentative).

More recent wine literature (Amerine *et al.* 1980, Benda 1982, Peynaud 1984, Sponholz 1993) still broadly applies Vaughn's classification and differentiates between wine spoilage reactions that are caused by acetic acid bacteria, which require oxygen for growth, and those caused by lactic acid bacteria, which grow optimally under reduced oxygen content. More specific terms are also used to describe a particular spoilage according to the metabolite causing the defect, eg. acetic acid, mannitol, diacetyl and acrolein. This section examines some of the major wine spoilage defects caused by both acetic acid bacteria and lactic acid bacteria.

Table 2.1 Modern French classification of bacterial wine spoilage (adapted from Rankine and Bridson 1971, Lafon-Lafourcade 1983, Peynaud 1984)

Spoilage type	Characteristics
Acescence or souring	Production of acetic acid and increased volatile acidity
Tourne - wine that has turned	Breakdown of tartaric acid causing increased pH and volatile acidity, loss of colour, turbidity, sometimes CO_2 liberation, sauerkraut odour due to acetoin, and in advanced cases flavour may turn 'mousy'
Piqure lactique	Increased fixed and volatile acidity caused by lactic fermentation of sugar in the must
Fermentation mannitique	Lactic fermentation of traces of sugar in stuck wines, closely related to *Piqure lactique*
Amertume	Breakdown of glycerol with the formation of acrolein and resulting in bitterness

2.1.1 Wine spoilage by acetic acid bacteria

Acetic acid bacteria are an important group of aerobic food spoilage microorganisms, which are responsible for the vinegary spoilage of wine, otherwise known as asescence, vinegar or acetic souring, acetic spoilage or acetification (Vaughn 1955, Amerine *et al.* 1980, Peynaud 1984). The unique property of acetic acid bacteria associating them with acetification is their ability to oxidize ethanol to acetic acid (Drysdale and Fleet 1988); thus a high concentration of acetic acid is symptomatic of this spoilage.

Acetic acid is the major volatile acid in wine and has a bitter, acrid aftertaste which is perceptible at a concentration of 0.7-0.75 g/L (Benda 1982, Peynaud 1984) and becomes objectionable at 1.2-1.3 g/L or higher (Margalith 1981). A side reaction arising from acetic spoilage is the esterification of acetic acid and ethanol and subsequent formation of ethyl acetate (see below). Ethyl acetate also has a vinegary flavour, but a much lower flavour threshold (approximately 120 mg/L) than acetic acid (Peynaud 1984). The subject of acetic acid bacteria in winemaking has been comprehensively reviewed by Vaughn (1955), Lafon-Lafourcade and Joyeaux (1981), Lafon-Lafourcade (1983) and more recently by Drysdale and Fleet (1988). Two closely related genera of acetic acid bacteria are recognised, *Acetobacter* and *Gluconobacter*, which are both placed within the family *Acetobacteracea* (Buchanan and Gibbons 1984). The genus *Acetobacter* comprises the species *A. aceti*, *A. liquifaciens*, *A. pasteurianus*, *A. hansenii,* whereas *Gluconobacter oxydans* is the only species belonging to the genus *Gluconobacter*. The distinguishing feature between *Acetobacter* and *Gluconobacter* is that while members of both genera can oxidize ethanol to acetic acid, only *Acetobacter* spp. can further oxidize acetic acid to carbon dioxide and water (ie. over-oxidize ethanol); species of *Gluconobacter* cannot further oxidize acetic acid. Drysdale and Fleet (1988) also note that another species of *Acetobacter*, *A. methanolicus*, has been described (Uhlig, Karbaum and Steudel 1986), and that species of *Frateuria*, which are closely related to acetic acid bacteria (Swings, De Ley and Gillis 1984), are also of interest because of their ability to oxidize ethanol to acetic acid at low pH.

Acetic acid bacteria can grow and influence wine quality at three stages of vinification: on grapes prior to harvest, in fermenting must, and in wine during storage. Sound grapes contain a low natural population of ca. 10^2 cells/g, the predominant species being *Gluconobacter oxydans* (Drysdale and Fleet 1988, Sponholz 1993). Grapes that are mechanically damaged or infected by the mould *Botrytis cinerea* are prone to spoilage by yeasts and acetic acid bacteria, leading to much higher populations of ca. 10^6 cells acetic acid bacteria /g (mainly *A. aceti* and *A. pasteurianus*). The juice from such spoiled grapes can contain up to 3.9 g/L of acetic acid as well as lower amounts of ethyl acetate (2.9-53.9 mg/L) and, according to Sponholz (1993), also contain ethanol (3.9 g/L) and glycerol (2.0 g/L) from yeast metabolism. The latter ethanol and glycerol components can be further converted to, respectively, acetic acid and dihydroxyacetone by acetic acid bacteria. The oxidation of glycerol to dihydroxyacetone is a well established property of *G. oxydans* and *A. aceti*, but is not displayed by the majority of *A. pasteurianus* strains (Drysdale and Fleet 1988, Sponholz 1993). Grape must infected with *G. oxydans* has reportedly produced as much as 260 mg/L dihydroxyacetone, approximately half of which was carried over into the wine (Sponholz and Dittrich 1985). This compound could affect the sensory properties of wine itself from its own sweet aroma and cooling taste or, perhaps more significantly, by reaction with amino acids such as proline in the formation of potent 'crust-like' or bready aromas (Margalith 1981; see later section on ACTPY). Further products of acetic acid bacteria metabolism occurring in

acetified grape must are gluconic acid and ketogluconates, which arise from the direct oxidation of glucose and other hexoses.

Species of *Acetobacter* produce less gluconic acid in grape must than *Gluconobacter*, a characteristic possibly associated with the weaker carbohydrate metabolism of *Acetobacter* compared with that of *Gluconobacter* (Joyeaux *et al.* 1984b, Eschenbruch and Dittrich 1986). Drysdale and Fleet (1988) suggest that the latter pathway is probably more important in the formation of gluconic acid and ketogluconate by *B. cinerea* in the case of *Botrytis*- infected grapes. Acetic acid bacteria can also oxidatively metabolize hexoses and pentoses by the hexose monophosphate pathway to acetic and lactic acids, and further in the case of *Acetobacter* to carbon dioxide and water via the tricarboxylic acid cycle (Drysdale and Fleet 1988).

Overall, the composition and quality of must and wine can be detrimentally affected by infection of grapes with acetic acid bacteria. Table 2.2 shows the concentration of some major grape juice components as a function of infection by acetic acid bacteria.

It is suggested by several authors that the strictly aerobic physiology of acetic acid bacteria generally precludes their proliferation under the anaerobic conditions of alcoholic fermentation. Vaughn (1938, 1955), however, describes cases of 'rapid acetification' in warm fermenting musts whereby the ethanol produced by yeast was oxidized to acetic acid by *A. aceti*. When the population of acetic acid bacteria or the temperature increased sufficiently, the yeast fermentation stopped and the bacteria then oxidized glucose to gluconic acid. Interestingly, the resultant wines had a sweet-sour taste and, if glucose oxidation had proceeded sufficiently, were also mousy (discussed in a later section). Factors influencing rapid acetification include the species of yeasts, acetic acid bacteria and presence of LAB (Vaughn 1938), and the proportion of acetic acid bacteria to yeasts at the commencement of fermentation (Watanabe and Iino 1984). Wines with incomplete or stuck fermentation are also susceptible to acetification if stored in partially filled vessels (Amerine *et al.* 1980).

Table 2.2 Concentration of some key grape juice constituents as a function of infection by acetic acid bacteria (adapted from Sponholz and Dittrich 1985, Sponholz 1993).

Infected grapes (%)	Concentration (mg/L)				
	Ethanol	Acetic acid	Glycerol	Dihydroxy-acetone	Gluconic acid
0	103	23	0	4	41
10	259	200	1100	41	252
20	494	463	2000	71	520
50	577	1040	1900	184	1581
75	419	1690	1400	259	2586

The population of acetic acid bacteria remaining after alcoholic fermentation is generally less than 10^2 cells/mL and, provided the wine is kept free of oxygen, remains at 10^3-10^5 cells/mL in wines during storage (Drysdale and Fleet 1988, Sponholz 1993). Great potential for the rapid growth of acetic acid bacteria up to 10^8 cells/mL (predominantly *Acetobacter*) exists upon even momentary exposure of wine to air during pumping, transfer operations, or by storage of wine in partially filled and poorly filled tanks, etc. (Joyeux *et al.* 1984a, Drysdale and Fleet 1989b, Sponholz 1993). Such growth is characteristically accompanied by the metabolism of ethanol into acetic acid, whereby ethanol is first oxidized to acetaldehyde by alcohol dehydrogenase, and acetaldehyde is then oxidized to acetic acid by acetaldehyde dehydrogenase.

Recent studies were conducted by Drysdale and Fleet (1989a) on the growth and metabolism of acetic acid bacteria in wine at different levels of aeration. In this study, strains of *A. aceti* and *A. pasteurianus* were found to oxidize 50-60% of the ethanol with concomitant formation of 1.28-3.75 g/L acetic acid in wines receiving 100% oxygenation. Other reactions accompanying acetic spoilage include formation of a significant concentration of acetaldehyde, the esterification of acetic acid and ethanol into ethyl acetate and the oxidation of glycerol into dihydroxyacetone (Dupuy and Maugenet 1963, Yoshioka 1983, Sponholz *et al.* 1982, Drysdale and Fleet 1989a). Moreover, acetic acid bacteria also have the ability to oxidize

organic acids whereby strains of *A. aceti* and *A. pasteurianus* can completely oxidize lactic, pyruvic and acetic acids to carbon dioxide and water via the TCA cycle, and other acids, such as malic, succinic, citric and fumaric acids, are similarly metabolized (Stouthamer 1959, De Ley 1961, Cooksey and Rainbow 1962, De Ley *et al.* 1984). Strains of *G. oxydans*, however, do not have a functional TCA cycle and are thus unable to metabolize acetic and many other organic acids (Greenfield and Claus 1972). They also have a low tolerance to ethanol; only 42% of *G. oxydans* strains were able to grow in media containing 5% ethanol, thus explaining why this organism predominates in grapes and grape juice but is rarely isolated from wines (De Ley and Swings 1984), and further as to its inability to grow in the oxygenated wine study of Drysdale and Fleet (1989a). Major factors affecting the growth and metabolism of acetic acid bacteria in wine, such as pH, temperature, sulfur dioxide and oxygen, are further discussed by Lafon-Lafourcade (1983) and Drysdale and Fleet (1988, 1989a). Acetic acid bacteria are therefore a major risk to the spoilage of wine, especially in view of recent reports of their survival and metabolism in wine containing reduced levels of oxygen (Joyeux *et al.* 1984a, Drysdale and Fleet 1985, 1988). Furthermore, there is a lack of information regarding the ability of acetic acid bacteria to metabolize other wine components which are of relevance to oenologists, including their role in the formation of mousy off-flavour.

2.1.2 Wine spoilage by lactic acid bacteria

The lactic LAB comprise a group of bacteria that produces lactic acid as a major end-product of carbohydrate metabolism. They also constitute one of the most ubiquitous groups of bacteria and occur in a diversity of environments including many foods and alcoholic beverages (Carr 1975). Species of LAB occur in two taxonomic families, namely, the *Lactobacillaceae* and the *Streptococcaceae*. Lactic acid bacteria relevant to the wine industry occur in the genera *Lactobacillus*, *Pediococcus*, and *Leuconostoc*. The genus *Lactobacillus* comprises rod-shaped homofermentative and heterofermentative species, whereas species of *Pediococcus* and *Leuconostoc* are, respectively, homofermentative and heterofermentative coccoid organisms (Rogosa 1974, Wibowo *et al.* 1985, Garvie 1986a,b). In the winemaking process, lactic acid bacteria are generally recognized for their ability to carry out malolactic fermentation (MLF), the conversion of L-malic acid to L-lactic acid and carbon dioxide (Davis *et al.* 1985, Wibowo *et al.* 1985, Kunkee 1991). [It has recently been proposed (Dicks *et al.* 1995) that the principal species associated with MLF, *Leuconostoc oenos*, be reclassified to a new genus, *Oenococcus oeni*. The reclassification of *Leuconostoc oenos* to *O. oeni* is adopted for the purposes of this thesis.] Under certain conditions, however, LAB can also rapidly grow and metabolise a range of other wine substrates, the end-products of which may cause severe wine spoilage. Spoilage reactions carried out by LAB in wine include

acidification, mannitol taint, ropiness, formation of diacetyl and formation of acrolein and bitterness. These and other spoilage effects are briefly discussed in the following sections.

(i) Acidification

The fermentation of sugars by LAB can lead to detrimental increases in fixed and volatile acidity of wine due to the formation of D-lactic acid and acetic acid. Such acidification only occurs in wines containing fermentable sugar, eg. fermenting musts, wines with a 'stuck' fermentation or those containing as little as 2 g/L residual sugar (Benda 1982, Sponholz 1993). Differentiation between homo- and heterofermentative LAB becomes important when considering this spoilage. Homofermentative species of LAB convert glucose mainly to lactic acid via the Embden-Meyerhof-Parnas (EMP) pathway, while heterofermentative species produce less lactic acid and increased amounts of acetic acid, carbon dioxide, glycerol and ethanol via the phosphoketolase (or 6-phosphogluconate) pathway (Kandler 1983, Axelsson 1993) (Figure 2.1). Other hexoses, eg. fructose, mannose and galactose, can also be metabolized by these pathways after conversion to the corresponding glucose or glucose phosphate. Pentose sugars are metabolized via the phosphoketolase pathway by both hetero- and also homofermentative species, producing equimolar concentrations of acetic and lactic acids (Kandler 1983).

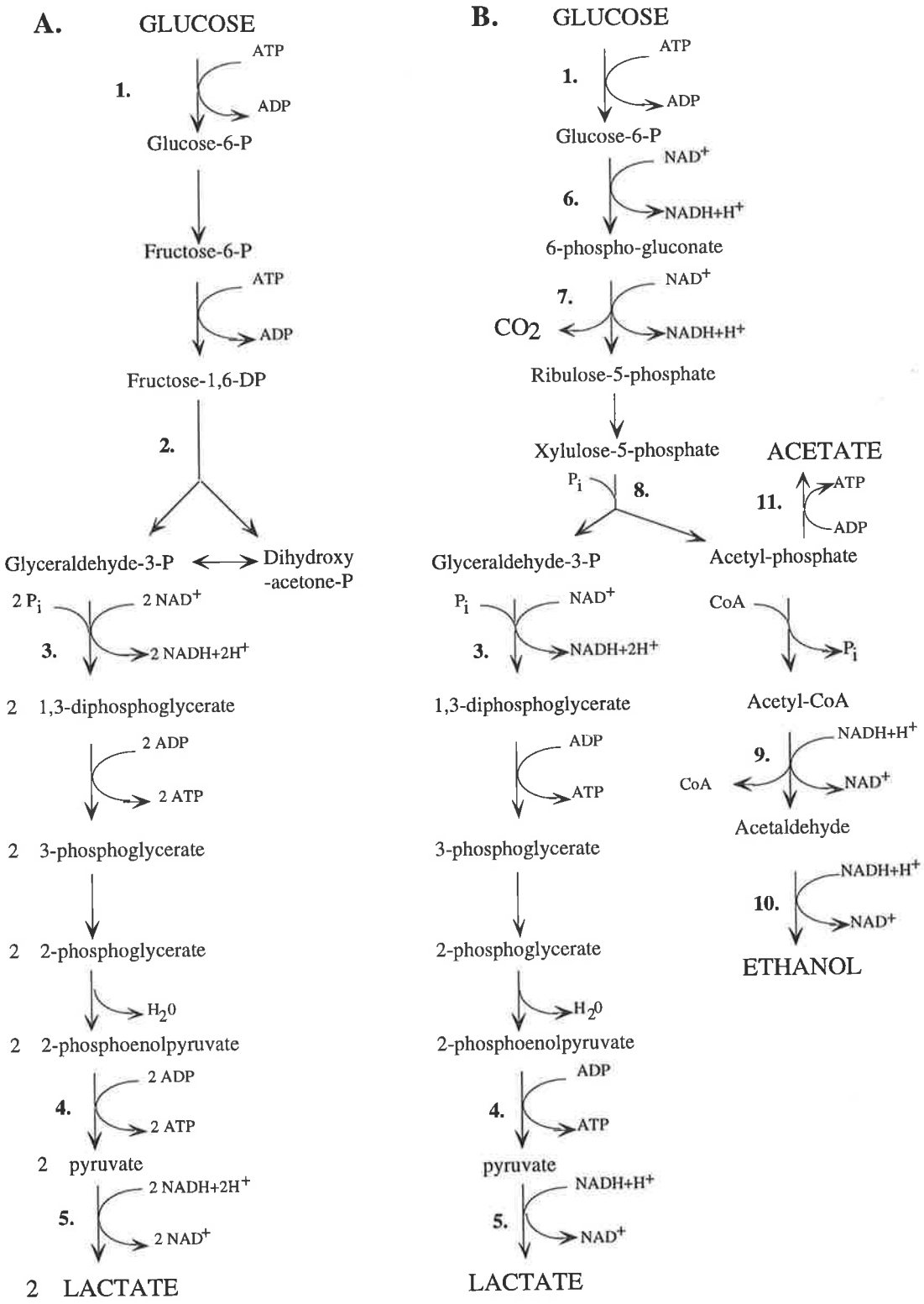
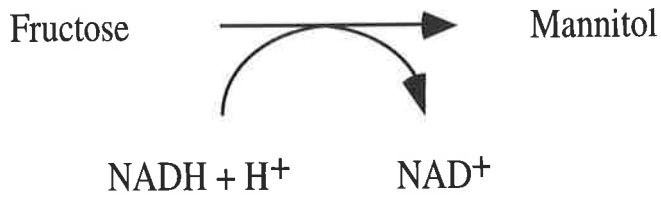


Figure 2.1 Fermentation pathways of glucose by lactic acid bacteria: (**A**) homolactic fermentation (glycolysis, Embden-Meyerhof pathway); (**B**) heterolactic fermentation (phosphoketolase/6-phosphogluconate pathway). Selected enzymes are numbered: 1. glucokinase; 2. fructose-1,6-diphosphate aldolase; 3. glyceraldehyde-3-phosphate dehydrogenase; 4. pyruvate kinase; 5. lactate dehydrogenase; 6. glucose-6-phosphate dehydrogenase; 7. 6-phosphogluconate dehydrogenase; 8. phosphoketolase; 9. acetaldehyde dehydrogenase; 10. alcohol dehydrogenase; 11. acetate kinase (adapted from Condon (1987) and Axelsson (1993))

Sponholz (1993) considers that acidification by LAB is an often under-estimated spoilage and that wines having high concentration of end-products often have a vinegary defect. Furthermore, Peynaud (1984) indicates different degrees of acidification may occur depending upon the quantity of sugar fermented. Milder forms can commonly occur in dry red wines, leading to a slight increase in volatile (and presumably also fixed) acidity and loss of wine freshness. Serious forms of acidification are more detrimental to wine quality and can be accompanied by the formation of mannitol (Peynaud 1984) (see below). Significantly, Van de Water (1994) reports many recent cases of acetic acid spoilage coupled with stuck fermentation caused by lactobacilli in red and white fermenting musts from California and Washington State. These spoilage incidents occurred after winemakers abandoned the addition of SO_2 to grapes before fermentation, in an effort to reduce the concentration of sulfite in wine. As a consequence, certain lactobacilli grew and stopped the yeast fermentation, leaving residual sugar concentration of approximately 5-10 g/L and an acetic acid concentration of 0.8-2 g/L or higher. Resulting wines had an unpleasant 'sweet-sour' finish. The overall descriptions of this spoilage are very similar to those of 'rapid acetification' caused by certain acetic acid bacteria (see previous section on spoilage by acetic acid bacteria). Van de Water (1994) further reported that that acetification by lactobacilli is again uncommon since re-introduction of SO_2 addition to wines before fermentation.

(ii) Mannitol taint

In addition to acidification, some heterofermentative species of LAB (eg. *L. brevis*) can produce a high concentration of mannitol by direct reduction of fructose (Wood 1961, Martinez *et al.* 1963). This reaction uses mannitol dehydrogenase (Axelsson 1993) and the pathway can be represented as:



Wines with this spoilage may contain approximately 9 g/L of mannitol together with an abnormally high concentration of acetic acid, D-lactic acid and also *n*-propanol and 2-butanol. Moreover, they typically have a vinegary, estery taste and may also exhibit slime formation and diacetyl taint (Sponholz 1993). Mannitol taint can occur in wines and distillation marcs that have high pH and a high concentration of fructose. It is now, however, generally uncommon, being effectively prevented by rational use of SO_2 (Peynaud 1984) and maintaining low pH (Sponholz 1993).

(iii) Ropiness and slime

Certain LAB have the ability to grow and produce extracellular polysaccharides in wine, causing a spoilage known as ropiness or slime. Affected wines typically have a slimy, viscous and oily character and may also 'rope' when poured (Dittrich 1987, Peynaud 1984). Moreover, dextran forming strains of LAB are widely known to cause serious spoilage by formation of slime in other foods and beverages, including *L. collinoides* and *P. cerevisiae* in cider, *L. vermiforme* in beer, *Leuc. dextranicum* and *Leuc. mesenteroides* in sugar manufacture and various species of *Lactobacillus* and *Leuconostoc* in meat and meat products (Sharpe and Pettipher 1983). The slime (extracellular dextran and levan) is generally produced by condensation of monomeric sugars from oligosaccharide carbon sources, such as sucrose. Sponholz (1993) considers, however, that ropiness in wine can precede more serious faults such as acidification and mannitol off-flavour.

(iv) Diacetyl production

Lactic acid bacteria are well known for their ability to produce the intensely aromatic diketone, diacetyl (2,3 butanedione) in a variety of fermented foods (Kempler 1983). The aroma threshold of diacetyl in wine is low and, depending upon wine type, may vary between 0.2-2.8 mg/L (Martineau *et al.* 1995). The formation of a high concentration of this compound (above approximately 7 mg/L), however, can produce an undesirable buttery aroma and flavour (Rankine *et al.* 1969, Benda 1982, Bertrand *et al.* 1984, Sponholz 1993, Bartowsky *et al.* 1997).

The small quantities (0.2-0.3 mg/L) of diacetyl that are normally produced by yeast during alcoholic fermentation generally increase in association with the growth of LAB and MLF (Pilone *et al.* 1966, Rankine *et al.* 1969, 1970, Zeeman *et al.* 1982, Postel and Meier 1983, Bertrand *et al.* 1984, Mascarenhas 1984, Sponholz 1993). Moreover, the amount of diacetyl produced varies with the LAB conducting MLF, whereby a small and unobjectionable concentration of diacetyl is produced by *O. oeni,* but a larger increase may occur if MLF is conducted by species of *Pediococcus* or *Lactobacillus* (Mayer 1974, Sponholz 1993). Furthermore, recent studies by Martineau and Henick-Kling (1995) and Bartowsky *et al.* (1997) have demonstrated significant variation in the abilities of *O. oeni* strains to produce diacetyl in wine.

Production of diacetyl by LAB may proceed via two pathways of which pyruvate is a common intermediate to both. The first pathway involves the metabolism of hexose and

pentose sugars, which are converted via pyruvate to acetolactate, which is then converted to diacetyl. The second pathway involves the metabolism of citric acid by citrate lyase which leads to the formation of acetic and oxaloacetic acids. Oxaloacetate is then metabolized to diacetyl through pyruvate (Weiller and Radler 1970, El-Gendy *et al.* 1983, Kempler 1983, Shimazu *et al.* 1985, Sponholz 1993). *Oenococcus oeni* has been most frequently associated with degradation of citric acid in wine and synthetic media, whereas no wine pediococci and only some wine lactobacilli display this characteristic (Davis *et al.* 1986a,b).

(v) Acrolein formation and bitterness

Although not generally occurring in present-day wines, the acrolein off-flavour and associated bitterness caused particular havoc toward the end of the last century in wines from the Burgundy region of France (Peynaud 1984). Acrolein is produced through the lactic fermentation of glycerol. Although not bitter itself, acrolein reacts chemically with wine polyphenols to produce a bitter substance (Rentschler and Tanner 1951). Margalith (1981) reports that 10 mg/L acrolein is sufficient to induce a bitter off-flavour. Moreover, red wines have a higher concentration of polyphenols than white wines and are thus also more susceptible to this spoilage (Benda 1982, Peynaud 1984). The degradation of glycerol can occur by two pathways. The first involves reactions in the reverse of the EMP pathway, leading to dihydroxyacetone phosphate, pyruvic acid and then lactic acid (Radler 1975). The second leads to the formation of acrolein and requires a key dehydratase enzyme which initially converts glycerol to 3-hydroxypropionaldehyde (Smiley and Sobolow 1962, Sliniger *et al.* 1983). Upon long storage in acidic solutions, as in wine, an equilibrium is further established between 3-hydroxypropionaldehyde and the unsaturated aldehyde, acrolein (Sponholz 1993).

The ability of LAB to degrade glycerol is not widespread. Davis *et al.* (1988) showed that 1% of *O. oeni*, 12% of *P. parvulus* and 31% of *Lactobacillus* species tested had this capability. The formation of acrolein from glycerol has been demonstrated in *Leuc. mesenteroides* and has also been implicated in spore-forming clostridia (Wilharm and Holz 1951, Hirano *et al.* 1962).

(vi) Decomposition of tartaric acid

The metabolism of tartaric acid by LAB can lead to complete spoilage of wine. Wines particularly susceptible are those with low acidity (Benda 1982) and a low concentration of sulfite (Peynaud 1984). The occurrence of this spoilage is now considered rare, however,

due to improved methods of vinification (Radler 1975, Peynaud 1984). Sponholz (1993) suggests that wines undergoing tartrate decomposition may already be spoiled by other serious faults such as degradation of glycerol and an increased concentration of lactic and acetic acids. Overall effects on wine quality from this spoilage include decreased fixed acidity, increased volatile acidity, loss of colour in red wines, production of CO_2, and in more advanced cases, formation of a disagreeable odour and mousy flavour (Peynaud 1984).

Significantly, the ability to degrade tartaric acid is not widespread and is restricted to only a few species of *Lactobacillus*, including strains of *L. plantarum* and *L. brevis* (Krumpermann and Vaughn 1966, Radler and Yannissis 1972). The reaction may proceed via two pathways depending on whether the bacteria are homo- or heterofermentative. The main enzyme common to both pathways is an inducible tartrate dehydratase, which dehydrates tartaric acid to oxaloacetic acid. End-products from the homofermentative strain, *L. plantarum* are lactic acid, acetic acid and CO_2, whereas the heterofermentative strain *L. brevis* shows a more complicated metabolism that additionally produces succinic acid (Radler 1975, Sponholz 1993).

(vii) The geranium off-odour

Sorbic acid (2,4-hexadienoic acid), a fungistatic agent used in wines to inhibit yeast, can be metabolized by certain strains of LAB and form an unpleasant odour similar to that produced by crushing leaves of the geranium plant (*Pelargonium* spp.) (Rapp and Pretorius 1989, Sponholz 1993). The formation of this off-flavour and its association with the activity of LAB in wines preserved with sorbic acid was originally recognized by Burkhardt (1973). The major compound responsible for the off-flavour was later identified as 2-ethoxyhexa-3,5-diene, which has a low odour threshold of 0.1 µg/L (Würdig *et al.* 1974, Crowell and Guymon 1975). A key initial step in off-flavour formation is the bacterial reduction of sorbic acid to sorbyl alcohol. Under acidic conditions, sorbyl alcohol is then isomerized to 3,5-hexadien-2-ol, which is further esterified with ethanol to produce 2-ethoxyhexa-3,5-diene (Figure 2.2).

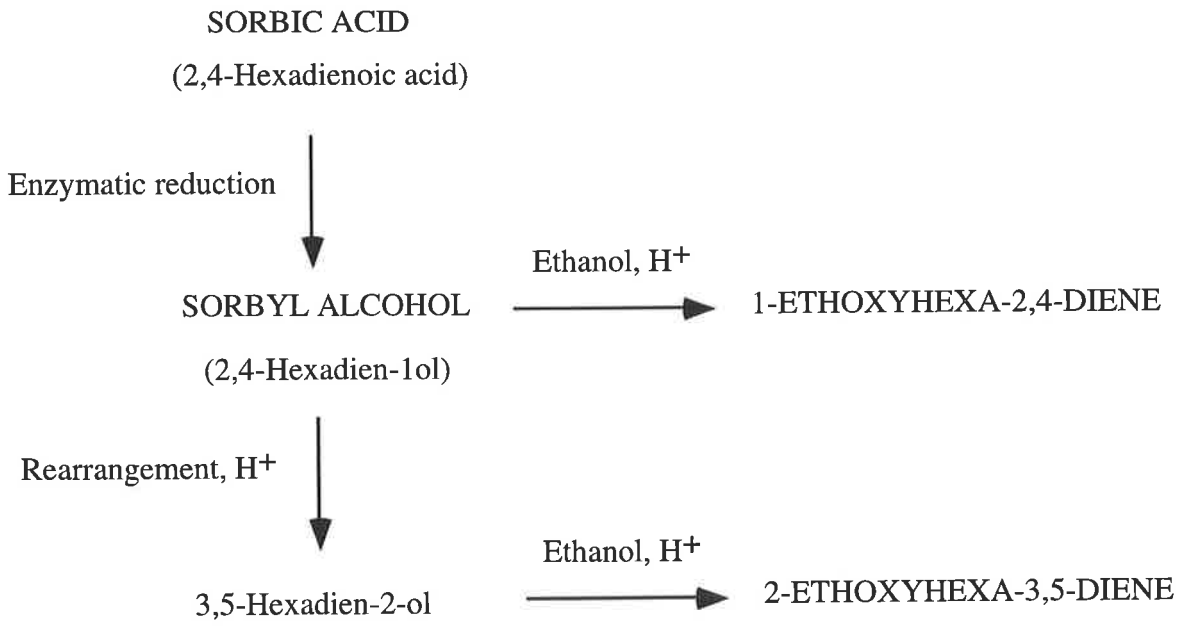


Figure 2.2 Degradation of sorbic acid in wine by lactic acid bacteria and formation of the geranium off-odour, 2-ethoxyhexa-3,5-diene (adapted from Rapp and Pretorius 1989)

Radler (1976) found that strains of *O. oeni* and some heterofermentative strains of *Lactobacillus* were capable of reducing sorbic acid to sorbyl alcohol, although other workers (Edinger and Splittstoeser 1986) did not find this ability in strains of *Lactobacillus* or *Pediococcus* (Rapp and Pretorius 1989, Sponholz 1993).

2.2 Mousy off-flavour wine spoilage

Mousy off-flavour has been recognized over the past century as a generally infrequent but devastating spoilage phenomenon in wines, ciders and other alcoholic beverages. It is characterized by a repulsive 'mousy-like' off-flavour which is often delayed in perception on the palate and, in severe cases, can render wine totally unpalatable. A second negative aspect to this off-flavour is that there are no known satisfactory methods for its removal, and affected

wines are generally unfit for blending or distillation purposes. Winemakers can therefore suffer major economic losses if wines exhibit this spoilage. Considerable study has been undertaken into the cause and nature of mousy off-flavour, and much controversy has arisen as to whether the off-flavour is of microbiological or chemical origin and, more recently, as to the identity of the causative compound. The following section gives a brief account of some of the early studies concerning mousy off-flavour and possible methods for its removal. These topics are also the subject of a comprehensive literature review by Tucknott (1977). A later section examines more recent literature on the subject.

(i) Early studies

One of the earliest references to the microbiological origin of mousy off-flavour and of its incidence in general was made in the late nineteenth century by Erckmann (1898; cited by Tucknott 1977), who aptly described it as 'a flavour, often left on the tongue as an aftertaste, reminiscent of the odour of mice kept in a confined space'. He associated the then frequently occurring disorder with the bacterial formation of acetamide. Mason (1889; cited by Tucknott 1977), however, had previously shown pure acetamide to be odourless and the erroneous association of mousy off-flavour with acetamide has unfortunately persisted to recent literature (Rankine *et al.* 1971). Nessler and Windisch (1908; cited by Tucknott 1977) were unable to determine the main cause of mousy off-flavour although, they considered that it was not produced by acetic acid bacteria after noticing the anaerobic formation of a high concentration of volatile acidity associated with the production of off-flavour. The first report directly linking mousy off-flavour with a causal microorganism was made by Müller-Thurgau and Osterwalder (1913), who isolated a homofermentative mannite-producing bacterium which they named *Bacterium mannitopoeum* from mousy wines. They established it to be a causal bacterium after reinoculation into sound, sterile wine reproduced mousy off-flavour. They also observed that the sensory perception of off-flavour varied according to individual sensitivity. Cruess (1918; cited by Tucknott 1977) was unable to isolate *B. mannitopoeum* from Californian grapes but supported Müller-Thurgau and Osterwalder's view on its role in the formation of mousy off-flavour. Douglas and Cruess (1936) later isolated and characterized *Lactobacillus hilgardii* from spoiled Californian wines, which was also capable of producing mousy off-flavour. Unlike *B. mannitopoeum*, *L. hilgardii* did not produce mannite from fructose. Wine spoilage by *L. hilgardii* was characterized by the formation of a silky cloudiness, followed by rapid increase in volatile acidity and finally the formation of a mousy odour and flavour, rendering wine fit only for low-grade distillation purposes. Pure cultures of the organism were found to produce the latter spoilage characteristics after inoculation into sound wine. Mousy odour was also produced by *L. hilgardii* after one week of culture in diluted grape juice or in diluted sweet fortified wine (Douglas and Cruess 1936). Further evidence of the microbiological origin of mousy off-flavour was again given by

Osterwalder (1948b; cited by Tucknott 1977) who, after refuting Schanderl's (1948; cited by Tucknott 1977) report that the off-flavour was only produced by physico-chemical means (see below), showed the presence of filamentous bacteria of the *B. mannitopoeum* type in mousy wines. Osterwalder (1948b) concluded that mousy off-flavour was generally produced in association with mannitic or glycerol fermentation.

In addition to lactobacilli, Vaughn (1938, 1955) reported the ability of acetic acid bacteria to produce mousy off-flavour. Vaughn's work demonstrated that a mousy off-flavour was produced by all twenty-seven strains of *A. aceti* tested in grape juice media and musts.

Other early reports linking mousy off-flavour with microbial activity are cited by Tucknott (1977) and include those of Kvasnikov (1951) and Kvasnikov and Kondo (1958) who, respectively, implicated *L. brevis* and heterofermentative LAB in off-flavour formation, and also Theron and Niehas (1947-48) and Zaslavskii (1955).

Most of the early studies on mousy off-flavour supported the general theory that the spoilage was caused by bacterial activity. However, reports by Drboglav (1940; cited by Tucknott 1977), Schanderl (1948) and others showed that a mousy character could also be chemically induced in wines by oxidative processes which cause high redox potentials. The role of microorganisms was considered by these authors to simply produce the physico-chemical conditions required for off-flavour to occur. In his studies on the formation of mousy off-flavour in Russian sparkling wines, Drboglav (1940) observed that a mousy off-flavour occurred at a redox potential of 0.46-0.55 volts and could be also induced by the addition of hydrogen peroxide. Similar conclusions were reached by Schanderl (1948) who, after many unsuccessful attempts to induce mousy off-flavour in German wines with *B. manitopoeum*, concluded that the off-flavour was not necessarily caused by bacteria but rather, was of purely physico-chemical origin and could be produced under conditions of high redox potential. Sound wines were found to have rH values between 18 to 21 and wines with rH values above 21 initially had an 'air-taste' which then became mousy. He further considered that the off-flavour could be induced or removed by raising or lowering the redox potential over the range of rH 21-25.

The reported physico-chemical origin of mousy off-flavour brought much criticism from Osterwalder (1948a, 1948b; cited by Tucknott 1977). Osterwalder (1948) interestingly pointed out that some non-mousy tastes were often confused with true mousy off-flavour of bacterial origin and, further, that the addition of hydrogen peroxide to wine was also previously known to produce a mousy taste atypical of true mousy off-flavour. He concluded that Schanderl could not differentiate an oxidized off-flavour from true mousy off-flavour.

Moreover, in contrast to Schanderl's view, Osterwalder also observed that the incidence of mousy off-flavour was low whereas the incidence of wine oxidation was widespread.

Further evidence for the role of physico-chemical processes in the formation of mousy off-flavour came, however, from Hennig (1951; cited by Tucknott 1977) who noted that ultrasonic treatment of an off-flavoured Chateau Lafite wine enhanced its mousy taste. Beridze and Kurdelashvili (1956, 1957; cited by Tucknott 1977) found that Gamma radiation of port and Madiera wines led to increased redox potential and subsequent formation of mousy off-flavour. Schanderl (1959; cited by Tucknott 1977) further reiterated his redox theory by demonstrating that a mousy flavour was formed in a wine one to two hours after the addition of hydrogen peroxide. Farkâs (1963; cited by Tucknott 1977) also noted that ultrasonic treatment in the presence of a copper membrane intensified mousy off-flavour, but in contrast to Schanderl and others observed a simultaneous fall in rH value.

Later studies by Unguryan, Ponomarchenko and Parfent-'eva (1968a,b,c; cited by Tucknott 1977) eluded to the combined effects of microbial and physico-chemical processes on off-flavour formation, whereby the formation of mousy off-flavour was often accompanied by a high concentration of LAB and was dependent upon the presence of oxygen (as hydrogen peroxide), wine pH and the concentration of tannins and pigments. The presence of iron was also reported to stimulate off-flavour development. Unguryan *et al.* (1968a) somewhat boldly derived an equation from their work relating off-flavour development to wine pH and to the concentration of dissolved oxygen (peroxide compounds) and of tannins and pigments:

$$I = rH / (tx10)$$

where I = Index of mousiness, (tx10) = sum of tannins and pigments (g/L) x 10.

They further correlated taste panel assessment scores of mousy wines and postulated that off-flavour developed between rH = 20–26 with an index rating upward from 3.7. Optimum physico-chemical conditions necessary for off-flavour formation were generally considered to be exposure to atmospheric oxidation, high pH (ca. 4.0), sufficient 'active' iron content and possibly a low concentration of tannins, pigments and sulfur dioxide. From a seemingly confusing lactic acid 'souring' (acidification) experiment, Unguryan *et al.* (1968b) also studied the effects of inoculated bacteria (LAB) on off-flavour production in wines of different tannin/pigment content and in which off-flavour had already been artificially induced. Their general conclusions were that mousy off-flavour was not a general indication of lactic acid 'souring' but frequently accompanies it because both processes require similar pH conditions. Unguryan and Ponomarchenko (1971; cited by Tucknott 1977) later highlighted the importance of oxidation in the production of off-flavour when considering the peroxidation of

white table wines. They suggested that the risk of oxidation and off-flavour formation could be reduced by preventing MLF in wines with high pH, and allowing three to four months lees contact time at less than 10°C. Reports by Amerine and Joslyn (1951) also suggest the association of mousy off-flavour with high pH wines and oxidative conditions.

Apart from the incorrect association of off-flavour with acetamide (Erckmann 1898, Rodopulo 1952, Dymchisin 1953 and others cited by Tucknott 1977), few suggestions as to the nature and identity of chemical compound(s) responsible for mousy off-flavour were made by early investigators. This was probably due to the unavailability of sufficiently sensitive analytical procedures and equipment at the time. Nevertheless, Villforth (1950; cited by Tucknott 1977) recognized that the mousy off-flavour compound was steam volatile at atmospheric pressure and that it was not an ester, since saponification of mousy distillates enhanced its odour. He further suggested that the causative compound could be a polymer of acetaldehyde or formaldehyde after noting that the off-flavour became weaker after the removal of aldehydes with 2,4-dinitrophenylhydrazine. Similar conclusions as to the aldehydic nature of mousy off-flavour were also reached by Schanderl (1948) and Farkâs (1963) after noting its binding capacity with sulfur dioxide. Unguryan *et al.* (1968c) further considered the off-flavour to be caused by a chemically unstable, unknown nitrogenous substance ($R\text{-}NH_2$) at extreme rH values.

Several methods have been proposed for the removal of mousy off-flavour from wine, most of which are suggested by supporters of the physico-chemical induction of off-flavour and claim that it can be removed by reversal or elimination of the physico-chemical conditions which cause it, ie. high redox potential, and/or presence of metal ions. There is little evidence in the literature, however, of methods to remove off-flavour from wines in which it has been induced through microbiological spoilage.

Schanderl (1959) suggested that slight mousiness could be removed by reduction processes, or for more severe cases by the addition of large amounts of sulfite or nascent hydrogen. Other reports confer with the use of sulfite for off-flavour reduction or removal, although Schanderl (1959) further proposed reducing redox potential and hence elimination of the off-flavour by inoculating the film yeast *Candida mycoderma* . In this study the redox potential of a wine was raised to rH 28 with hydrogen peroxide, which induced a strong mousy flavour. Twenty-eight days after inoculation with *Candida valida*, the wine rH was reduced to 19 with concomitant disappearance of mousy flavour. Although this experiment successfully eliminated mousiness from wine, other undesirable spoilage products associated with the growth of *Candida valida* would be expected to be produced, eg. high volatile acidity. A wine yeast strain with desirable winemaking properties would perhaps be more appropriate if the wine base was sweetened with grape juice prior to inoculation.

Unguryan *et al.* (1968) also suggested the use of sulfur dioxide as a reducing and binding agent to remove mousiness from dry wines. Moreover, in contrast to Schanderl's approach to lower the redox potential by growth of *Candida valida*, Unguryan *et al.* (1968) advocated the use of fresh yeast sediment, although the mechanisms of this process are unclear. Addition of 5-15% sediment was suggested, after which the wine is either left to stand or heated to 40-45 °C in a pasteurizer and allowed to cool naturally. No indication is made, however, as to how long the wine should be left in contact with sediment before mousiness disappears. Another method was claimed by Unguryan *et al.* (1968) to eliminate mousy off-flavour from three commercial wines by firstly, lowering their iron content with ferrocyanide, adding bentonite, filtering, further sulfiting and finally pasteurizing at 65°C.

Some workers have reported the development and use of ion exchange resins for the removal of mousy off-flavour. Farkâs' (1963) consideration that the mousy compound to be a nitrogenous compound ($R\text{-}NH_2$) led to the suggestion that off-flavour could be removed by the addition of the H^+ form of a cation exchange resin to off-flavoured wine. By this process reduction of the nitrogenous base to $-NH_4^+$ ions which were exchanged for H^+ ions, or complete absorption of the $R\text{-}NH_2$ with the resins $-SO_3H$ groups was thought to occur. Farkâs (1963) further suggested that mousy wines which were heavily sulfited required sequential cation, anion, cation exchange resins and, importantly, for industrial application anion followed by cation exchange without heavy sulfiting. Mousy wines treated by these ion exchange methods were graded organoleptically as not off-flavoured by a taste panel. As an extension of Farkâs (1963) procedure for the elimination of mousy off-flavour, it is possible to consider the use of a basic anion exchange resin in the bisulfite form similar to that described by Williams *et al.* (1981) for the removal of carbonyls in the deodorization of grape marc spirit. Another ion exchange method for the removal of mousy off-flavour was reported by Nilov *et al.* (1971; cited by Tucknott 1977). They suggest preparing a fibre filter ion exchanger by treating cellulose tissue, gauze or cardboard with orthophosphoric acid and urea. This exchange material was claimed to be advantageous, because it had optimal porosity and exchange properties, was biologically inert, and did not affect the flavour and bouquet of the product. Significantly, this exchange procedure prevented recurrence of the off-flavour by removing heavy metal ions. The exchange material could be regenerated with 0.5% hydrochloric acid. Further, exchange material in the salt form did not remove the mousy compound, suggesting it exists as a zwitterion.

Walter (1928; cited by Tucknott 1977) claimed that, in contrast to reductive processes, strong oxidation by ozone treatment through the use of a Siemans Ozonizer successfully and permanently removed mousy off-flavour from wine without any deleterious effect on wine quality. Although no other reports of ozonolysis have been made in this regard, this finding concurs with other reports (Unguryan *et al.* 1968b) indicating the disappearance of off-flavour

after further oxidation of wines in which off-flavour had been artificially induced. Another procedure advocated to remove mousy off-flavour is fortification with alcohol. Episkoposyan (1959; cited by Tucknott 1977) found that off-flavoured wine fortified to 15% v/v showed no change in flavour after 180 days, but when raised to 20%, 25% and 30% v/v alcohol, the mousy flavour disappeared after 60, 30 and 20 days, respectively. This effect was more rapid in oak casks than in glass bottles, but pasteurization or acidification with 1g/L citric acid did not affect the disappearance time. Episkoposyan (1959) concluded that fortification to 30-35% v/v alcohol as a possible method to remove mousy off-flavour and, after the off-flavour has disappeared, that such wines could be blended with ordinary strong and dessert wines without recurrence of mousiness.

Later studies by Tucknott (1977) also recommend that a secondary yeast fermentation be further investigated for the removal of mousy off-flavour. Although no detail was provided, a possible microorganism for this role would be an appropriate winemaking yeast strain (*Saccharomyces* spp.). Such wine yeast are well known for their ability to reduce many carbonyl compounds (eg. diacetyl, acetaldehyde) and related compounds. In an analogous situation, Umezo *et al.* (1979) investigated the oxidative degradation of biogenic amines by nitrate reducing bacteria and lactobacilli in *saké* brewing. From this approach it is also feasible that certain strains of LAB could be used for the removal of mousy compounds from wine.

(ii) Recent studies on the nature and origin of mousy off-flavour

Greater progress has been made towards elucidating the cause and nature of mousy off-flavour since the mid-1970's with the availability use of improved analytical techniques. Two of the recent investigators were Tucknott from the UK and Heresztyn and co-workers from the Australian Wine Research Institute, who utilised microbiological skills in combination with more advanced analytical techniques to gain greater knowledge of this spoilage phenomenon. This next section summarises the latter more recent studies on mousy off-flavour.

Significant advancement in mousy off-flavour research came from a major study by Tucknott (1977) who examined three related aspects of mousy off-flavour in fermented beverages (cider), namely, its perception by tasters, the role of microorganisms and its chemical nature. Preliminary surveys by Tucknott revealed that the off-flavour occurred in wines from numerous countries including Germany, France, Switzerland, Hungary, Yugoslavia, Malta, USA, South America, and Australia. Further taste panel assessment of mousy wines demonstrated that there was considerable variation in sensitivity to the off-flavour between tasters, from extremely sensitive to seemingly anosmic or odour blindness. Similar findings

were also noted much earlier by Müller-Thurgau and Osterwalder (1913). Tucknott demonstrated that this variability could be related to the pH of the tongue surface or saliva, whereby panelists with low saliva / tongue surface pH had less perception of the off-flavour than those with higher saliva / tongue surface pH. This effect was thought to be due to the release of the volatile compound at higher pH from an odourless bound form at low pH. Anosmia to the off-flavour was suggested to be a possible genetic effect. Panel training increased recognition but not sensitivity to the off-flavour.

Although the cause of mousy off-flavour was much debated, Tucknott regarded the role of microorganisms important, at least in part, since off-flavour could not be induced physico-chemically in unfermented juices. In order to establish more fully the nature of this role, different strains of yeast and bacteria previously isolated from mousy ciders were screened for their ability to produce off-flavour (determined by taste assessment) in juice-based culture media. Of the strains tested only *Brettanomyces anomalus* produced off-flavour whereas LAB and AAB did not, thus conflicting with earlier reports (Müller-Thurgau and Osterwalder 1913 and others) that the latter bacteria were off-flavour-producing organisms. Further investigation by Tucknott led to the major discovery that a mousy taste could be produced by lactobacilli if symbiotically cultured with a fermenting yeast (*Saccharomyces* spp.) and that no off-flavour was produced when these organisms were grown separately. This requirement for yeast in off-flavour formation by lactobacilli was found to be caused by ethanol, hence explaining the exclusive occurrence of off-flavour in fermented wines and not in juices. Incorporation of ethanol (or propanol) into a juice-based culture medium similarly facilitated off-flavour production by lactobacilli and served as a convenient method for further screening of LAB in pure culture. It is surprising that Tucknott did not use this test medium to confirm whether or not AAB were able to produce off-flavour. Furthermore, Tucknott was not successful in formulating a totally synthetic medium which facilitated off-flavour formation by LAB and found it necessary to incorporate yeast extract for off-flavour to develop. Such a synthetic medium was, however, developed for *Brettanomyces* spp. and from which it was discovered that the amino acid lysine was a precursor in off-flavour production. Moreover, aerobic growth conditions were also conducive to off-flavour formation.

In addition to the latter microbiological studies, Tucknott examined the chemical nature of mousy off-flavour. Previous studies in this area were limited by the unavailability of suitable analytical techniques and only indicated that the off-flavour compound was possibly aldehydic and/or nitrogenous and displayed the properties of a steam volatile base (Villforth 1951, Farkâs 1963). Tucknott, however, utilized more advanced analytical techniques including gas chromatography (GC), gas chromatography-mass-spectrometry (GC-MS), infra-red (IR) and nuclear magnetic resonance (NMR) spectroscopy and made significant advancement in elucidating the identity of the off-flavour compound. Tucknott initially investigated the compound responsible for the mousy odour of impure acetamide since he considered there

may be similarities between it and the naturally occurring compound causing mousy off-flavour. The isolated impurity had a mousy odour and was identified as 2,4,6-trimethyl-1,3,5-triazine (Figure 2.3) by comparison of analytical data with those of authentic reference material. Other related alkyl substituted s-triazines were also found to be mousy but less intense than 2,4,6-trimethyl-1,3,5-triazine. The triazines were water soluble, weakly basic volatiles, indicating they could exist in an alcoholic beverage either free or as a salt.

Tucknott further examined a considerable range of procedures for extracting and isolating the causative mousy compound from ciders and fermentation media. Preliminary experiments indicated that mousy aroma could be extracted from cider by sequential distillation, strong acidic cation exchange and/or solvent extraction, but this method failed, however, to yield sufficient quantity of compound for instrument detection. Further experiments were thus undertaken to optimize the extraction procedure and interference from organic compounds was also reduced by use of a synthetic medium fermented with *Brettanomyces anomalus* as source material. This medium contained lysine as a sole nitrogen source. One such small-scale fermentation (8L, pH 2.5) was extracted by, firstly, concentration to dryness by rotary evaporation, followed by cation exchange and final continuous liquid/liquid extraction (15 hours). Concentration of the ether phase by distillation gave an extract with a strong mousy and meaty aroma, although Tucknott noted considerable losses may have occurred during this stage. A mousy, biscuity region was also noted in the extract by GC-sniff assessment (Carbowax 20M glass column) but further concentration for GC-MS analysis resulted in poor resolution of peaks and loss of the mousy odour. Interestingly, poor chromatography was considered to be the result of an over-abundance of nitrogenous compounds extracted by ion exchange. Extraction of a second small scale fermentation was therefore attempted in which the ion exchange step was replaced with a series of selective solvent extraction and drying stages. This approach proved the most successful and, together with the use of a superior glass GC column treatment (Ucon Fluid LB550X and KOH), produced a moderate sized mousy smelling peak. Mass spectral analysis of this peak indicated that it matched with 2-ethyl Δ^1-piperideine (2-ethyl-3,4,5,6-tetrahydropyridine) (Figure 2.4) and was subsequently verified as such by comparison with synthesized authentic reference material. This was the first report of the compound occurring in a natural product or processed foodstuff. 2,4,6-trimethyl-1,3,5-triazine was not detected in the extracts.

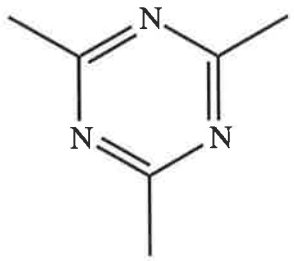


Figure 2.3 Chemical structure of 2,4,6-trimethyl-1,3,5-triazine, the mousy impurity of acetamide.

Tucknott was unable to further confirm the compound's identity by IR and NMR analyses because of insufficient material and, consequently, carried out additional and much larger scale synthetic medium fermentations (450L and 225L) in an effort to achieve this aim. These experiments were unsuccessful, however, mainly due to poor growth of the *Brettanomyces anomalus* yeast and possible losses of the compound during scaled-up extraction and processing procedures. It is interesting, however, that these larger scale extractions produced other mousy smelling regions by GC-sniff assessment, but were not sufficiently concentrated to enable identification. Despite these drawbacks, several interesting and overall conclusions were reached by Tucknott concerning 2-ethyl-Δ^1-piperideine. First, that the odour of pure 2-ethyl-Δ^1-piperideine was dry, green, hemlock-like and becoming strongly mousy only after exposure to air. Furthermore, it was related to the piperidine based alkaloids, eg. coniine (2-propyl-piperidine) and coniceine (2-propyl-Δ^1-piperideine), of which Hemlock (*Conium maculatum L.*) is a major source. This plant was described as having a mousy odour when bruised (Long 1924; cited by Tucknott 1977).

Tucknott's report of the role of 2-ethyl-Δ^1-piperideine as the cause of mousy off-flavour was later disputed by Craig and Heresztyn (1984), who showed a complete absence of this compound in mousy wines from Australia. Craig and Heresztyn (1984) developed a relatively sensitive method of estimating 2-ethyl-Δ^1-piperideine in wine, using more advanced high performance (SCOT) glass GC columns and more sensitive techniques of mass spectrometry (multiple ion detection) than those available to Tucknott. They also adopted a simplified and gentler extraction procedure, ie. concentration (5X) by rotary evaporation and then continuous liquid/liquid extraction in Freon 11 for 24 hours to remove interfering neutral

components, followed by a similar continuous liquid/liquid extraction of the basified aqueous phase and final concentration of solvent. The results of this study were in direct contrast to those of Tucknott whereby 2-ethyl-Δ^1-piperideine could not be detected in the mousy extracts. Craig and Heresztyn further indicated that if this compound was present it could only have occurred at concentrations below the detection limit of 20 µg/L. This concentration was also significantly lower than the flavour threshold of 2-ethyl-Δ^1-piperideine in white wine (0.15 mg/L), also determined by these authors, thus leading them to conclude that mousy off-flavour was possibly caused by other compounds. They did support Tucknott's view, however, that the causative compound was an organic base, since extracts of the basified wines all had a mousy odour. It is somewhat surprising, however, that Craig and Heresztyn did not perform GC-sniff assessment on mousy wine extracts since they used this technique to assess the mousy aroma of synthetic 2-ethyl-Δ^1-piperideine. Such information would have at that time been useful for further identifying the mousy compound from off-flavoured wines.

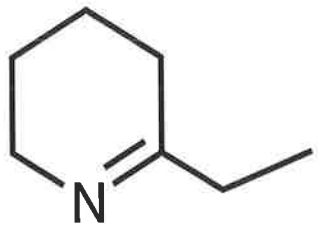
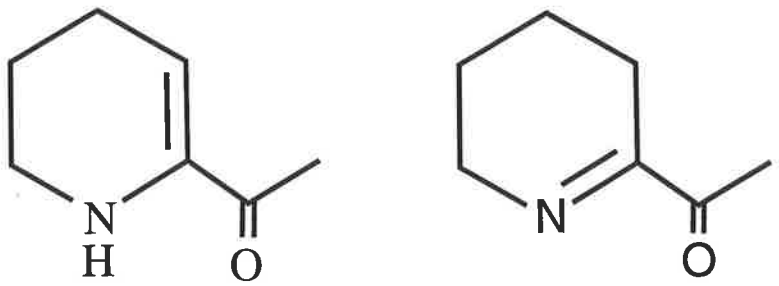


Figure 2.4 Chemical structure of 2-ethyl Δ^1-piperideine identified by Tucknott (1977) as the cause of mousy off-flavour in a synthetic medium fermented by *Brettanomyces anomalus*.

A short time later, workers from the same group (Strauss and Heresztyn 1984) identified the tautomeric 2-acetyl-tetrahydropyridine as a major compound responsible for mousy off-flavour in wine. This study utilized a procedure of strong cation exchange coupled with liquid/liquid extraction using Freon 11 to extract and concentrate mousy organic bases from off-flavoured wines. All other analytical methods were similar to the previous study of Craig and Heresztyn with the exception that a larger wine sample was used ie. 800mL compared to 300 mL. GC-sniff assessment of mousy wine extracts produced two strong mousy smelling regions of the chromatogram which had longer retention times than that of synthetic 2-ethyl Δ^1-piperideine. Significantly, two compounds having similar mass spectra were found in the mousy regions which were consistent with the two tautomeric structures of 2-acetyl-tetrahydropyridine (ACTPY), ie. the enamine 2-acetyl-1,4,5,6-tetrahydropyridine (I) and its imino tautomer, 2-acetyl-3,4,5,6-tetrahydropyridine (II) (Figure 2.5). These identities were confirmed by synthesis of a mixture of authentic compounds (I) and (II) followed by co-chromatography and mass spectral comparison. GC-sniff assessment of the synthetic materials further confirmed their strong mousy odour. Interestingly, injection of a deuterated mixture of synthetic compounds (I) and (II) enabled confirmation that enamine (I) was the first tautomer to elute from the GC column. In conclusion to their study, Strauss and Heresztyn further substantiated the association of ACTPY with mousy off-flavour after reporting its absence from sound wines and from wines exhibiting microbiological spoilage other than mousy off-flavour.

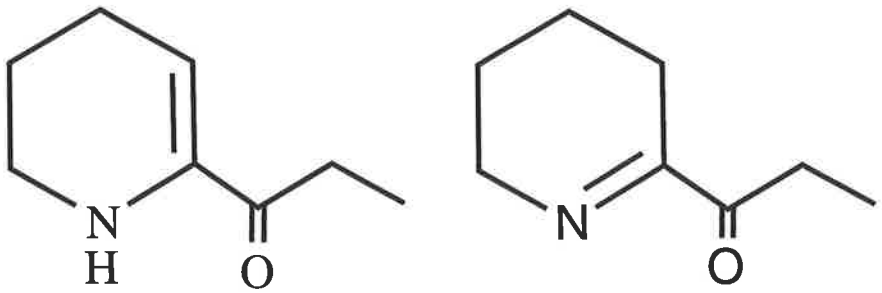
A final study by Heresztyn (1986) examined the formation of mousy off-flavour and substituted tetrahydropyridines by species of *Brettanomyces* and *Lactobacillus* isolated from 10 mousy wines. Preliminary screening revealed that 3 *Brettanomyces* (*Dekkera*) yeasts (*B. intermedius*(a), *B. lambicus* and *B. intermedius*) and 2 lactobacilli (*L. brevis* and *L. cellobiosus*) reproduced the off-flavour (subjectively assessed) after reinoculation into sound wine or growth media. These results generally supported the findings of Tucknott regarding the role of *Brettanomyces* and *Lactobacillus* in the formation of mousy off-flavour, and extended them to reveal other species with this ability. Strong off-flavour producing bacteria (*L. brevis*) and yeast (*B. intermedius*(a)) strains were further studied for the production of off-flavour and also mousy compounds (determined by GC-MS analysis) after culture in, respectively, a synthetic medium and a grape juice medium. The results of these studies were highly significant, demonstrating that both organisms were capable of producing strong off-flavour and relatively abundant levels of ACTPY. Furthermore, *L. brevis* did not produce off-flavour or ACTPY tautomers in the absence of ethanol. Substitution of ethanol with propanol in the *L. brevis* culture medium, however, resulted in the recurrence of off-flavour together with the novel formation of propionyl analogues of the two ACTPY tautomers, ie. 2-propionyl-1,4,5,6-tetrahydropyridine and 2-propionyl-3,4,5,6-tetrahydropyridine (Figure 2.6). GC-sniff assessment of a synthetic preparation of the two propionyl tetrahydropyridines confirmed their mousy aroma properties. Incorporating 5% v/v n-

propanol into the medium fermented with *B. intermedius* (a) similarly lead to the formation of the two propionyl tetrahydropyridines, thus confirming the essential role of ethanol in the synthesis of ACTPY by both yeast and bacteria. Heresztyn also found that formation of ACTPY by *B. intermedius* (a) was dependent upon the presence of lysine and that substitution of lysine with proline did not result in a mousy product.



I **II**

Figure 2.5 Tautomers of 2-acetyl-tetrahydropyridine (2-acetyl-1,4,5,6-tetrahydropyridine (I) and 2-acetyl-3,4,5,6-tetrahydropyridine (II)) identified by Strauss and Heresztyn (1984) as a cause of mousy off-flavour in wine



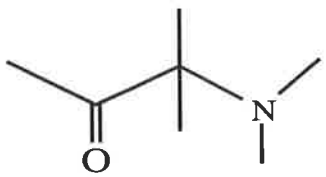
III **IV**

Figure 2.6 Tautomers of 2-propionyl-tetrahydropyridine (2-propionyl-1,4,5,6-tetrahydropyridine (III) and 2-propionyl-3,4,5,6-tetrahydropyridine (IV)) produced by *Brettanomyces intermedius* (a) and *Lactobacillus brevis* (Heresztyn 1984)

Overall, Heresztyn extended Tucknott's conclusions regarding the roles of ethanol and lysine in the formation of mousy off-flavour to implicate these compounds as precursors of ACTPY. However, a major weakness of this work was the absence of quantitative data and use of different extraction procedures for bacteria (ion exchange) and yeast (liquid/liquid) cultures. Correction of these limitations would enable quantification of ACTPY in wines and culture media and also allow comparative assessment of ACTPY production by different microorganisms. Furthermore, a deficiency of both Heresztyn's and Tucknott's work was the lack of a synthetic culture medium for study of ACTPY production by lactobacilli, which precluded them from determining amino acid and other nutritional precursors in the synthesis of ACTPY by these organisms. Amendment of the latter shortcomings would therefore be objectives for future studies on bacteriological formation of mousy off-flavour.

2.3 Occurrence of 2-acetyltetrahydropyridine and related compounds in other foods

In contrast to the undesirable sensory property associated with ACTPY in wine spoiled by mousy off-flavour, this compound forms part of the 'cracker-like' group of odour compounds which include 2-acetylpyridine, 2-acetylpyrazine (Teranishi *et al.* 1975) and the lower analogue, 2-acetyl-1-pyrroline (ACPY) (Buttery *et al.* 1982). Folkes and Gramshaw (1981) suggested that heterocyclics with the structural feature:



where the nitrogen atom and the adjacent carbon atom form part of a ring structure, exhibit cracker-like or roasty odours (Schieberle 1991).

Significantly, numerous reports have identified ACTPY and/or ACPY and related compounds of this group as potent and desirable odorants of a variety of cereal-based and other processed foods. Hunter *et al.* (1969) identified ACTPY as an important aroma component of baked bread (crust), although more recent studies have shown that ACPY is a major aroma compound ('roasty') of wheat bread crust while ACTPY contributes to a lesser extent (Schieberle and Grosch 1983, 1985, 1987a, 1987b, 1994, Schieberle 1990b). 2-Acetyl-1-pyrroline is also a major aroma component ('popcorn-like') of cooked rice and is important to

the aroma of the more aromatic rice varieties (Buttery *et al.* 1982, 1983b, 1988). Furthermore, both ACPY and ACTPY, as well as 2-propionyl-pyrroline are primary odorants of popcorn (Schieberle 1991), while ACTPY, 2-acetylpyridine and 2-propionyl-tetrahydropyridine have been isolated from malt and beer (Tressl *et al.* 1981a). These and other examples of the latter compounds contributing 'cracker-like' and similar 'roasty' aromas to foods are summarized in Table 2.3.

The potency of ACPY, ACTPY and related compounds is reflected in their extremely low odour threshold values, which are summarised in Table 2.4. It is worth noting that higher homologues of ACPY and 2-propionyl-pyrroline, ie. 2-butanoyl- and 2-hexanoyl-1-pyrroline, have been reported to have a 10^5 higher odour threshold value than the latter (Schieberle 1991). Teranishi *et al.* (1975) also note that odour thresholds of the 'cracker-like' compounds in general could be influenced by ring structure. Another feature of ACTPY is that it is an unstable compound, being very sensitive to air (Büchi and Wüest 1971) and decomposing even at low temperature to give several of products including 2-acetylpyridine (Buttery *et al.* 1971). Buttery *et al.* (1988) similarly indicate ACPY to be unstable. These compounds also appear to be highly reactive. Helak *et al.* (1989) have demonstrated ring enlargement reactions leading to formation of 3-substituted 7*H* -cyclopenta[b]pyridin-7-ones as Maillard reaction products after aldol condensation of ACTPY with aldehydes. The susceptibility of monomeric piperideine and pyrrolidine molecules to trimerization (Bock and Dammel 1987, De Kimpe and Stevens 1993, De Kimpe *et al.* 1993) further suggests that ACPY and ACTPY analogues may undergo similar polymerization reactions. The sensory qualities of these end-products would be negligible in comparison with the parent/precursor compounds.

Table 2.3 . Occurrence of 2-acetyltetrahydropyridine and related 'cracker-like' odour compounds in foods and other sources

Source	Odour compound	Odour description	Reference
Wheat bread crust	2-acetyl-1-pyrroline*	Roasty, cracker-like	Schieberle (1990b)
	2-acetyl-tetrahydropyridine	Roasty	Schieberle and Grosch (1987a,b,)
		Cracker-like	Hunter *et al.* (1969)
Rye bread crust	2-acetyl-tetrahydropyridine	Cracker-like	Schieberle and Grosch (1985)
	2-acetyl-1-pyrroline		Schieberle and Grosch (1987b)
	2-acetylpyridine	Crust-like	Schieberle and Grosch (1983, 1985)
Popcorn	2-acetyl-tetrahydropyridine*	Roasty	Schieberle (1990b)
	2-acetyl-1-pyrroline*	Roasty, popcorn-like	Schieberle (1990b, 1991)
	2-propionyl-1-pyrroline*	Roasty	Schieberle (1990b)
Cooked and aromatic rices	2-acetyl-1-pyrroline*	Popcorn-like	Buttery *et al.* (1982, 1983b)
Malt, beer	2-acetyl-tetrahydropyridine	Bready, cereal	Tressl *et al.* (1981a)
	2-propionyl-tetrahydropyridine	Bready, cereal	" " "
	2-acetylpyridine	Bready, cereal	" " "
Cooked beef	2-acetyl-1-pyrroline	Roasty	Gasser and Grosch (1988)
Potato chips	2-acetylpyridine	Cracker	Buttery *et al.* (1971)
Cupuaçu fruit (*Theobroma grandiflorum* Spreng.)	2-acetyl-1-pyrroline†	Bready	Fischer *et al.* (1994)
Pandan leaves (*Pandanus amaryllifolius* Roxb.)**	2-acetyl-1-pyrroline	Scented rice	Buttery *et al.* (1983a)

* Primary odorant of the food material
† Presence of 2-acetyl-1-pyrroline was reported to be thermally related after simultaneous steam distillation extraction
** Pandan leaves are a natural source of 2-acetyl-1-pyrroline (Buttery *et al.* 1983a)

Table 2.4 Odour threshold values of 2-acetyl-tetrahydropyridine and related 'cracker-like' odour compounds.

Compound	Odour threshold values			
	Water (μg/L)	Reference	Air (ng/L)	Reference
2-acetyl-1,4,5,6-tetrahydropyridine	1.6	Teranishi *et al.* (1975)	0.06	Schieberle and Grosch (1991)
2-acetyl-1-pyrroline	0.1	Buttery *et al.* (1983b)	0.02	Schieberle (1991), Schieberle and Grosch (1991)
2-propionyl-tetrahydropyridine	-	-	0.02	Schieberle (1991)
2-acetylpyridine	19	Teranishi *et al.* (1975)	-	-

Unlike the microbiological formation of mousy N-heterocycles in alcoholic media (Tucknott 1977, Heresztyn 1986), the 'cracker-like' N-heterocycles in the aforementioned food systems are thermally generated minor end-products of the Maillard reaction. This is a non-enzymatic browning reaction which involves Strecker degradation of amino acids with a reducing sugar (carbonyl compound). Hunter *et al.* (1971, 1973) produced US patents describing the synthesis of ACTPY from the reaction of proline and dihydroxyacetone, and suggested its usefulness in flavoring bread and other bakery products. Tressl *et al.* (1981b) also reported the thermal generation of ACTPY and a range of other bread-, cracker-like and similar aromas in various model solutions of proline (and hydroxyproline) and reducing sugars. Schieberle (1990c) later found that ACTPY and ACPY were thermally generated in model systems containing, respectively, proline/fructose and ornithine/fructose.

The reasons for the apparent disparity (desirability or undesirability) in the sensory properties ascribed to ACTPY in wine, compared to that of other food systems described above are not known. However, variations in the sensory properties and perception of these N-heterocycles may arise from different food matrixes. Furthermore, Bartowsky and Henschke (1995) recently indicate that while some wine tasters recognize mousy off-flavour as mouse urine, others may perceive it as 'cracker biscuit', thus highlighting the differences that may arise between individuals in the perception of these compounds.

With the knowledge that a range of structurally similar N-heterocycles exhibit cracker-like sensory properties, further investigations of the formation of mousy off-flavour in wine should also consider the potential occurrence of N-heterocycles other than ACTPY which may also contribute to this wine spoilage. Furthermore, the general lack of knowledge concerning the abilities of wine bacteria to produce mousy off-flavour and the causative mousy compounds requires more rigorous screening of these organisms for this metabolism. To achieve these aims, a sensitive procedure for the extraction and quantitation of mousy compounds from wine is required. In addition, study of the substrates, precursors and possible pathway(s) involved in mousy off-flavour metabolism by wine bacteria also necessitates the development of appropriate microbiological techniques using chemically-defined test media.

3. SURVEY OF WINE LACTIC ACID BACTERIA FOR PRODUCTION OF MOUSY OFF-FLAVOUR

3.1 INTRODUCTION

As described in the Literature Review, Tucknott (1977) and Heresztyn (1986) demonstrated that certain *Lactobacillus* spp., including strains of *L. brevis* , *L. hilgardii* and *L. cellobiosus*, were capable of producing mousy off-flavour in ethanolic media. It remains unknown, however, as to how widespread the ability to produce mousy off-flavour is amongst wine lactobacilli, and whether this phenomenon is strain and / or species dependent. Moreover, it is also unknown whether *O. oeni*, the main bacterium responsible for MLF, or other wine LAB such as Pediococci could also be a cause of mousy off-flavour. Identification of those LAB which possess the potential to produce mousy off-flavour would be of considerable benefit to the wine industry and, furthermore, provide fundamental information for further study of factors which govern off-flavour production.

In this chapter, strains of representative genera and species of wine LAB were surveyed for the ability to produce mousy off-flavour during growth in a range of undefined test media, including a grape juice medium and various wine-based media. In order to avoid tasting the mousy off-flavour in bacterial culture media, qualitative assessments of mousy off-flavour were performed using an alkaline test strip procedure (Heresztyn 1986, Costello *et al.* 1993, Grbin *et al.* 1996). By this method, mousy off-flavour components were rendered volatile, and hence could be detected as a mousy odour by sniffing the test strips.

3.2 MATERIALS AND METHODS

3.2.1 Bacteria strains and method of preculture

Strains of LAB used in experiments described in this chapter are listed in Table 3.1. In the first experiment, one strain of *Leuconostoc mesenteroides*, 6 strains of *O. oeni*, 10 strains of *Pediococcus* spp. and 17 strains of *Lactobacillus* spp. were screened for the production of mousy off-flavour in a grape juice medium. In subsequent experiments, representative strains were selected to further investigate the effects of media composition on the production of mousy off-flavour. In a final experiment, another *Lactobacillus* sp. (*Lactobacillus brevis* IV-1) was included for testing in wine media. This was an important strain to test as it had previously been shown by Heresztyn (1986) to produce mousy off-flavour (and the off-

flavour compound ACTPY) in a grape juice medium. For each experiment, LAB strains were pre-cultured in 10 mL of MRSA medium (see Appendix 3.1 for composition of MRSA medium) for 7 days at 27°C prior to inoculation into test media.

Table 3.1 Species and strains of lactic acid bacteria screened for the production of mousy off-flavour in various media

Genus / species	Strain reference †	Test media††
Leuconostoc mesenteroides	Lc1a	G
Oenococcus oeni	Lc5a	G,M,C
" "	Lc5b	G,M,C,W
" "	Lc5c	G
" "	Lc5g	G
" "	Lc5p	G
" "	Lc5db	G
Pediococcus damnosus	P1a	G,M,C
" "	P1b	G
" "	P1c	G
Pediococcus pentosaceus	P3a	G
" "	P3b	G
Pediococcus parvulus	P6a	G
" "	P6f	G,M,C
" "	P6h	G
" "	P6k	G
" "	P6m	G
Lactobacillus plantarum	L11a	G,M,C,W
Lactobacillus fermentum	L15a	G
Lactobacillus cellobiosus	L16a	G
Lactobacillus brevis	L17a	G,M,C,W
" "	L17b	G,M,C
" "	L17c	G
" "	IV-1	W
Lactobacillus buchneri	L18a	G
" "	L18b	G
" "	L18c	G
Lactobacillus hilgardii	L21a	G
" "	L21b	G
" "	L21c	G,M,C,W
" "	L21d	G
" "	L21e	G
" "	L21f	G
" "	L21g	G
" "	L21h	G

† All strains used in this chapter were obtained from a culture collection held at the Australian Wine Research Institute, Urrbrae, South Australia.

†† Test media in which strains were screened: G = GJ medium, M = MRS-FMEt medium, C = Carr-MEt medium, W = wine media (see section 3.2.2 for media formulations)

3.2.2 Test media used for screening lactic acid bacteria for the production of mousy off-flavour.

Seven different test media were used for screening strains of LAB for the production of mousy off-flavour. These media, described below, comprised a grape juice medium, two undefined media and four wine-based media, and were collectively referred to as off-flavour-assessment media.

(i) Grape juice medium

Grape juice (GJ) medium consisted of (per litre):

Grape juice (sulfite reduced)	500 mL
Yeast extract	5g
Ethanol (96% v/v, redistilled)	52 mL (5% v/v)
pH	4.5

Doradillo grape juice (1992 vintage), obtained from Berri Renmano Estate, Renmark, South Australia, was used in the preparation of GJ medium and was selected because of its neutral sensory (aroma and flavour) properties. Prior to the preparation of GJ medium, H_2O_2 (3% v/v stock) was sequentially added to the Doradillo juice until the concentrations of free and total sulfur dioxide were reduced to 0 mg/L and less than 10 mg/L, respectively. The chemical properties of the Doradillo juice are shown in Table 3.2.

Table 3.2 Chemical properties of Doradilla grape juice* used in the preparation of grape juice (GJ) medium.

Property	Value
pH	3.26
Free SO_2	0 mg/L
Total SO_2	7 mg/L
Sugar (glucose plus fructose)	186 g/L

* H_2O_2 (3% v/v) added to reduce the concentrations of free and total sulfur dioxide

(ii) Modified de Man, Rogosa and Sharpe (MRS-FMEt) medium

de Man, Rogosa and Sharpe medium was modified by the addition of fructose, L-malic acid and ethanol (MRS-FMEt medium) and consisted of (per litre):

Component	Amount
Yeast extract (Oxoid)	4g
Bacteriological peptone (Oxoid)	10g
Lab Lemco powder (Oxoid)	8g
D-Glucose	20g
D-Fructose	10g
L-Malic acid	3.0g
Tween 80	1mL
Sodium acetate tri-hydrate	5g
Tri-ammonium citrate	2g
K_2HPO_4	2g
$MgSO_4.7H_20$	0.2g
$MnSO_4.4H_20$	0.05g
Ethanol (96% v/v, redistilled)	52 mL (5% v/v)
pH	4.5

(iii) Modified Carr (Carr-MEt) medium

A modification of a medium described by Carr *et al.* (1976), Carr-MEt medium, consisted of (per litre):

Component	Amount
Yeast extract (Oxoid)	4g
Casamino acids (Difco)	5g
D-Fructose	50g
L-Malic acid	5g
Citric acid	2g
KH_2PO_4	5.5g
KCl	4.25g
$CaCl_2.2H_2O$	1.66g
$MgSO_4.7H_2O$	1.25g
$FeSO_4.7H_2O$	0.043g
$MnSO_4.H_2O$	0.025g
Tween 80	1mL
Ethanol (96% v/v, redistilled)	52 mL (5% v/v)
pH	4.5

(iv) Wine media

Four different wine media were prepared using four commercially vinified table wines. The wines were selected from a range of seventeen white wines and one red wine on the basis that they did not exhibit mousy off-flavour (determined by the alkaline paper test strip method) or other sensory properties which could potentially interfere with mousy off-flavour assessment (eg. oxidative aromas). The range of wines assessed included three sparkling wine base blends and a sultana wine, which were obtained in bulk (20L, 1992 vintage) from wine companies in Victoria and South Australia. The other thirteen wines were purchased from a local retail outlet as packaged products and comprised the varieties Rhine Riesling, Chenin Blanc and Shiraz-Cabernet blends. Prior to selection, fourteen wines required the addition of H_2O_2 (3% v/v stock) to reduce the concentration of sulfite to a range which would not suppress the growth of LAB, that is, less than 30 mg/L total SO_2 and 0-2 mg/L free SO_2. However, an apparent mousy off-flavour rapidly developed in seven of these peroxide-treated wines (See Literature Review for further information on the induction of mousy off-flavour by chemical oxidation), thus rendering them unsuitable for use. From the eighteen wines that were assessed, three wines did not require peroxide addition and were used in wine media: a Chardonnay-Pinot Noir sparkling wine base, a Crouchen (preservative-free) wine and Rhine Riesling (preservative-free) wine. A peroxide-treated Rhine Riesling wine which did not exhibit mousy aroma was used as a fourth wine in wine media. Some properties of the wines selected for use in wine media are listed in Table 3.3.

Table 3.3 Properties of commercial white wines used in the preparation of wine media

Wine	Free sulfur dioxide (mg/L)	Total sulfur dioxide (mg/L)	Alcohol (% v/v)	Level of mousy off-flavour *
Rhine Riesling (H_2O_2 treated)	0	30	11.3	0
Chardonnay-Pinot Noir sparkling wine base	2	22	11.5	0
Crouchen (preservative free)	0	2	12.2	0
Rhine Riesling (preservative free)	0	22	10.9	0

* Level of mousy off-flavour determined by three or more assessors using the alkaline paper strip method. Off-flavour levels: 0 = no off-flavour; 1 = weak; 2 = moderate; 3 = strong.

Each wine medium was adjusted to a final alcohol concentration of 5% v/v by appropriate dilution with Milli-Q purified water, and to a pH value of 4.5 in order to standardise these parameters with the first three test media. Further, each wine was supplemented with a synthetic medium base consisting of carbohydrates, amino acids, vitamins and other components (Table 3.5) to stimulate the growth of LAB. This supplement was based upon the components of a synthetic medium described by Lonvaud-Funel *et al.* (1988) for the culture of LAB, with the exception that the final concentrations of glucose and fructose were respectively increased to 5 g/L and 7.5 g/L to encourage more profuse cell growth, and the concentrations of the remaining compounds were reduced to compensate for the presence of these compounds derived from wine. Tween 80 was also added to the synthetic supplement. The four wine media are listed in Table 3.4.

Table 3.4 Wine-based test media used in the assessment of mousy off-flavour production by LAB

• Chardonnay-Pinot Noir wine medium
• Rhine Riesling wine medium
• Rhine Riesling (sulfite reduced) wine medium
• Crouchen wine medium

Table 3.5. Composition of synthetic medium (adapted from Lonvaud-Funel *et al* . 1988) used for the nutritional supplementation of wine media

Compound	Concentration†	Stock solution
Sugars	**(g/L)**	
Glucose	5.0	
Fructose	7.5	
Organic acids	**(g/L)**	
L-malic	1.5	
Citric	0.1	20x, H_2O
Succinic	0.3	
L-Tartaric	1.5	
Mineral salts	**(g/L)**	
A.		
KH_2P0_4	0.75	66.67x, H_2O
K_2HP0_4	0.75	
B.		
$MgSO_4.7H_20$	0.1	
$MnSO_4.4H_2O$	0.005	200x, H_2O
$FeCl_3$	0.001	
NaCl	0.005	
Amino Acids	**(mg/L)**	
A.		
L-Alanine	50	
L-Arginine	50	
Asparagine	50	
Cysteine	50	
Glycine	50	
L-Histidine	50	
L-Isoleucine	50	
L-Leucine	50	
L-Lysine	50	10x, H_2O
L-Methionine	50	
L-Phenylalanine	50	
L-Proline	50	
L-Serine	50	
L-Threonine	50	
L-Tryptophan	50	
L-Valine	50	
B.		
L-Glutamic acid	50	
C.		
L-Tyrosine	50	

Compound	Concentration†	Stock solution
Vitamins	**(mg/L)**	
A.		
Thiamin.HCl	0.112	
Riboflavin	0.100	
Pyridoxine.HCl	0.122	
Pantothenic acid calcium salt	0.109	100x H_2O
Nicotinic acid	0.100	
p-aminobenzoic acid	0.100	
meso-Inositol	10.00	
B.		
Folic acid	0.005	1,000x 1N $NaHCO_3$
C.		
d-Biotin	0.004	1,000x H_2O
D.		
Cyanocobalamine	0.00025	100,000x H_2O
Purines and pyrimidines		
A.		
Guanine	5	100x 1N KOH
B.		
Adenine	5	
Uracil	5	0.1N KOH
Thymidine	5	
Xanthine	5	
Other components		
Tween 80	0.5mL	

† Concentrations given are expressed as final concentrations in the wine media

3.2.3 Media sterilisation

All off-flavour assessment media described in this chapter were sterilised by filtration with a 0.22 μm sterile membrane filter and stored at 2 °C until required.

3.2.4 Inoculation and incubation of off-flavour-assessment media

Aliquots (30 mL) of off-flavour assessment media were dispensed into 50 mL sterile glass screw cap culture bottles. Off-flavour-assessment media were then inoculated (2% v/v) with respective LAB strains which had been precultured in MRSA medium for 7 days at 27 °C. Experiments undertaken in GJ, MRS-FMEt and Carr-MEt media utilised single test cultures, while those undertaken in wine media were performed in duplicate. Inoculated media were incubated statically and aerobically at 22 - 23 °C. Samples (1 - 2 mL) were taken periodically to test for the production of mousy off-flavour, cell growth and in some cases degradation of L-malic acid.

3.2.5 Qualitative assessment of mousy off-flavour by alkaline test strip

Mousy off-flavour was determined subjectively by assessing the aroma of an alkaline paper test strip which had been dipped into samples (1mL, clarified by centrifugation 30 sec at 10,000 x *g* using a bench-top centrifuge) of culture media (Heresztyn 1986, Costello *et al.* 1993, Grbin *et al.* 1996). Alkaline paper test strips were prepared from chromatography paper which had been soaked in 0.1N NaOH, dried in a warm air oven and cut into strips of approximately 0.5 x 7 cm. Chromatography paper was preferred over filter paper (eg. Whatman No.1) for the preparation of alkaline test strips as it yielded a neutral aroma after dipping into Milli-Q purified water. The latter filter papers, on the other hand, were unsuitable for this purpose since they produced 'wet hessian / cardboard' aromas after dipping into Milli-Q purified water, which could potentially interfere with mousy off-flavour assessment. Moreover, as the experiments of this chapter were of a screening nature and, in most cases contained a large number of samples, a sensory panel was not utilised and off-flavour assessment was generally performed by the author. To assist in standardising off-flavour assessment, an aqueous solution of ACTPY (diluted to a concentration perceivable as mousy) was used as a reference in initial experiments described in this chapter. Further, the assessment of sample test strips was regularly verified by one or more laboratory colleagues familiar with mousy off-flavour, especially in cases where assessment was difficult because of a low level of off-flavour and / or the occurrence of other fermentation aromas.

3.2.6 Chemical and microbiological analyses

The concentration of alcohol in table wines was measured by a Near Infra-red spectrometry (NIRS) procedure as described by Baumgarten (1987), using an Infra-Alyzer 260™ (Bran + Luebbe). This instrument was calibrated for the determination of alcohol in table wines.

Free and total sulfur dioxide were determined by an aspiration method described by Rankine and Pocock (1975).

Growth of LAB strains in off-flavour assessment media was measured by several different means. In initial experiments using Grape Juice medium, growth was monitored by visual assessment of turbidity and, in subsequent experiments using MRS-FMEt and Carr-MEt media, by absorbance (650 nm) using a Beckman (DU-64) spectrophotometer (1cm cell). In the final experiment using four wine media with synthetic supplement, growth was measured by absorbance (650 nm) using a Molecular Devices Kinetic Microplate Reader system and multiwell plates dispensed with duplicate 0.3mL aliquots of culture suspension.

L-malic acid was determined using the enzymatic procedure described by Boehringer Mannheim (Anon. 1995).

3.3 RESULTS

3.3.1 Production of mousy off-flavour by lactic acid bacteria in grape juice medium

The production of mousy off-flavour (as determined by the alkaline test strip procedure) and other aroma characteristics during growth of LAB strains in GJ medium is shown in Table 3.6. The formation of mousy off-flavour over a 13 day period is also presented graphically in Figures 3.1, 3.2 and 3.3. In general, the results show that most *Lactobacillus* spp., *O. oeni* and *Lc. mesenteroides* produced varying levels of mousy off-flavour during growth in GJ medium, whereas negligible off-flavour was detected from test cultures of *Pediococcus* spp. Of the six species of *Lactobacillus* tested (Figure 3.1), the highest levels of off-flavour were produced by *L. hilgardii* and *L. brevis*, whereby seven of the eight *L. hilgardii* strains (L21a, L21b, L21c, L21e, L21f, L21g, and L12h) and two of the three *L. brevis* strains (L17b and L17d) produced moderate to strong levels of off-flavour on one or more occasion during the 13 day test period. In particular, two strains of *L. hilgardii*, L21g and L21c, produced strong off-flavour successively on days 5 and 13. Only one of the *L. hilgardii* strains tested, L21b, yielded a off-flavour level of zero, and this only occurred on one occasion at 13 days (Figure 3.1). The three strains of *L. buchneri* (L18a, L18b and L18c) generally produced weak off-flavour, although no off-flavour was produced by strains L18a and L18b at day 5. *Lactobacillus plantarum* L11a initially produced moderate off-flavour, although subsequently did not produce detectable off-flavour at days 5 or 13. The remaining strains of Lactobacillus, ie. *L. fermentum* L15a and *L. cellobiosus* L16a, did not produce off-flavour at any stage of incubation.

Figure 3.2 and Table 3.6 show that the production of mousy off-flavour was a general feature for *Lc. mesenteroides* and *O. oeni* strains during growth in GJ medium. Of all the LAB strains tested, *Lc. mesenteroides* Lc1a produced the highest overall levels of off-flavour over the thirteen day incubation period. *Oenococcus oeni* generally produced weak to moderate levels of off-flavour over this time, although some strain variation in this ability was exhibited. For example, mousy off-flavour production was not initially observed with strains Lc5b, Lc5p and Lc5a, while one strain (Lc5c) produced strong mousy off-flavour on one occasion at day 13.

In contrast to the results for most *Lactobacilli*, *Leuconostoc* and *Oenococcus* strains, the data show that virtually no mousy off-flavour was detected from the ten *Pediococcus* spp. during culture in GJ medium (Figure 3.3). However, *P. parvulus* P6f produced a weak level of off-flavour on one occasion at day five.

During the assessment of alkaline test strips, other aromas were frequently observed either in addition to, or separate from mousiness (Table 3.6). Of these aromas, that of bready commonly occurred in association with mousiness. Bready, and also beer and honey aromas were detected in addition to mousiness during growth of some *O. oeni* strains. Bready aroma was also detected during alkaline test strip assessment of *P. pentosaceus* P3a, *P. pentosaceus* P3b and *P. parvulus* P6f cultures at 2 days, although at 5 days most *Pediococcus* spp. produced a diacetyl aroma. Further, of the lactobacilli tested, a bready aroma was exhibited by three *L. buchneri* strains (L18a, L18b and L18c) and *L. hilgardii* L21c at two days, and by *L. hilgardii* L21d at day 5, while butyric aroma was detected in three cultures of *L. brevis* at 5 days.

Further to the aromas detected by alkaline test strip described above, mousiness and other fermentation aromas were also observed in the GJ culture medium itself (Table 3.6). For example, the aroma of GJ medium cultured with *Lc. mesenteroides* was predominantly mousy, while aromas occurring with *O. oeni* cultures included mousy, honey, butyric and bready. Diacetyl aroma was initially observed in cultures of *Pediococcus* spp., although other odours including butyric (*P. pentosaceus* P3b, *P. parvulus* strains P6a and P6f), honey (*P. pentosaceus* P3a and *P. parvulus* P6m), toasty (*P. parvulus* P6k) and also mousy (*P. parvulus* P6a) were observed at 13 days. Various fermentation odours were produced by *Lactobacillus* spp. in GJ medium. Most strains of *L. brevis*, *L. buchneri* and *L. hilgardii* initially displayed pungent, butyric aromas, whereas *L. plantarum* L11a exhibited bready aroma, and *L. fermentum* L15a and *L. cellobiosis* L16a both produced honey-like aromas. The aromas produced by respective lactobacilli over subsequent sampling days further diversified to include mousy, as well as apple and acetic.

3.3.2 Production of mousy off-flavour by lactic acid bacteria in MRS-FMEt and Carr-MEt media.

The growth and production of mousy off-flavour and other aroma characteristics of eight strains of LAB in MRS-FMEt and Carr-MEt media are shown in Table 3.7 and Figure 3.4. Studies using MRS-FMEt medium revealed that of the eight strains tested, six strains (*O. oeni* strains Lc5a and Lc5b, *P. cerevisiae* P1a, *P. parvulus* P6f and *L. brevis* L17b, and *L. hilgardii* L21c) exhibited weak to moderate levels of off-flavour production within the first 3 days of growth, and a seventh strain (*L. brevis* L17a), produced moderate off-flavour at 5 days. Only one strain, *L. plantarum* L11a, failed to produce off-flavour at any stage of growth in MRS-FMEt medium. Of the seven strains which produced off-flavour in MRS-FMEt medium, maximum levels of off-flavour were attained by five strains (*O. oeni* strains Lc5a and Lc5b, *P. cerevisiae* P1a, *P. parvulus* P6f and *L. brevis* L17b) at day 3.

Table 3.7 and Figure 3.4 reveal that six of eight LAB strains were capable of producing off-flavour in Carr-MEt medium (*O. oeni* strains Lc5a and Lc5b, *L. brevis* L17a and L17b, *L. hilgardii* L21c, and *L. plantarum* L11a). With the exception of *L. brevis* L17b, which only produced weak off-flavour at day thirteen, these strains generally produced higher levels of off-flavour, that is, strong off-flavour within 3 to 5 days, than the weak to moderate levels produced over the same period of time in MRS-FMEt medium. The two *Pediococci* (*P. cerevisiae* P1a and *P. parvulus* P6f), however, did not produce mousy off-flavour in Carr-Et medium. Moreover, the relatively sufficient growth of strains indicates that the apparent inability to produce off-flavour was not related to their growth response in this medium.

The apparent lack of correlation between cell growth and off-flavour formation mentioned above was further evident in the diverse levels of cell density (absorbance) exhibited by LAB strains at respective stages of maximum off-flavour production. Furthermore, although higher cell densities were produced by LAB during growth in MRS-FMEt medium than in modified Carr medium, the levels of off-flavour produced by most strains were generally lower in MRS-FMEt than in Carr-MEt medium.

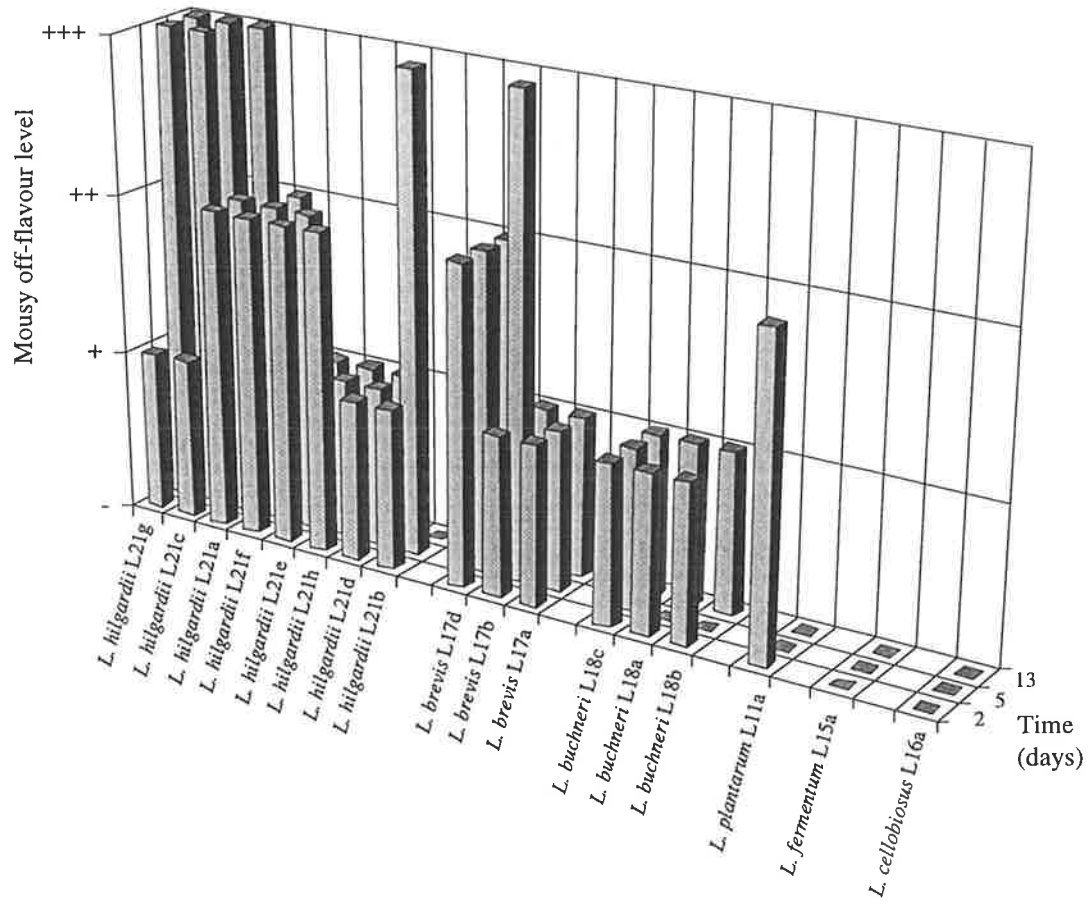


Figure 3.1 Production of mousy off-flavour by *Lactobacillus* spp. during growth in grape juice medium. Mousy off-flavour assessed by the level of mousy odour detected using the alkaline test strip method. Off-flavour level: -, no off-flavour; +, weak; ++, moderate; +++, strong

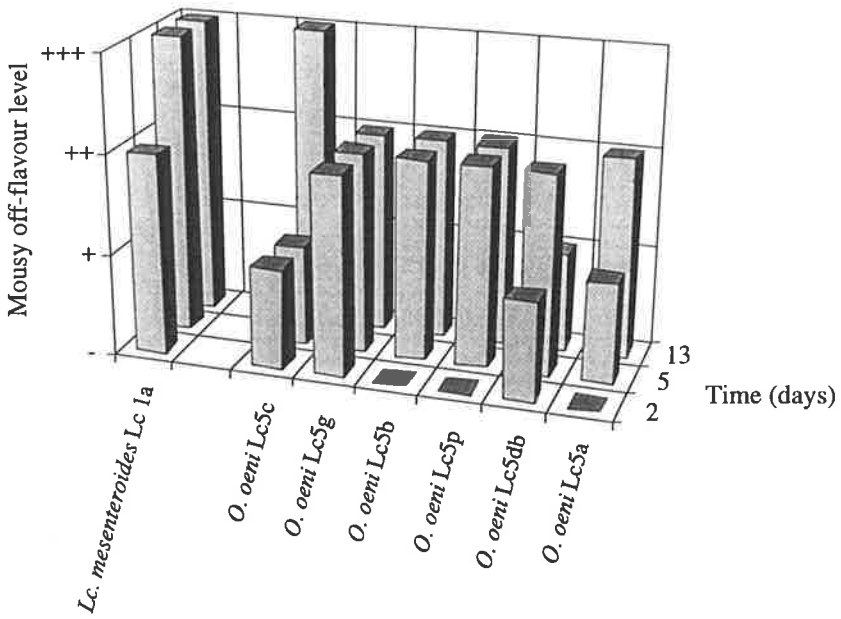


Figure 3.2 Production of mousy off-flavour by *Lc. mesenteroides* and *O. oeni* during growth in grape juice medium. Mousy off-flavour assessed by the level of mousy odour detected using the alkaline test strip method. Off-flavour level: -, no off-flavour; +, weak; ++, moderate; +++, strong

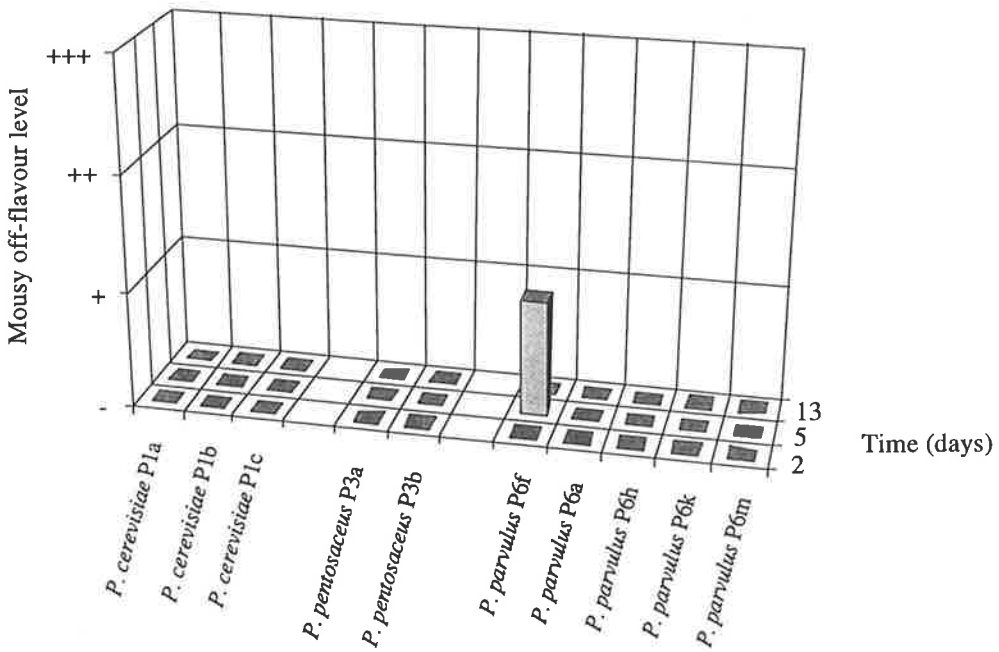


Figure 3.3 Production of mousy off-flavour by *Pediococcus* spp. during growth in grape juice medium. Mousy off-flavour assessed by the level of mousy odour detected using the alkaline test strip method. Off-flavour level: -, no off-flavour; +, weak; ++, moderate; +++, strong

Table 3.6 Production of mousy off-flavour by lactic acid bacteria during growth in grape juice (GJ) medium.

Bacteria strain		Time (days) after inoculation								
		2			5			13		
Genus / species	Strain Ref.	Growth†	Mousy off-flavour††	Aroma of culture medium‡	Growth	Mousy off-flavour	Aroma of culture medium	Growth	Mousy off-flavour	Aroma of culture medium
Leuconostoc mesenteroides	Lc1a	+	++	mousy	+++	+++	mousy	+++	+++	mousy, butyric
Oenococcus oeni	Lc5a	+/-	-	n/d**	++	+ beery, bready	mousy	+++	++	butyric
" "	Lc5b	+/-	-	n/d	++	++	mousy	+++	++	mousy
" "	Lc5c	+	+ bready*	n/d	++	+ sweet, honeyed	sweet, honeyed	+++	+++	mousy, sweet, pungent
" "	Lc5g	+	++	n/d	++	++ sweet, honeyed	sweet, honeyed	+++	++	mousy, sweet, pungent
" "	Lc5p	+/-	-	n/d	++	++	n/d	+++	++	bready
" "	Lc5db	+	+ bready	n/d	+	++	n/d	+++	+	pungent
Pediococcus cerevisiae	P1a	+	-	diacetyl	+	- diacetyl, solvent	diacetyl	++	-	buttery
" "	P1b	+	-	diacetyl	+	- diacetyl, solvent	diacetyl	++	-	buttery
" "	P1c	+	-	diacetyl	+	- diacetyl	diacetyl	++	-	buttery
Pediococcus pentosaceus	P3a	+	- bready	diacetyl	+	- diacetyl	diacetyl	++	-	honeyed
" "	P3b	++	- bready	acetic	++	- diacetyl	diacetyl	+++	-	butyric
Pediococcus parvulus	P6a	+	-	diacetyl	+	- diacetyl	diacetyl	+	-	mousy, butyric
" "	P6f	+	- bready	diacetyl	+	+ diacetyl	diacetyl	+	-	butyric
" "	P6h	+	-	diacetyl	+	- diacetyl	diacetyl	+	-	diacetyl
" "	P6k	+	-	diacetyl	+	- diacetyl	diacetyl	++	-	toasty
" "	P6m	+	-	diacetyl	+	- diacetyl	diacetyl	++	-	honey
Lactobacillus plantarum	L11a	++	++	bready	++	- honeyed	sweet, honeyed	+++	-	sweet
Lactobacillus fermentum	L15a	+	-	honeyed	+++	- sweet, appley	sweet, honeyed	+++	-	mousy, pungent
Lactobacillus cellobiosus	L16a	++	-	honeyed	+++	- sweet, appley	sweet, honeyed	+++	-	acetic
Lactobacillus brevis	L17a	++	+	pungent, butyric	+++	+	butyric	+++	+	butyric
" "	L17b	+	+	pungent, butyric	++	+++	sweet, honeyed	+++	+	butyric
" "	L17d	++	++	pungent, butyric	++	++ butyric, honeyed	n/d	+++	++	mousy
Lactobacillus buchneri	L18a	++	+ bready	pungent, butyric	+++	- butyric	butyric	+++	+	n/d
" "	L18b	++	+ bready	pungent, butyric	+++	- butyric	butyric	+++	+	sweet
" "	L18c	++	+ bready	pungent, butyric	+++	+	appley	+++	+	sweet
Lactobacillus hilgardii	L21a	+	++	mousy, pungent, butyric	+++	++	appley	+++	+++	sweet
" "	L21b	++	+ bready	pungent, butyric	++	+++	bready	+++	-	honeyed
" "	L21c	+	+	pungent, butyric	+++	+++	mousy, appley	+++	+++	mousy, butyric
" "	L21d	+	+	pungent, butyric	+++	+ bready	butyric	+++	+	appley
" "	L21e	+	++	pungent, butyric	+++	++	appley	+++	+	appley
" "	L21f	++	++	pungent, butyric	+++	++	sweet, honeyed	+++	++	n/d
" "	L21g	++	+	honeyed	+++	+++	appley	+++	+++	n/d
" "	L21h	++	++	n/d	+++	+ appley	honeyed	+++	+	buttery
Uninoculated control		-	- grape, neutral	grape, neutral	-	- stewed fruit	stewed fruit	-	-	stewed grape

† Level of growth estimated by visual assessment of culture turbidity: -, no growth; +/-, possible; +, weak; ++, moderate; +++, strong

†† Level of mousy off-flavour determined by aroma using alkaline test strip procedure.
Level of mousy off-flavour: -, none; +, weak; ++, moderate; +++, strong. Perception of mousy off-flavour was assessed with reference to a dilute aqueous solution of ACTPY.

* Other aroma characteristics observed from alkaline test strip are indicated

** n/d: no distinctive aroma observed

‡ Descriptors given to fermentation aromas of culture medium

Of the various fermentation aromas produced by LAB in MRS-FMEt and Carr-MEt media (Table 3.7), that of mousy initially was most evident, either singly or in combination with other aromas, in the latter medium at 3 days with 4 strains (*O. oeni* Lc5a, *O. oeni* Lc5b, *L. plantarum* L11a and *L. brevis* L17a), and also at 13 days with *O. oeni* Lc5a and *L. hilgardii* L21c. Mousy fermentation aroma was also noted, to a lesser extent, in MRS-FMEt medium at three days of growth with *L. brevis* strains L17a and L17b.

3.3.3 Production of mousy off-flavour by lactic acid bacteria in wine media

Four representative LAB strains (*O. oeni* Lc5b, *L. plantarum* L11a, *L. brevis* L17a and *L. hilgardii* L21c) previously shown to produce off-flavour in either GJ, MRS-FMEt or Carr-MEt medium, as well as a fifth strain (*L. brevis* IV-1) that was reported to produce mousy off-flavour in a grape juice medium (Heresztyn 1986), were tested for the production of mousy off-flavour in four wine media (Table 3.8, Figures 3.5 - 3.8). Of the latter strains, *O. oeni* Lc5b and *L. hilgardii* L21c consistently produced weak to strong levels of off-flavour at most sampling periods in each wine medium. Furthermore, *O. oeni* Lc5b produced maximum (strong) off-flavour after 3 days of growth and sustained moderate to strong levels of off-flavour for 10 days in each wine medium. Off-flavour production by *O. oeni* Lc5b declined to weak to moderate at 17 days, and further to within the range of no off-flavour to weak off-flavour at 25 days. A similar pattern of off-flavour production was exhibited by *L. hilgardii* L21c, except that strong off-flavour was attained by most replicates at 5 days in Chardonnay-Pinot Noir and Rhine Riesling (sulfite reduced) wine media, and at 10 days in Crouchen wine medium. Lower maximum levels of off-flavour (weak to moderate) were produced by this strain at days 5 and 10 in Rhine Riesling wine medium. The range of off-flavour levels produced by *L. hilgardii* L21c generally decreased during the final stages of incubation (days 17 to 25), although the off-flavour produced in Chardonnay-Pinot Noir and Rhine Riesling (desulfited) wine media (weak to strong) in this period were generally higher than those of Crouchen and Rhine Riesling wine media (no off-flavour to weak off-flavour).

Although the three remaining Lactobacilli tested in the four wine media (*L. plantarum* L11a, *L. brevis* L17a and *L. brevis* IV-1) exhibited similar patterns of growth to *L. hilgardii* L21c, each strain generally produced no off-flavour or, in some instances, a maximum of only weak off-flavour.

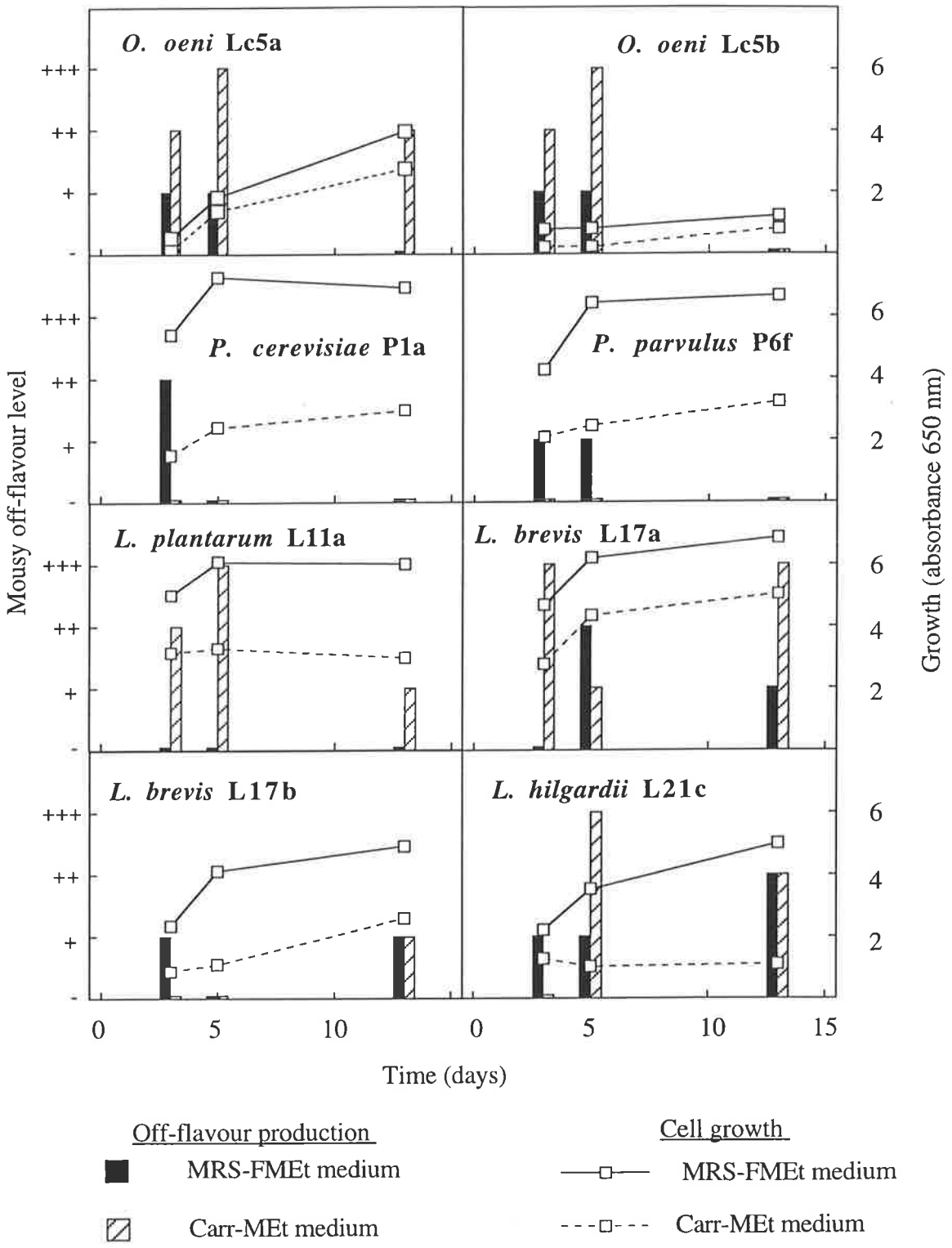


Figure 3.4 . Growth (lines) and production of mousy off-flavour (bars) by lactic acid bacteria in MRS-FMEt medium and Carr-MEt medium. Mousy off-flavour assessed by the level of mousy odour detected using the alkaline test strip method. Off-flavour level: -, none; +, weak; ++, moderate; +++, strong.

Table 3.7 Production of mousy off-flavour and other aroma characteristics by lactic acid bacteria during growth in MRS-FMEt and Carr-MEt media

Medium	Bacteria strain		Time (days) after inoculation: 3			5		13		
	Genus / species	Strain	Growth* (a.u.)	Mousy off-flavour†	Aroma of culture medium††	Growth (a.u.)	Mousy off-flavour†	Growth (a.u.)	Mousy off-flavour†	Aroma of culture medium
MRS-FMEt	*Oenococcus oeni*	Lc5a	0.53	+	n/d**	1.85	+	3.96	-	lactic
	" "	Lc5b	0.80	+	n/d	0.82	+	1.22	-	lactic
	Pediococcus damnosus	P1a	5.42	++	diacetyl	7.27	-	6.93	-	diacetyl
	Pediococcus parvulus	P6f	4.28	+	diacetyl	6.43	+	6.65	-	diacetyl
	Lactobacillus plantarum	L11a	5.01	-	butyric	6.10	-	6.02	-	butyric
	Lactobacillus brevis	L17a	4.68	-	mousy, butyric	6.20	++	6.85	+	butyric
	" "	L17b	2.34	+	mousy, butyric	4.13	-	4.92	+	diacetyl
	Lactobacillus hilgardii	L21c	2.21	+	n/d	3.53	+	5.00	++	diacetyl
	Uninoculated control		0.03	- meaty	strong peptone	0.02	-	0.03	- meaty	meaty
Carr-MEt	*Oenococcus oeni*	Lc5a	0.13	++	mousy	1.40	+++	2.76	++	mousy,butyric
	" "	Lc5b	0.22	++	mousy	0.23	+++	0.80	-	butyric
	Pediococcus damnosus	P1a	1.53	-	n/d	2.42	-	2.96	-	diacetyl
	Pediococcus parvulus	P6f	2.10	-	n/d	2.47	-	3.23	-	diacetyl
	Lactobacillus plantarum	L11a	3.15	++	mousy	3.30	+++	2.98	+	butyric
	Lactobacillus brevis	L17a	2.78	+++	mousy	4.34	+	5.03	+++	butyric
	" "	L17b	0.88	-	pungent	1.11	-	2.60	+	butyric
	Lactobacillus hilgardii	L21c	1.28	-	n/d	1.02	+++	1.11	++	mousy, diacetyl
	Uninoculated control		0.00	-	sl. yeasty, peptone	0.01	-	0.01	- peptone	peptone

* cell growth measured by absorbance at 650 nm, 1 cm pathlength cell. a.u. = absorbance units

† Level of mousy off-flavour determined by the level of mousy odour detected using the alkalkine test strip procedure;
Level of mousy off-flavour: -, none; +, weak; ++, moderate; +++, strong.

†† Fermentation aromas of culture medium determined at days 3 and 13

Figures 3.5 to 3.8 and Table 3.8 also reveal that most LAB strains completely degraded L-malic acid during the growth in each of the four wine media. Exceptions to this were wine media cultured with *L. brevis* L17a, in which L-malic acid was only partially degraded to concentrations in the range 0.39 - 0.71 g/L after 25 days .

In addition to mousiness, aromas detected by alkaline test strip from the four wine media during the growth of LAB ranged from honey and vinous at day 3, to butterscotch and bandaid, as well as bready and cracker between days 5 to 25. Of these, bready and cracker were mainly observed in association with mousy off-flavour.

The range of aromas observed directly from the four wine media during the growth of LAB were similar to the above aromas detected by alkaline test strip and, in addition, included those of creamy, acetic, spicy and butyric. Moreover, mousy aroma, as well as cracker and bready aromas were generally observed directly in wine media cultured with LAB strains which produced weak to strong levels of mousy off-flavour as detected by alkaline test strip.

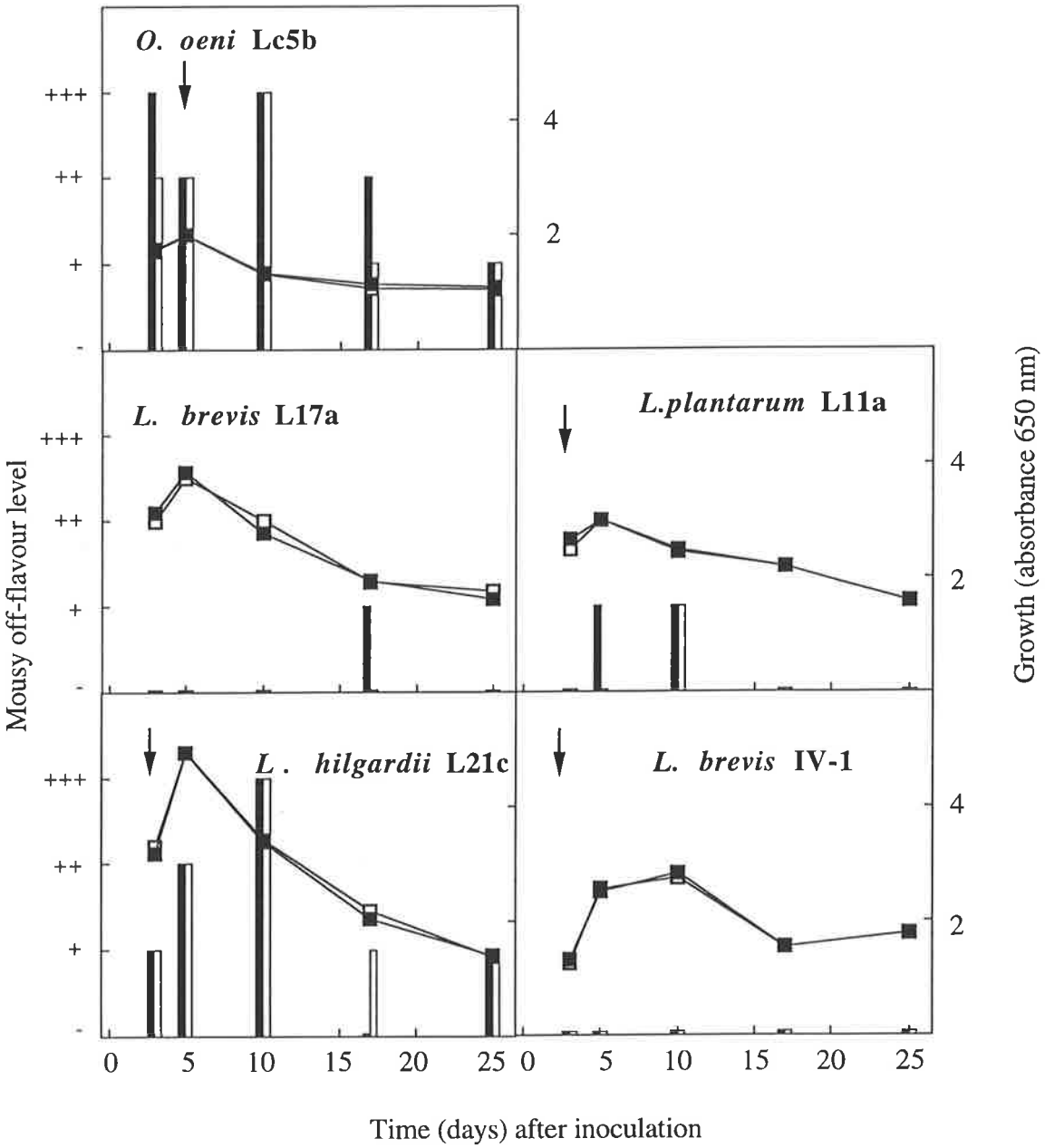


Figure 3.5 Growth (lines) and production of mousy off-flavour (bars) by lactic acid bacteria strains in Crouchen wine medium. Mousy off-flavour assessed by the level of mousy odour detected using the alkaline test strip method. Off-flavour level: -, none; +, weak; ++, moderate; +++, strong. Data from duplicate experiments are presented. Arrows indicate completion of L-malic acid catabolism

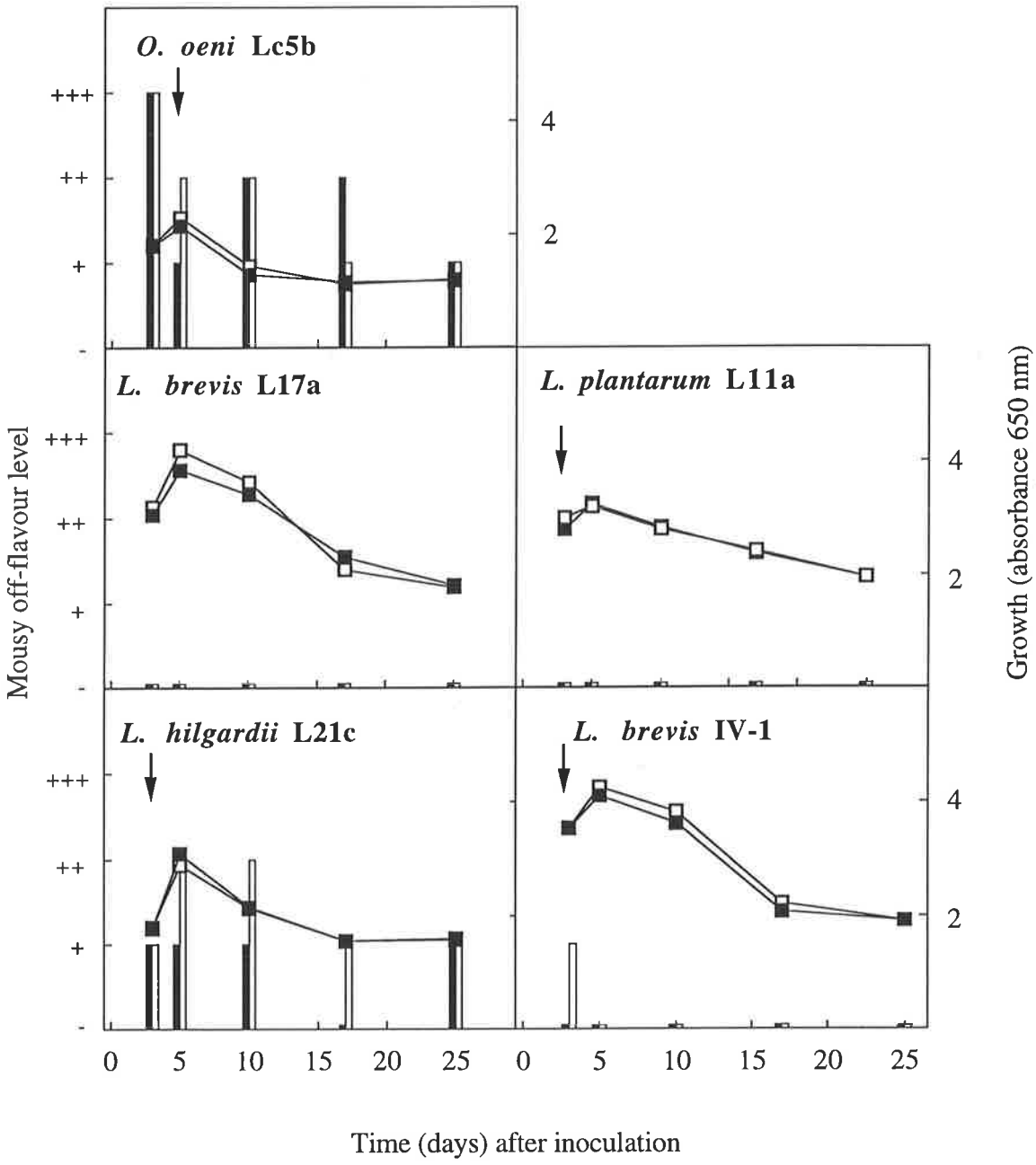


Figure 3.6 Growth (lines) and production of mousy off-flavour (bars) by lactic acid bacteria strains in Rhine Riesling wine medium. Mousy off-flavour assessed by the level of mousy odour detected using the alkaline test strip method. Off-flavour level: -, none; +, weak; ++, moderate; +++, strong. Data from duplicate experiments are presented. Arrows indicate completion of L-malic acid catabolism

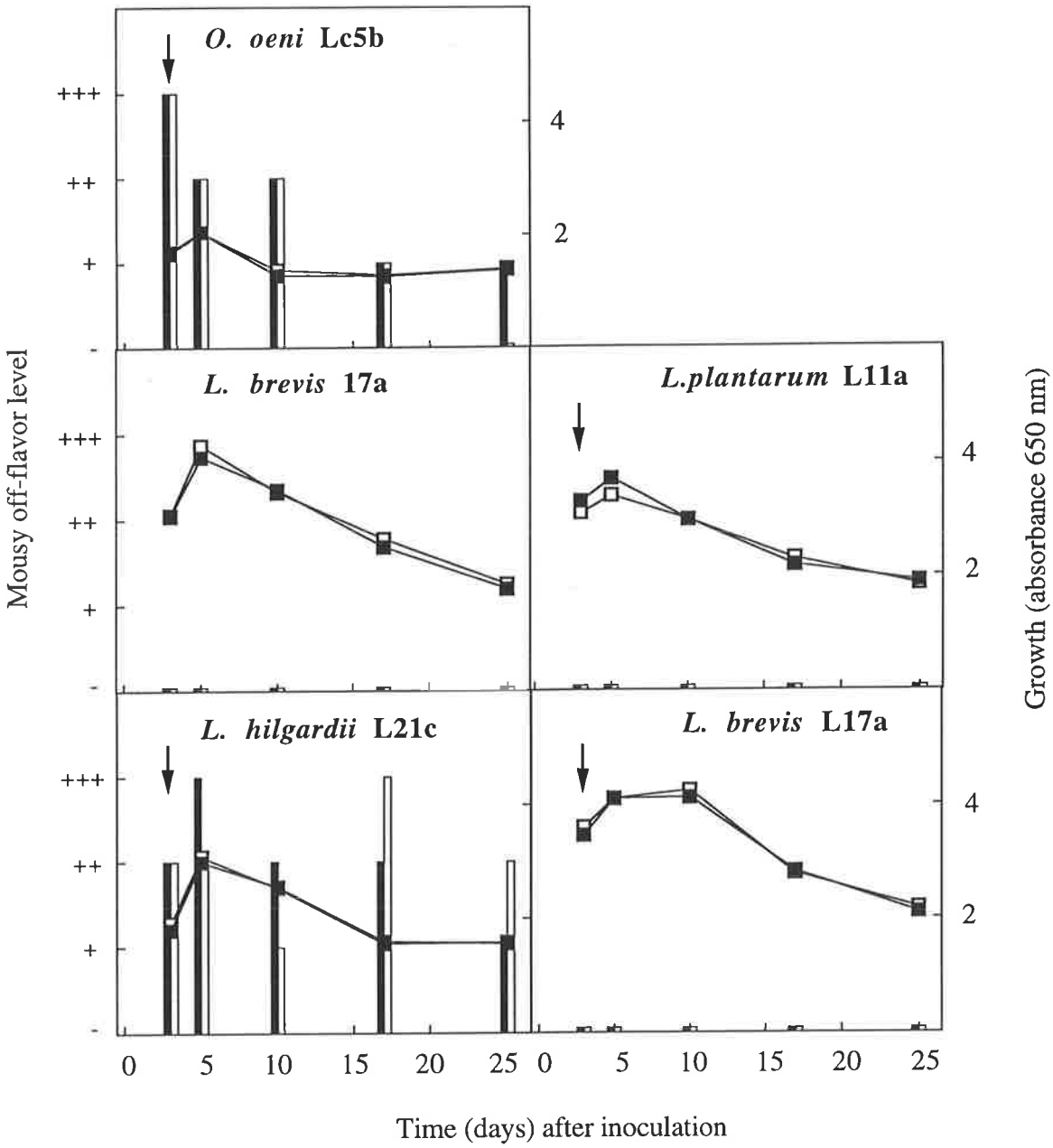


Figure 3.7 Growth (lines) and production of mousy off-flavour (bars) by lactic acid bacteria strains in Chardonnay-Pinot Noir wine medium. Mousy off-flavour assessed by the level of mousy odour detected using the alkaline test strip method. Off-flavour level: -, none; +, weak; ++, moderate; +++, strong. Data from duplicate experiments are presented. Arrows indicate completion of L-malic acid catabolism

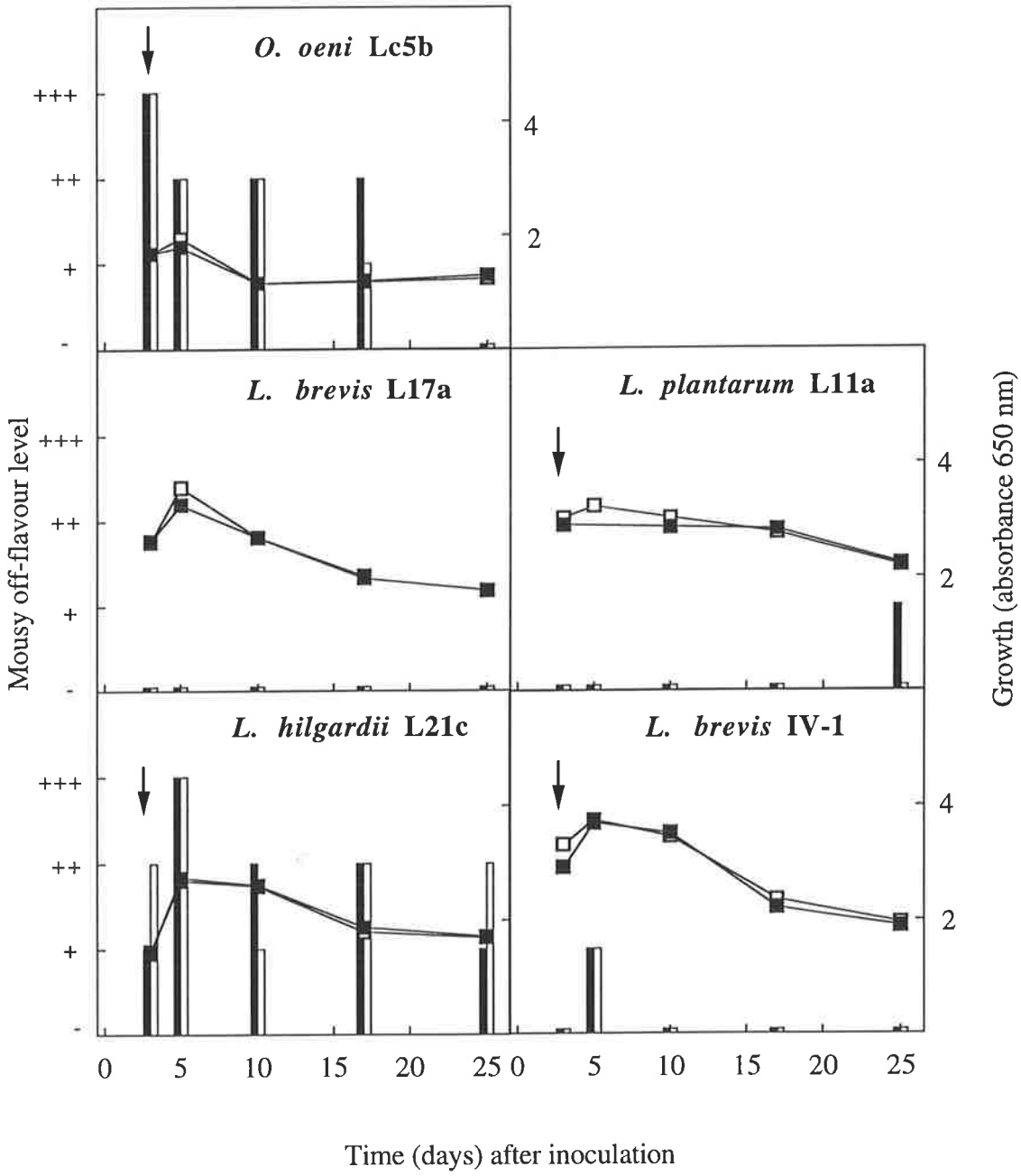


Figure 3.8 Growth (lines) and production of mousy off-flavour (bars) by lactic acid bacteria strains in RhineRiesling (sulfite reduced) wine medium. Mousy off-flavour assessed by the level of mousy odour detected using the alkaline test strip method. Off-flavour level: -, none; +, weak; ++, moderate; +++, strong. Data from duplicate experiments are presented. Arrows indicate completion of L-malic acid catabolism

Table 3.8 Production of mousy off-flavour and other aroma characteristics by lactic acid bacteria during growth in wine media (p. 1 of 4)

Wine medium	Bacteria strain		Replicate	Time (days) after inoculation											
				3				5				10			
	Genus / species	Strain	No.	Growth* (a.u.)	Malic acid (g/L)	Mousy off-flavour†	Aroma of culture medium††,†††	Growth (a.u.)	Malic acid (g/L)	Mousy off-flavour	Aroma of culture medium	Growth (a.u.)	Malic acid (g/L)	Mousy off-flavour	Aroma of culture medium
Crouchen	*Oenococcus oeni*	Lc5b	#1	1.76	0.00	+++	mousy	2.00	0.01	++	mousy	1.34		+++	mousy, acetic
	" "	"	#2	1.72	0.00	+++	mousy	2.00	0.01	++	mousy	1.32		+++	mousy
	Lactobacillus plantarum	L11a	#1	2.66	0.00	- (h)**	butterscotch	3.00	0.11	+ (br)	honey	2.48		+ (b)	honey
	" "	"	#2	2.48	0.01	-	butterscotch	3.00	0.05	- (br)	n/d	2.44		+ (b)	honey
	Lactobacillus brevis	L17a	#1	3.14	1.19	-	vinous	3.85	0.91	-	vinous	2.78	0.67	-	vinous
	" "	"	#2	2.98	1.18	-	vinous	3.75	1.08	-	vinous	3.00	0.69	-	vinous
	Lactobacillus brevis	IV-1	#1	3.18	0.00	-	vinous	4.95	0.02	- (ba)	n/d	3.38		-	vinous
	" "	"	#2	3.30	0.00	-	vinous	4.95	0.04	- (ba)	n/d	3.42		-	vinous
	Lactobacillus hilgardii	L21c	#1	1.32	0.00	+	mousy, vinous	2.50	0.02	++ (br)	sw	2.82		+++ (cr)	mousy
	" "	"	#2	1.26	0.00	+	mousy, vinous	2.55	0.02	++ (br)	sw	2.74		+++ (cr)	mousy
	Uninoculated control		#1	0.01	2.10	-	vinous, oily	0.01	2.10	-	vinous, oily	0.01	2.13	-	vinous, oily
	" "		#2	0.01		-	vinous, oily	0.01	2.24	-	vinous, oily	0.01		-	vinous, oily
Rhine Riesling	*Oenococcus oeni*	Lc5b	#1	1.80	0.00	+++	mousy, creamy	2.15	0.05	+	n/d	1.28		++	mousy
	" "	"	#2	1.82	0.00	+++	mousy	2.30	0.03	++	n/d	1.44		++	mousy
	Lactobacillus plantarum	L11a	#1	3.00	0.01	- (h)	creamy	3.20	0.05	-	honey	2.80		- (cr)	butterscotch
	" "	"	#2	2.80	0.01	- (h)	creamy	3.25	0.05	-	honey	2.84		- (cr)	butterscotch
	Lactobacillus brevis	L17a	#1	3.06	1.14	-	vinous	3.85	1.00	- (v)	sw	3.42	0.62	-	vinous
	" "	"	#2	3.20	1.15	-	mousy, vinous	4.20	1.01	- (v)	sw	3.64	0.53	-	vinous
	Lactobacillus brevis	IV-1	#1	3.54	0.01	-	vinous	4.10	0.06	-	n/d	3.62		-	vinous
	" "	"	#2	3.54	0.01	-	vinous	4.25	0.03	-	n/d	3.82		-	butterscotch
	Lactobacillus hilgardii	L21c	#1	1.78	0.00	+	mousy, vinous	3.10	0.01	+ (cr)	sw	2.16		+	vinous
	" "	"	#2	1.80	0.00	+	mousy, vinous	2.90	0.02	++ (cr)	sw	2.14		++ (cr)	n/d
	Uninoculated control		#1	0.01	2.08	-	vinous, oily	0.00	2.06	-	vinous, oily	0.01	2.15	-	vinous, oily
	" "		#2	0.01	2.03	-	vinous, oily	0.01	2.17	-	vinous, oily	0.01	2.15	-	vinous, oily

* Cell growth measured by absorbance at 650 nm.

† Mousy off-flavour assessed by the level of mousy odour detected using the alkaline test strip method. Level of mousy taint: -, no taint; +, weak; ++ moderate; +++, strong.

** Abbreviations refer to other aroma characteristics observed during assessment of alkaline test strip: b, butterscotch; v, vinous; h, honey; br, bready; cr, cracker; ba, bandaid; sw, sweet.

†† Descriptors of fermentation aromas

††† n/d = no distinctive aroma

Table 3.8 Production of mousy taint and other aroma characteristics by lactic acid bacteria during growth in wine media (p. 2 of 4)

Wine medium	Bacteria strain		Replicate	Time (days) after inoculation							
				17				25			
	Genus species	Strain	No.	Growth* (a.u.)	Malic acid (g/L)	Mousy off-flavour†	Aroma of culture medium†,**	Growth (a.u.)	Malic acid (g/L)	Mousy off-flavour	Aroma of culture medium
Crouchen	*Oenococcus oeni*	Lc5b	#1	1.14		++ (br)**	mousy	1.08		+ (br)	acetic
	" "	"	#2	1.06		+ (br)	mousy, acetic	1.04		+ (br)	acetic
	Lactobacillus plantarum	L11a	#1	2.18		-	butterscotch	1.58		-	honey
	" "	"	#2	2.18		-	butterscotch	1.58		-	honey
	Lactobacillus brevis	L17a	#1	1.94	0.54	+ (br)	vinous	1.62	0.54	-	vinous
	" "	"	#2	1.92	0.54	- (ba)	vinous	1.76	0.54	-	vinous
	Lactobacillus brevis	IV-1	#1	2.04		-	vinous, oily	1.78		-	acetic
	" "	"	#2	2.18		-	vinous, oily	1.78		-	acetic
	Lactobacillus hilgardii	L21c	#1	1.54		- (cr)	mousy	1.40		+ (cr)	vinous
	" "	"	#2	1.54		+ (cr)	mousy, cracker	1.38		+ (cr)	vinous
	Uninoculated control		#1	0.01	2.20	-	vinous, oily	0.01	2.29	-	vinous, oily
	" "		#2	0.01	2.17	-	vinous, oily	0.01	2.23	-	vinous, oily
Rhine Riesling	*Oenococcus oeni*	Lc5b	#1	1.16		++ (br)	mousy	1.18		+	acetic
	" "	"	#2	1.12		+ (br)	mousy	1.20		+	acetic
	Lactobacillus plantarum	L11a	#1	2.42		- (ba)	butterscotch	1.96		-	honey
	" "	"	#2	2.38		- (ba)	butterscotch	1.96		-	honey
	Lactobacillus brevis	L17a	#1	2.32	0.42	- (ba)	n/d	1.82	0.39	-	n/d
	" "	"	#2	2.10	0.43	- (ba)	n/d	1.78	0.39	-	n/d
	Lactobacillus brevis	IV-1	#1	2.08		-	n/d	1.92		-	n/d
	" "	"	#2	2.22		-	n/d	1.92		-	n/d
	Lactobacillus hilgardii	L21c	#1	1.56		- (cr)	cracker	1.58		+ (cr)	vinous
	" "	"	#2	1.56		+ (cr)	cracker	1.60		+ (cr)	vinous
	Uninoculated control		#1	0.01	2.18	-	vinous, oily	0.02	2.19	-	vinous, oily
	" "		#2	0.01	2.17	-	vinous, oily	0.02	2.15	-	vinous, oily

* Cell growth measured by absorbance at 650 nm.

† Mousy off-flavour assessed by the level of mousy odour detected using the alkaline test strip method. Level of mousy taint: -, no taint; +, weak; ++ moderate; +++, strong.

** Abbreviations refer to other aroma characteristics observed during assessment of alkaline test strip: b, butterscotch; v, vinous; h, honey; br, bready; cr, cracker; ba, bandaid; sw, sweet.

Table 3.8 Production of mousy off-flavour and other aroma characteristics by lactic acid bacteria during growth in wine media (p. 3 of 4)

Wine medium	Bacteria strain		Replicate	Time (days) after inoculation											
				3				5				10			
	Genus / species	Strain	No.	Growth* (a.u.)	Malic acid (g/L)	Mousy off-flavour†	Aroma of culture medium††,†††	Growth (a.u.)	Malic acid (g/L)	Mousy off-flavour	Aroma of culture medium	Growth (a.u.)	Malic acid (g/L)	Mousy off-flavour	Aroma of culture medium
Chardonnay-Pinot Noir	*Oenococcus oenos*	Lc5b	#1	1.66	0.01	+++	mousy	2.05	0.04	++ (cr)	mousy	1.28		++ (br)	n/d
	" "	"	#2	1.70	0.01	+++	mousy	2.05	0.02	++	n/d	1.38		++ (br)	n/d
	Lactobacillus plantarum	L11a	#1	3.30	0.01	- (b)	butterscotch	3.70	0.03	-	vinous	2.98		- (cr)	butterscotch
	" "	"	#2	3.10	0.02	- (h)	butterscotch	3.40	0.02	-	vinous	2.98		-	butterscotch
	Lactobacillus brevis	L17a	#1	3.06	1.83	- (v)	vinous	4.10	1.10	- (v)	vinous	3.52	0.90	-	vinous
	" "	"	#2	3.08	1.44	- (v)	vinous	4.30	1.63	- (v)	vinous	3.48	0.89	-	vinous
	Lactobacillus brevis	IV-1	#1	3.46	0.00	- (v)	vinous	4.10	0.04	-	n/d	4.12		-	n/d
	" "	"	#2	3.60	0.00	- (v)	vinous	4.10	0.02	-	n/d	4.24		-	n/d
	Lactobacillus hilgardii	L21c	#1	1.80	0.00	++	vinous	3.00	0.05	++ (cr)	n/d	2.56		++ (cr)	vinous
	" "	"	#2	1.92	0.00	++	vinous	3.10	0.03	++ (cr)	mousy	2.54		+	vinous
	Uninoculated control		#1	0.02	3.17	-	vinous, oily	0.00	3.25	-	vinous, oily	0.02	3.25	-	vinous, oily
	" "		#2	0.02	3.16	-	vinous, oily	0.01	3.29	-	vinous, oily	0.02	3.27	-	vinous, oily
Rhine Riesling (sulfite reduced)	*Oenococcus oenos*	Lc5b	#1	1.68	0.02	+++	mousy	1.80	0.04	++ (cr)	spicy	1.16		++ (br)	n/d
	" "	"	#2	1.68	0.02	+++	mousy	1.95	0.06	++ (cr)	spicy	1.16		++ (br)	n/d
	Lactobacillus plantarum	L11a	#1	2.92	0.01	- (h)	butterscotch		0.07	-	fruity	2.88		-	honey
	" "	"	#2	3.04	0.01	- (h)	butterscotch	3.25	0.09	-	fruity	3.04		-	honey
	Lactobacillus brevis	L17a	#1	2.66	1.44	- (v)	vinous, oily	3.30	1.36	- (v)	n/d	2.72	0.91	-	vinous
	" "	"	#2	2.64	1.53	- (v)	creamy, vinous	3.60	1.43	- (v)	n/d	2.72	0.80	-	vinous
	Lactobacillus brevis	IV-1	#1	2.92	0.03	- (v)	vinous	3.70	0.05	+	butyric	3.52		-	n/d
	" "	"	#2	3.32	0.01	- (v)	vinous	3.75	0.05	+	butyric	3.46		-	n/d
	Lactobacillus hilgardii	L21c	#1	1.42	0.01	+	creamy, vinous	2.75	0.06	+++ (cr)	butyric	2.62		++ (cr)	vinous
	" "	"	#2	1.46	0.05	++	creamy, vinous	2.70	0.06	+++ (cr)	butyric	2.60		+ (cr)	vinous
	Uninoculated control		#1	0.03	2.41	-	vinous, oily	0.01	2.48	-	vinous, oily	0.03	2.42	-	vinous, oily
	" "		#2	0.03	2.41	-	vinous, oily	0.01	2.46	-	vinous, oily	0.03	2.43	-	vinous, oily

* Cell growth measured by absorbance at 650 nm.

† Mousy off-flavour assessed by the level of mousy odour detected using the alkaline test strip method. Level of mousy taint: -, no taint; +, weak; ++ moderate; +++, strong.

** Abbreviations refer to other aroma characteristics observed during assessment of alkaline test strip: b, butterscotch; v, vinous; h, honey; br, bready; cr, cracker; ba, bandaid; sw, sweet.

†† Descriptors of fermentation aromas

††† n/d = no distinctive aroma

Table 3.8 Production of mousy-off-flavour and other aroma characteristics by lactic acid bacteria during growth in wine media (p. 4 of 4)

Wine medium	Bacteria strain		Replicate	Time (days) after inoculation							
				17				25			
	Genus / species	Strain	No.	Growth (a.u.)	Malic acid (g/L)	Mousy off-flavour†	Aroma of culture medium††,†††	Growth (a.u.)	Malic acid (g/L)	Mousy off-flavour	Aroma of culture medium
Chardonnay-Pinot Noir	*Oenococcus oenos*	Lc5b	#1	1.26		+ (br)	bready	1.38		+	acetic
	" "	"	#2	1.28		+ (br)	bready	1.40		+/-	acetic
	Lactobacillus plantarum	L11a	#1	2.18		- (ba)	honey	1.90		- (v)	honey
	" "	"	#2	2.30		- (ba)	honey	1.84		- (v)	honey
	Lactobacillus brevis	L17a	#1	2.52	0.63	- (ba)	vinous	1.78	0.61	- (ba)	n/d
	" "	"	#2	2.66	0.63	- (ba)	vinous	1.86	0.56	- (ba)	n/d
	Lactobacillus brevis	IV-1	#1	2.82		-	n/d	2.10		- (ba)	vinous
	" "	"	#2	2.78		-	n/d	2.18		- (ba)	vinous
	Lactobacillus hilgardii	L21c	#1	1.60		++ (cr)	n/d	1.56		+	vinous
	" "	"	#2	1.56		+++ (cr)	n/d	1.58		++ (cr)	vinous
	Uninoculated control		#1	0.02	3.21	-	vinous, oily	0.03	3.08	-	vinous, oily
	" "		#2	0.02	3.22	-	vinous, oily	0.03	3.12	-	vinous, oily
Rhine Riesling (sulfite reduced)	*Oenococcus oenos*	Lc5b	#1	1.20		++ (br)	bready	1.30		+/- (br)	n/d
	" "	"	#2	1.18		+ (br)	bready	1.24		+/- (br)	n/d
	Lactobacillus plantarum	L11a	#1	2.84		-	butterscotch	2.24		- (ba)	sweet
	" "	"	#2	2.78		-	butterscotch	2.20		- (ba)	sweet
	Lactobacillus brevis	L17a	#1	2.00	0.76	-	n/d	1.78	0.71	- (ba)	n/d
	" "	"	#2	2.04	0.75	-	n/d	0.00	0.68	-	n/d
	Lactobacillus brevis	IV-1	#1	2.22		- (ba)	vinous	1.90		-	vinous
	" "	"	#2	2.36		- (ba)	vinous	1.96		-	vinous
	Lactobacillus hilgardii	L21c	#1	1.88		++ (cr)	bready	1.72		+	vinous
	" "	"	#2	1.80		++ (cr)	bready	1.70		++ (cr)	vinous
	Uninoculated control		#1	0.04	2.45	-	vinous, oily	0.05	2.34	-	vinous, oily
	" "		#2	0.04	2.43	-	vinous, oily	0.05	2.30	-	vinous, oily

* Cell growth measured by absorbance at 650 nm.

† Mousy off-flavour assessed by the level of mousy odour detected using the alkaline test strip method. Level of mousy taint: -, no taint; +, weak; ++ moderate; +++, strong.

** Abbreviations refer to other aroma characteristics observed during assessment of alkaline test strip: b, butterscotch; v, vinous; h, honey; br, bready; cr, cracker; ba, bandaid; sw, sweet

†† Descriptors of fermentation aromas

††† n/d = no distinctive aroma

3.4 DISCUSSION

This chapter presents a survey of representative genera and species of wine LAB for the ability to produce mousy off-flavour in a range of ethanolic growth media. Although mousy off-flavour was assessed indirectly by the alkaline paper test strip method, several significant results have nevertheless emerged. Primarily, the findings of Tucknott (1977) and Heresztyn (1986) that certain *Lactobacillus* spp. are capable of producing mousy off-flavour have been confirmed and extended to suggest that this phenomenon is more widespread amongst LAB, particularly amongst *Lactobacillus* spp. and, of major significance to the wine industry, *O. oeni* and also *Lc. mesenteroides*.

Of the six species of *Lactobacillus* tested in GJ medium, the highest levels of mousy off-flavour (medium to strong) were produced by most strains of *L. hilgardii*, *L. brevis,* and also *L. plantarum* L11a. Further, *L. hilgardii* and *L. brevis* strains generally exhibited off-flavour production throughout the incubation period. These results are in agreement with those of Tucknott (1977) and Heresztyn (1986), who similarly reported high levels of off-flavour production by isolates of *L. hilgardii* and *L. brevis*, respectively, in ethanolic juice-based media. Moreover, the sustained and generally high levels of off-flavour produced by strains of these species further implicates their potential role as common causal agents of mousy off-flavour in wine. This supports the much earlier findings of Douglas and Cruess (1936) who originally isolated and described *L. hilgardii* as a main cause of spoilage and also mousiness in California wine, and of Vaughn (1955) who considered *L. brevis* (and other LAB) as a common wine microorganism that was also capable of causing of spoilage (including mousiness).

Of the three other species of *Lactobacillus* tested in GJ medium, only strains of *L. buchneri* (L18a, L18b and L18c) produced off-flavour (weak), while *L. fermentum* L15a and *L. cellobiosus* L16a were not observed to produce off-flavour. Interestingly, Heresztyn (1986) indicated that an LAB isolate identified as *L. cellobiosus* did in fact produce mousy off-flavour. This apparent discrepancy could be due to strain variation as already noted here with other *Lactobacillus* spp. or, alternatively, off-flavour formation by *L. cellobiosus* could be intrinsically low and thus near the detection limit of the alkaline paper strip method. Under the conditions used in this study, off-flavour scores ranging from zero to weak would be anticipated for the *L. cellobiosus* isolate from Heresztyn (1986). Clearly, a greater number of strains of *L. cellobiosus* and also of *L. fermentum* and *L. plantarum* would require screening to clarify the extent of mousy off-flavour production within these species.

The effects of different test media (MRS-FMEt, Carr-MEt and the 4 wine media) on the abilities of *Lactobacillus* spp. to produce off-flavour were varied according to the species and strains involved. For example, *L. hilgardii* L21c consistently produced moderate to high

levels of off-flavour in all seven media, yet *L. plantarum* L11a only produced moderate to high off-flavour levels in Carr-MEt and GJ media and exhibited little or no off-flavour formation in either MRS-FMEt or the 4 wine-based media. Further, *L. brevis* L17a produced moderate to high levels of off-flavour in Carr-MEt and MRS-FMEt media, weak off-flavour in GJ medium, and almost none in the 4 wine media. Reasons for these variations in off-flavour formation in response to different medium compositions are at this stage unknown. Moreover, the generally satisfactory growth of strains in each medium suggests that such variations in off-flavour formation were not due to differences in cell growth. Furthermore, with the exception of *L. brevis* L17a, the metabolic activities of 3 other *Lactobacillus* spp. (*L. plantarum* L11a, *L. brevis* IV-1 and *L. hilgardii* L21c) were apparently not impeded in the 4 wine media as the latter strains completely degraded L-malic acid within 3 days. One possible explanation for the variations in off-flavour formation, however, could be related to the compositional differences between media, including different concentrations of sugars, organic acids, nitrogenous components and salts. These compositional differences could in turn cause species and / or strain specific responses in the activities of metabolic pathways leading to the formation of off-flavour. Alternatively, under certain nutritional conditions, off-flavour formation may in fact be occurring but its detection could be masked by other fermentation aromas (see below), or the causative off-flavour compounds could be further metabolised to non-volatile compounds.

A significant finding from the screening studies was the relatively high levels of mousy off-flavour exhibited by *Lc. mesenteroides* and also, of particular importance to the wine industry, strains of *O. oeni*. Further testing of *O. oeni* revealed that this species consistently produced off-flavour in each of the 7 different off-flavour-assessment media. These findings support much earlier indications by Vaughn (1955) that *Leuconostoc* spp. (in addition to other wine bacteria) were capable of causing mousiness in wine by the metabolism of glucose and fructose. The association of *O. oeni* and *Lc. mesenteroides* with mousy off-flavour, however, appears to conflict with the generally accepted view that *O. oeni* is a preferred bacterium to conduct MLF since, in part, it is relatively devoid of off-flavour formation (Davis *et al.* 1985, Henschke 1993, Bartowsky and Henschke 1995). This apparent divergence in sensory properties associated with *O. oeni* could be due to greater expression of off-flavour compound metabolism in off-flavour-assessment media compared to that which normally occurs in wine. Such metabolic differences are likely to arise from intrinsic differences in the physico-chemical properties of off-flavour-assessment media compared to those of wine, eg. higher sugar concentration and pH, presence of oxygen. To verify this assumption, further testing of *O. oeni* (and other representative LAB strains) should be carried out in wine. Nevertheless, the current study has identified the potential for off-flavour formation by these bacteria under certain conditions. Furthermore, the results indicate that winemakers should not only exercise care in selecting appropriate malolactic bacteria to minimise the risk of

mousy off-flavour formation during MLF, control over the physico-chemical conditions under which MLF is induced should also be exercised.

In contrast with the results of mousy off-flavour formation by *Lactobacillus* spp., *O. oeni* and *L. mesenteroides* described above, most *Pediococcus* spp. were generally lacking in the ability to produce off-flavour. These results indicate that off-flavour formation by this genus could be intrinsically low and, furthermore, suggests that two broad groups of LAB may exist with respect to off-flavour producing ability, ie. off-flavour producers and non- or low-off-flavour producers. These groups could be further defined according to their mode of glucose metabolism whereby most mousy off-flavour producing LAB, ie. *Lactobacillus* spp., *O. oeni* and *L. mesenteroides*, are heterofermentative species, and those lacking off-flavour production, ie. *Pediococcus* spp., are homofermentative bacteria. Obviously, such categorisation would be dependent upon the species and strain of LAB involved as well as the composition of the test medium. For example, it would be difficult to categorise *L. plantarum* L11a into this scheme as this homofermentative bacterium was capable of off-flavour production in GJ and Carr-MEt media. Nevertheless, a possible link between off-flavour production and the mode of glucose metabolism is highly significant and may provide valuable insight towards elucidating the biochemical basis of this spoilage.

Since the production of mousy off-flavour by some strains of Pediococci was apparent, eg. *P. parvulus* P6f and *P. damnosus* P1a in MRS-FMEt medium, an alternative explanation to the apparent lack of off-flavour producing ability by this species could be that off-flavour formation may indeed have occurred and, as previously suggested for some non-off-flavour producing *Lactobacillus* spp., the detection of off-flavour by alkaline test strip could have been masked by other fermentation aromas, eg. by diacetyl (Tables 3.6 and 3.7). This supports the findings of Vaughn (1955) who suggested that the homofermentative *Micrococcus* (*Pediococcus*) spp., as well as other LAB, were capable of producing mousiness in wine in association with the lactic fermentation of sugars. Interference or masking of off-flavour by fermentation aromas could also account for some of the variations in off-flavour levels observed between different genera, species and strains of LAB, and also between sampling points of respective strains. Further, since homofermentative bacteria produce lactic acid as a major product of glucose metabolism, it is possible that a greater decrease in pH may occur in media cultured with Pediococci than with heterofermentative bacteria, thus rendering alkalisation and hence also detection of basic off-flavour compounds more difficult with the alkaline test strip. It follows, therefore, that the potentially greater amounts of lactic acid produced from the higher sugar contents of GJ medium (93 g/L) and Carr-MEt medium (50 g/L) compared to that of MRS-FMEt medium (30 g/L) may explain why off-flavour formation was detected in the latter medium, but not the former two media, after culture with *Pediococcus* spp. The confusion as to whether mousy off-flavour formation is lacking or, alternatively, is a general feature of *Pediococcus* spp. requires clarification by

further quantitative studies of the production of mousy compounds by this genus, as well as other LAB generally.

Although a number of important features of mousy off-flavour formation by LAB have been highlighted in this chapter, the alkaline test strip method cannot be viewed as an infallible procedure for definitive and comparative assessment of mousy off-flavour. The inherent problems associated with this method, eg. potential variability in assessment between sample lots, media and individuals, and masking of off-flavour by other aromas, necessitates the development of a reliable and sensitive analytical procedure for the quantification and study of the compounds which are causative of mousy off-flavour.

3.5 Summary

The ability of wine LAB to produce mousy off-flavour during growth in ethanolic media has been demonstrated using a qualitative alkaline test strip procedure. This method provided an indirect assessment of mousy off-flavour by rendering the mousy components volatile under alkaline conditions. Importantly, off-flavour formation was not restricted to certain species or strains, but was associated with a diversity of LAB, particularly amongst *Lactobacillus* spp., strains of *O. oeni* and also *Lc. mesenteroides*, although was comparatively lacking in *Pediococcus* spp. The screening of a range of LAB for the ability to produce off-flavour has suggested a possible link between off-flavour formation by a particular LAB species / strain and its pathway of hexose metabolism.

While considerable variation in off-flavour producing ability was observed between different species and strains of LAB, and to some extent between different media formulations, these trends overall are of major significance to the wine industry.

4. ANALYSIS AND QUANTIFICATION OF MOUSY OFF-FLAVOUR COMPOUNDS

4.1 INTRODUCTION

Although mousy off-flavour has been recognized as a wine spoilage phenomenon for a century (Erckmann 1898), it has only been the relatively recent studies of Tucknott (1977) and Strauss and Heresztyn (1984) which have respectively identified two N-heterocyclic bases, 2-ethyltetrahydropyridine (ETPY) and 2-acetyltetrahydropyridine (ACTPY), as causative off-flavour compounds. Moreover, it is likely that the identification of these compounds was facilitated by concurrent developments in GC and GC-MS technologies over recent decades. However, a major drawback of the aforementioned studies was the lack of quantitative data. Consequently, the development of a quantitative method for the analysis of mousy off-flavour compounds is pivotal for gaining greater understanding of the chemical nature of this spoilage phenomenon. Equally, such a procedure is required for the undertaking of the remaining microbiological studies of this thesis.

The study of mousy off-flavour should also assess the possibility that compounds other than ACTPY and ETPY are associated with this spoilage. This is implicated by the range of structurally related N-heterocyclic bases which also exhibit mousy aroma. These include 2-methyl- and 2-propionyltetrahydropyridine, 2-ethylpiperideine (Craig and Heresztyn 1984), and the analogue of ACTPY, 2-acetyl-1-pyrroline (Seitz *et al.* 1993). Significantly, the latter compound was recently considered to cause mousy aroma in wetted pearl millet (Seitz *et al.* 1993), and was also detected in the culture medium of bacteria previously isolated from the fermentation of cocoa (Romanczyk *et al.* 1995).

Preliminary experiments of this chapter investigated the GC analysis and extraction of ACTPY. These studies highlighted the considerable chemical and chromatographic instability of this compound. Further experiments concentrated on the development of a sensitive and reliable procedure for the analysis of ACTPY and other mousy off-flavour compounds from wine. In a final experiment, mousy compounds were identified and quantified from a survey of mousy wines. The chemical experiments and development of methods for the analysis of mousy compounds described in this chapter were performed in this laboratory under the guidance of Dr. Markus Herderich, and in collaboration with Mr. Paul Grbin.

4.2 Materials And Methods

4.2.1 Synthesis of mousy off-flavour compounds

4.2.1.1 2-Acetyltetrahydropyridine

Synthesis and initial purification

Reference ACTPY was synthesized and purified using procedures based upon those of Hunter and Walden (1971, 1973). Sodium bisulfite (60g), proline (40g) and dihydroxyacetone (20g) were ground in a mortar, transferred to a baking tray and heated in an oven at 92 °C for 30 minutes. The mixture fused and rose during the heating period and subsequently dried to a friable cake-like sinter material. Approximately 50g of the sinter was accurately weighed and dissolved in 100 mL of distilled water, which was then cooled in an ice-bath. 4N NaOH (100 mL) was then slowly added with stirring. This basified solution was extracted with pentane (3 x 100 mL) and the combined extracts were clarified by boiling for approximately three minutes with decolourizing charcoal (activated charcoal -Ajax Chemicals, 1g) followed by filtration through diatomaceous earth filter aid. Excess solvent was removed by distillation, leaving a light amber coloured concentrated extract.

Approximately 3g of concentrated pentane extract was purified by vacuum distillation (Büchi GKR-50 Betriebsanleitung), from which the following three fractions were obtained:

Fraction	(1)	B.Pt.	107 °C at 5 mm Hg:	0.0416g
	(2)		120 °C at 13mm Hg:	1.5258g
	(3)		130 °C at 7mm Hg:	0.0883g

In addition to possessing the highest net weight of distillate, GC-MS analysis revealed that Fraction (2) also contained the highest relative concentration of ACTPY. This second fraction (referred to as VD/F-2) was stored at -20 °C and was used as the main source of reference ACTPY for preliminary studies assessing methods for the extraction of ACTPY, and also as starting material for further purification.

Further purification of 2-acetyltetrahydropyridine

It was noted that the vacuum distilled ACTPY (VD/F-2) changed in colour and appearance from light brown liquid to a darker brown viscous mass during storage. Further purification of this fraction was therefore undertaken, and for which several procedures were attempted

including column fractionation, ether extraction, preparation of a bisulfite adduct and further vacuum distillation.

(i) Column fractionation

A column fractionation procedure similar to that described by Schieberle and Grosch (1983) for separation of flavour compounds (including ACTPY) from the crust of rye bread was evaluated. A column (13 cm x 0.7 cm) was packed with a slurry of silica gel (ca. 12 g) (Merck 9385, Kieselgel 60, 230-400 mesh) in hexane. Approximately 25-30 mg of ACTPY (VD/F-2) was dissolved in 10 mL of 5:95 diethyl ether - hexane (v/v), and then eluted through the column with 30 mL of each of 5:95 diethyl ether - hexane (v/v; fraction 1), 30:70 diethyl ether - hexane (v/v; fraction 2), 70:30 diethyl ether-hexane (v/v; fraction 3) and diethyl ether (fraction 4). Gas chromatography analysis revealed that ACTPY was eluted in fraction 3. This fraction was collected and concentrated by rotary evaporation (20 °C), transferred to a 10 mL volumetric flask (tared) and further dried under a gentle stream of nitrogen to constant weight. This concentrate was then made up to 10 mL with dichloromethane and the content of ACTPY was determined by GC using 2-acetylpyridine as an internal standard.

(ii) Ether extraction

2-Acetyltetrahydropyridine (VD/F-2; 120 mg) was extracted with diethyl ether (5 x 10 mL), and the combined extracts concentrated by rotary evaporation, dried under a gentle stream of nitrogen and weighed to constant weight. The content of ACTPY in the extract was determined by GC using 2-acetylpyridine as an internal standard.

(iii) Preparation of a bisulfite salt of 2-acetyltetrahydropyridine

A method similar to that described by Hunter and Walden (1971, 1973) was used for the preparation of a bisulfite salt (adduct) of ACTPY. Ninety milligrams of ACTPY (VD/F-2) was dissolved in 20 mL pentane / dichloromethane (1:1), which was then washed with 5 x 20 mL of aqueous sodium bisulfite (5 g/L). The combined aqueous extracts were dried by freeze-drying yielding a faint yellow coloured powder (0.212 g) which, according to Hunter and Walden (1971, 1973), comprised the ACTPY-bisulfite complex and excess sodium bisulfite. The dried ACTPY-bisulfite was stored desiccated at -25 °C.

Free ACTPY was conveniently released from the bisulfite adduct by direct extraction in ethanol. A precipitate which formed was separated by centrifugation, and the content of

ACTPY in the supernatant was immediately determined by GC using 2-acetylpyridine as an internal standard.

(iv) Second vacuum distillation

2-Acetyltetrahydropyridine (VD/F-2) was further purified by a second vacuum distillation process (0.5-1 mm Hg; 0 - 66 °C) in which volatiles were collected in glass traps cooled with liquid nitrogen. The following four fractions were obtained from this second distillation process:

Fraction	(1)	B.Pt.	20 °C: 0.021 g
	(2)		45 °C: 0.019 g
	(3)		66 °C: 0.016 g
	(4)		60 °C: 0.0185 g

Toluene sulfonic acid (in dichloromethane) was added to the residue of ACTPY (VD/F 2) prior to distillation of Fraction 4 to encourage de-polymerization of ACTPY. Each fraction was made up to 50 mL with ethanol and the content of ACTPY was determined by GC using 2-acetylpyridine as an internal standard. Fraction 2 (referred to as VD/F 2.2) yielded the highest recovery of ACTPY (85% w/w; see results section Table 4.1) and was therefore used as a source of reference compound in subsequent experiments throughout this study.

4.2.1.2 2-Ethyltetrahydropyridine

2-Ethyl-3,4,5,6-tetrahydropyridine (ETPY) was synthesized by Mr. Paul Grbin in this laboratory using a method similar to that described by Grundon and Reynolds (1963) and Tucknott (1977).

4.2.1.3 2-Acetylpyrolline

An authentic reference sample of 2-acetyl-1-pyrolline (ACPY) was kindly provided by Dr. Ron Buttery of the Agricultural Research Service, Western Regional Center, US Department of Agriculture, Albany, CA. The identity of ACPY was confirmed by comparison of mass spectral data of the reference sample to published data (Buttery *et al.* 1982, 1983b).

4.2.2 Development of a procedure for quantitative extraction of mousy compounds

Prior to extraction, samples of wine and fermentation media were clarified where necessary by either centrifugation (12 min at 10,000-12,000 x *g*) or filtration (0.22µm membrane).

4.2.2.1 Assessment of extraction methods

(i) Simultaneous steam distillation / extraction.

One millilitre of dichloromethane stock solution of ACTPY (VD/F 2; 2.07 mg) was added to 500 mL phosphate buffer (0.2 M, pH 8.0), which was then distilled and continuously extracted for 2 h with 50 mL of diethyl ether using the simultaneous steam distillation / extraction apparatus (SDE) described by Nickerson and Likens (1966). After drying over $MgSO_4$ and addition of internal standard (1.14 mg 4-dimethylaminopyridine), the diethyl ether extract was concentrated to 1 - 2 mL by distillation at ca. 40 °C using a Vigreux column (ca. 1 x 10 cm).

(ii) Ion exchange chromatography

Ion exchange chromatography was performed using a modification of a procedure for the extraction of amino acid esters from wine (Heresztyn, 1984), and of mousy compounds from growth media and wine (Heresztyn, 1986). Prior to the ion exchange procedure, 490 mL of citrate buffer (0.1M, pH 3.0) was spiked with a 10 mL solution containing ACTPY (1 mL dichloromethane stock solution of ACTPY (VD/F 2; 2.07 mg) dissolved in 9 mL redistilled ethanol). The spiked buffer solution was then eluted at ca. 5 mL / min through a column (9 x 1.5 cm) of Dowex 50W strongly acidic cation-exchange resin in the hydrogen ion form. The resin was washed with 150 mL citrate buffer (containing 10 % v/v ethanol) to remove interfering acidic and neutral components. Basic material was then eluted from the resin with 50 mL saturated NaCl solution, followed by 170 mL saturated NaCl adjusted to pH 9.5 with Na_2CO_3. The combined NaCl eluates were adjusted to pH 9.5 with Na_2CO_3 and then extracted with Freon F11 (3 x 100 mL) in a separatory funnel. After drying over $MgSO_4$ and addition of internal standard (1.14 mg 4-dimethylaminopyridine), the Freon F11 extract was concentrated by distillation at ca. 35 °C using a Vigreux column (ca. 1 x 10 cm) and sequentially replaced with 1-2 mL diethyl ether.

For the above experiments, the content of ACTPY in extracts and also in standard reference solutions was determined by GC analysis using 4-dimethylaminopyridine as an internal standard. The recovery of ACTPY for each extraction method was calculated from the

concentrations of ACTPY in sample extracts compared with those of respective standard reference solutions.

4.2.2.2 Continuous liquid-liquid extraction procedures

(i) Continuous liquid-liquid extraction with subsequent simultaneous steam distillation and extraction

Pre-clarified (0.22µm filter) samples of wine (ca. 300 mL) were adjusted to pH 2.5 with HCl, then concentrated (ca. 25% volume reduction) by rotary evaporation (20 °C) to remove ethanol and acidic and neutral volatile compounds. The concentrated samples were then adjusted to pH 8.0 with NaOH and continuously extracted for 16 h with ca. 200 mL Freon 11 (37 °C bath temperature). The organic phase was dried over $MgSO_4$, then concentrated and sequentially replaced with 1 - 2 mL diethylether by distillation at ca. 37 °C using a Vigreux column (ca. 1 x 15 cm). The CLLE extract was then added to 500 mL Clark and Lubs buffer (0.2 M, pH 8.0) and further extracted using the SDE procedure described above to obtain a final ether extract of volatile bases. 3-Acetylpyridine was used as a first internal standard and was added to the acidified wine prior to rotary evaporation. A second internal standard (4-acetylpyridine) was added prior to concentration of the ether extract obtained from SDE.

The extraction recovery of ACTPY for this procedure was determined by extraction of a white wine (sparkling wine base) that was spiked with 28.2 µg/L ACTPY and 33.1 µg/L 3-acetylpyridine. An ethanolic stock solution consisting 31.1 µg/mL ACTPY (from VD/F 2.2) and 36.4 µg/mL 3-acetylpyridine was used as the spike solution. The concentration of ACTPY in the stock spike solution were determined by GC analysis (Carbowax 20 CAM column) using 3-acetylpyridine as an internal standard, and quantification of ACTPY in the CLLE-SDE extract was determined by GC-MS analysis. Unspiked white wine served as a control, and extractions were performed in duplicate.

(ii) Optimized continuous liquid-liquid extraction technique

Samples (250 - 300 mL) of clarified wine were saturated with NaCl and adjusted to pH 2.5 with HCl. The first internal standard (4-acetylpyridine) was added, and acidic and neutral components were subsequently removed by extraction with 3 x 100 mL Freon 11. The wine sample was then adjusted to pH 8.0 with 5N NaOH and immediately transferred to the CLLE apparatus (bath temperature 37 °C) for continuous overnight (16 h) extraction of basic compounds with Freon 11 (ca. 200 mL). After drying over Na_2SO_4, the second internal standard (3-acetylpyridine) was added to the organic phase, which was then concentrated by

careful distillation (bath temperature 37 °C) using a water-jacketed Vigreux column (1 x 15 cm). During the concentration process, Freon 11 was sequentially replaced with approximately 0.5 mL dichloromethane. Prior to GC-MS analysis, the extract was further concentrated (10-fold) into 10 µL of *iso*-octane under a gentle stream of nitrogen. A flow diagram of the extraction and quantification procedure is shown in Figure 4.1. During the course of this study, all Freon 11 from initial acid extraction and final concentration stages was collected and recycled for subsequent use by distillation.

The recoveries of both ACTPY and ACPY using this procedure were determined by extraction of replicate 300 mL samples of white wine (Rhine Riesling) which were spiked with 150 µL of an ethanolic solution containing 45.5 µg/mL ACTPY (from VD/F 2.2), 43.3 µg/mL ACPY and 79.5 µg/mL 4-acetylpyridine. 3-Acetylpyridine (74.3 µg/mL; 50 µL) was used as the second internal standard and was also added to the spike solution prior to GC-MS analysis. The concentrations of ACTPY and ACPY in the latter spike solution and CLLE wine extracts were determined by prior GC-MS analysis. Unspiked white wine served as a control and extractions were performed in duplicate.

4.2.3 Instrumentation

4.2.3.1 Gas chromatography with flame ionization detection (GC-FID)

Gas chromatography was carried out using a Varian 3300 gas chromatograph equipped with a flame ionisation detector (FID) and connected to an LDC / Milton Roy CI-10B integrator. The carrier gas [Helium (He)] flow rate was 25 mL / min and injections (0.1-1 µL) were made using a split ratio of 1:25. The HRGC column for the analysis of taint compounds was a Carbowax 20 CAM column (30 m, 0.25 mm internal diameter, 0.25 µm film thickness, J & W Scientific), and the following temperature protocol was used: 60 °C for 3 min, then 60 - 220 °C at 5 °C/min and then held at 220 °C for a further 10 min. The temperature of both the injector and detector was 220 °C. The Carbowax 20 CAM column was used for the analysis of mousy compounds throughout the remainder of this study.

4.2.3.2 Gas chromatography-mass spectrometry (GC-MS)

For analysis of wine samples and other extracts, gas chromatography-mass spectrometry (GC-MS) was carried out using a Finnigan TSQ 70 mass spectrometer (Finnigan MAT, San Jose, Calif., USA) connected to a Varian 3400 GC. The GC was fitted with the Carbowax 20 CAM column, utilising the temperature protocol described above. Sample injections were splitless

(0.5 min). Electron impact mass spectral (EI-MS) analyses were carried out at an ionization energy of 70 eV. Ion abundances in the range 40-200 *m/z* were monitored.

4.2.4 Analysis of mousy off-flavour compounds

4.2.4.1 Quantification by gas chromatography

In preliminary experiments of this chapter, GC-FID analysis was used to monitor the purity of reference ACTPY, and the efficiency of ion exchange and SDE for its extraction from aqueous media. Concentrations were calculated from the ratio of peak areas of ACTPY tautomers to those of the internal standard (IS) using the following general equation:

Concentration target compound =
{(Peak area of target compound) / (peak area of IS)} x IS concentration

The concentration of ACTPY was determined by summation of respective tautomers and, for the purposes of this study, it was assumed that target compounds and internal standards gave the same relative responses per unit weight in the FID detector.

4.2.4.2 Identification by gas chromatography-mass spectrometry.

Target mousy compounds were detected in sample extracts by GC-MS using selected ion (molecular and fragment ion) chromatograms for ETPY (*m/z*, 111,110), ACPY(*m/z* 111, 83) and ACTPY (*m/z* 125, 82), and further identified by comparison of retention time and mass spectral data with those of the synthetic compounds and published data. Retention data of the reference taint compounds were calculated from the retention times of alkanes.

4.2.4.3 Quantification by gas chromatography-mass spectrometry

The concentration of mousy compounds from initial GC-MS analyses (SDE extractions of wine) were calculated from the ratio of total ion responses [reconstructed ion chromatograms (RIC)] of target compounds to IS (2-acetylpyridine) using the following general equation:

Concentration of target compound =
{(RIC of target compound) / (RIC of IS)} x IS concentration

The total ion responses per unit weight of target compound and IS were assumed equivalent in the latter analyses.

Subsequent quantification by GC-MS also calculated the ratio of individual ion responses of target compounds to IS (4-acetylpyridine or 3-acetylpyridine) using the following general equation:

Concentration of target compound =
{(peak area of target ion) / (peak area of IS ion)} x IS Concentration x RRf

where RRf = Relative Response Factor.

Relative response factors were calculated from the ratio of the RIC responses of target compound and IS to respective ion responses of target compounds and IS:

RRf = {(RIC of target compound) / (RIC of IS)} /
{(Ion response of target compound) / (Ion response of IS)}

Relative response factors were determined from replicate GC-MS analyses of reference solutions containing similar concentrations of ACPY, ACTPY and internal standards, and were used for calculations with analyses which employed the optimized continuous liquid-liquid extraction technique.

The concentrations of ACTPY and ACPY were determined by summation of respective tautomers.

4.2.4.4 Fast atom bombardment mass spectrometry

Fast atom bombardment mass spectrometry (FAB MS-MS) of freeze-dried preparations of the bisulfite adduct of ACTPY was undertaken by Dr. Markus Herderich in this laboratory using a triple stage quadropole Finnigan TSQ 70 mass spectrometer. Xenon was the bombardment gas, the ionization voltage was 8 KeV, ion current < 0.5mA and the collision cell pressure 1.800 millitor. The sample matrix used was glycerol.

4.2.4.5 Gas chromatography and coupled sniff assessment

Gas chromatography-sniff (GC-sniff) assessment of 2-acetyl-1-pyrroline was carried out by Dr. Markus Herderich and Mr. Paul Grbin in this laboratory using a Varian 3300 GC fitted with a Carbowax 20 CAM column, an FID and external sniff cup. An SGE Vitreous Silica outlet splitter separated the sample between the FID and sniff port. Both FID and sniff cup were connected to the splitter via 30 cm x 0.75 mm deactivated fused silica columns (LC Packings, Switzerland), split ratio 1:1. The temperature program was 80 °C for 3 min, then from 80 °C to 180 °C at 10 °C/min. The column temperature to the sniff cup was maintained at 150 °C by a heated transfer line, and water saturated air was passed through the sniff cup at 100 mL/min. Other GC conditions were as described above for GC analysis.

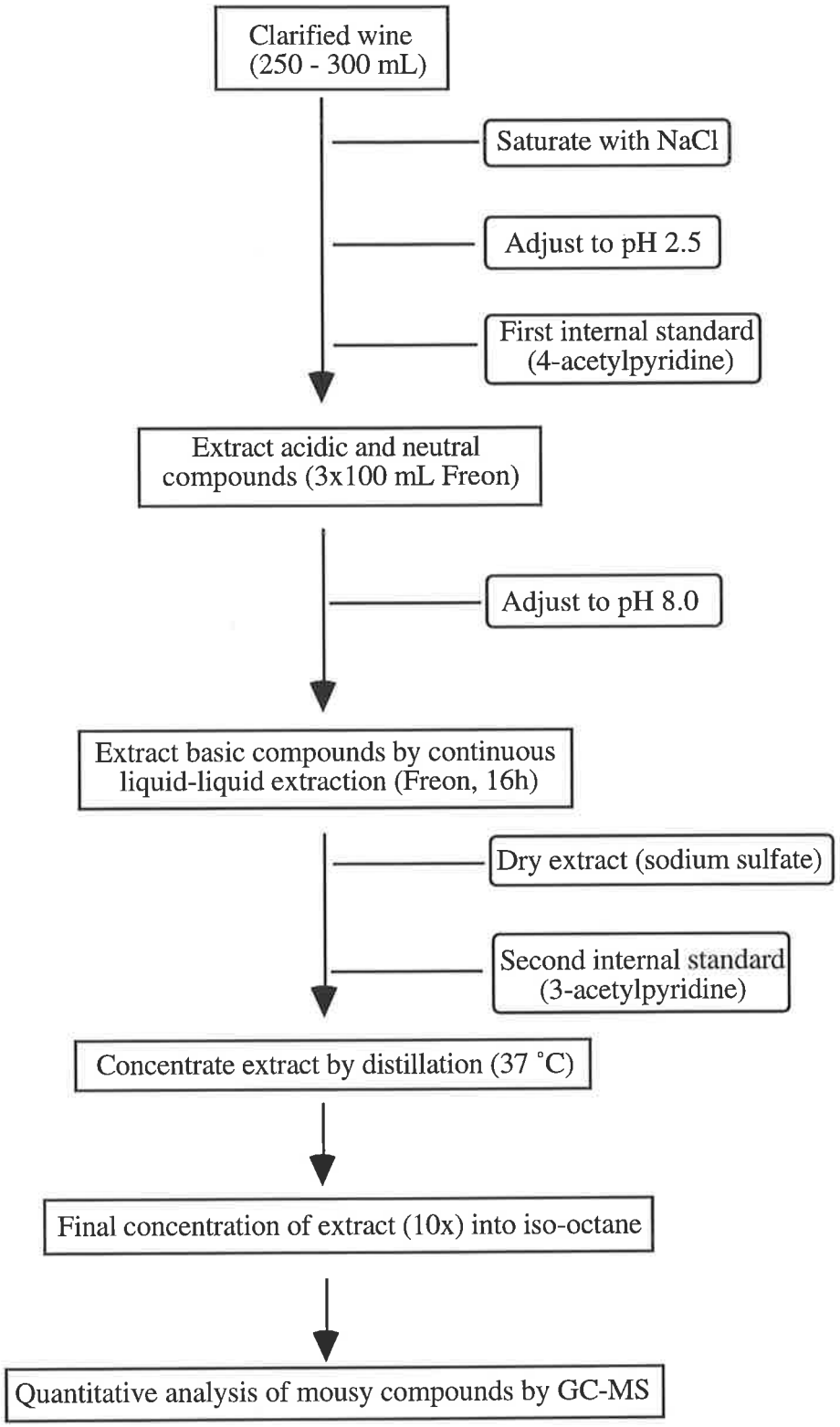


Figure 4.1 Flow chart of the continuous liquid-liquid extraction (CLLE) method for the quantititative analysis of mousy compounds

4.3 RESULTS

4.3.1 Analysis and properties of mousy off-flavour compounds

4.3.1.1 Analysis, purification and stability of 2-acetyltetrahydropyridine

(i) Analysis by gas chromatography

The separation and resolution of the two tautomers of ACTPY using the DB1701 column was variable and often poor, particularly after long-term use of the column which resulted in tautomers merging into one broad co-elution. In contrast, the Carbowax 20 CAM consistently produced clear separation and sharp resolution of ACTPY tautomers. It was noted, however, that inadvertent exposure of the Carbowax 20 CAM column to air would cause a deterioration of column performance, resulting in impaired resolution of ACTPY.

Four different substituted pyridines were assessed for their suitability as internal standards for the quantitation of ACTPY. The first of these, 4-N,N-dimethylaminopyridine, was inappropriate with the Carbowax 20 CAM column as it coeluted with the second tautomer of ACTPY (II). Another, 2-acetylpyridine, could not be routinely used as it was found to be a degradation product of ACTPY. Two other compounds, 3- and 4-acetylpyridine did not suffer any such drawbacks and were subsequently utilised as internal standards for the remainder of this work.

(ii) Purification and stability

The unstable nature of ACTPY became evident after initial vacuum distillation, whereby the appearance of the distillate (VDF.2) changed from a faint yellow coloured liquid to a darker brown viscous mass after storage for several weeks at minus 20 °C, indicating degradation during this period. Several attempts were therefore made to further purify the degraded ACTPY, including use of column fractionation, ether extraction, formation of a sodium bisulfite adduct and further vacuum distillation. None of these procedures, however, produced a highly purified material (Table 4.1). Interestingly, in addition to the relatively low purity of ACTPY derived from column fractionation (17.3%) and extraction with diethyl (36.9%), the appearance of these extracts was also noted to progressively change from a faint yellow to a darker brown during final concentration and drying stages. A significant improvement in ACTPY purity was subsequently achieved by substituting the latter concentration and drying stages with immediate dilution of purified fractions into ethanol. This approach was used in conjunction with vacuum distillation, and from which the second

fraction (VD/F 2.2) yielded a relatively high purity of 81.5%, calculated as a percentage of the original mass of the fraction.

Formation of the bisulfite adduct of ACTPY produced a relatively low level of purity (6.7 % w/w), with the remaining 93.3% of material presumably comprising excess sodium and sodium bisulfite ions. Despite such a low purity, it would be anticipated that ACTPY in the bisulfite adduct form would remain relatively stable under appropriate desiccated storage. On the other hand, a 22.5% reduction in purity of the vacuum distilled ACTPY (VD/F 2.2) was noted after storage for 48 days in ethanol at -20 °C (Table 4.2).

Table 4.1 Purity of 2-acetyltetrahydropyridine (ACTPY)* after column fractionation, ether extraction, formation of bisulfite adduct and further vacuum distillation.

Purification stage	Purity of ACTPY (% w/w)
Column fractionation [a]	
Fraction #3	17.3†
Ether extraction[a]	36.9†
Sodium bisulfite adduct[b]	6.7††
Vacuum distillation[b]	
Fraction #1 (VD/F 2.1)	34.0†††
#2 (VD/F 2.2)	81.5†††
#3 (VD/F 2.3)	62 5†††
#4 (VD/F 2.4)	6.2†††

* ACTPY starting material derived from initial vacuum distillation (VD/F 2)

† Purity expressed as the percentage (%w/w) of ACTPY in the final dried, extract / fraction .

†† ACTPY analysis determined on ethanol extract of the bisulfite adduct. Purity expressed as percentage concentration of ACTPY (%w/w) in the dried bisulfite adduct.

Table 4.2 Degradation of purified 2-acetyltetrahydropyridine (ACTPY)† during storage in ethanol at -20 °C

Time (days) at -20 °C	ACTPY†† (μg/mL)	Relative purity of ACTPY††† (%)
0	310	100
1	305	98
31	230	74
48	226	72

† Purified ACTPY derived from second vacuum distillation, Fraction #2 (VD/F 2.2) diluted in 50 mL ethanol

†† Concentration of ACTPY in diluted distillate determined by GC analysis using a Carbowax 20 CAM column and 2-acetylpyridine (0.085 mg) as internal standard.

††† Relative purity of ACTPY calculated as a percentage of the initial ACTPY concentration at day zero.

4.3.1.2 Analysis of mousy compounds by gas chromatography-mass spectrometry

(i) Identification

Representative mass spectra of the three reference compounds, ACPY, ACTPY and ETPY are shown in Figures 4.2 - 4.4. Additional chromatographic and MS data for the three reference compounds are also shown in Table (4.3). In addition to the previously noted separation of ACTPY into two distinct tautomers, separation of ACPY and of ETPY into two apparent isomers was also observed. However, the latter second peaks of ACPY and ETPY contributed only a small proportion of the total amount of each compound. For the purposes of this study, the latter peaks were tentatively assigned as tautomers and hence were summed for quantification purposes.

(ii) Quantification

The response factors determined for ACPY, ACTPY and the first internal standard were relatively consistent (Appendix 4.1). Moreover, the quantitation of these compounds using selected (molecular) ion chromatography and respective RRf values also provided little variation between replicate analyses of a standard reference mixture (Appendix 4.2), thus substantiating the reliability of this method of quantification.

4.3.1.3 Fast atom bombardment mass spectrometry of the bisulfite adduct of 2-acetyltetrahydropyridine

The MS of the bisulfite salt of ACTPY showed a positive daughter ion of *m/z* 208, and other positive daughter ions *m/z* 127, *m/z* 109, *m/z* 84 and *m/z* 55, which is consistent with an ACTPY-bisulfite adduct structure (data not shown).

4.3.1.4 Gas chromatography-sniff assessment of 2-acetylpyrroline

The reference sample of ACPY exhibited an intense mousy odour. Some variations in this characteristic were noted which may have been caused by minor by-products in the reference material. In order to exclude such interference, the sensory property of ACPY (tautomer I) was evaluated by GC-sniff assessment. Using this technique, even trace quantities (approximately 1 ng / injection) of the clearly separated ACPY were described by GC-sniff assessors as mousy (Table 4.3).

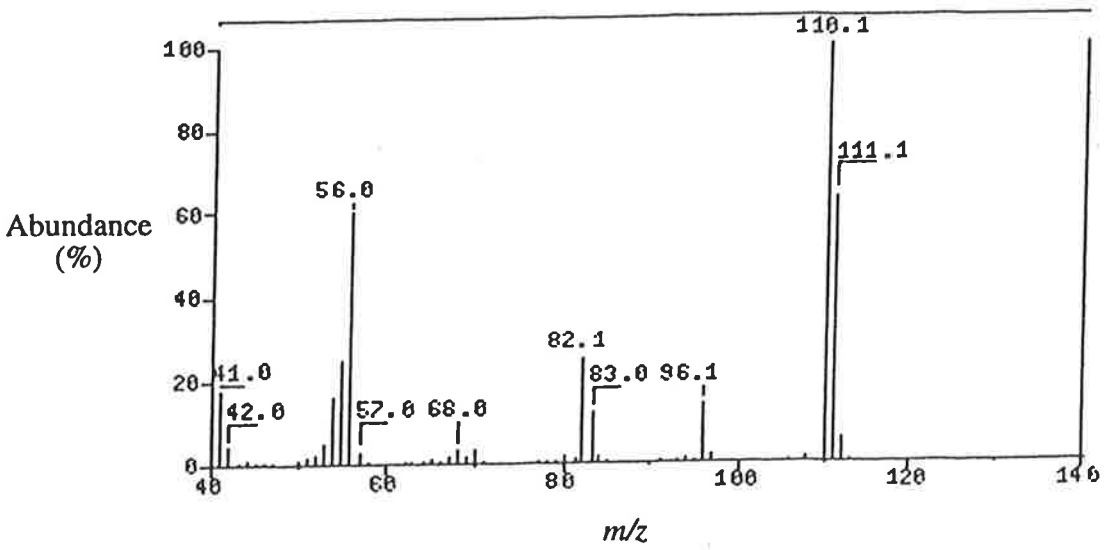
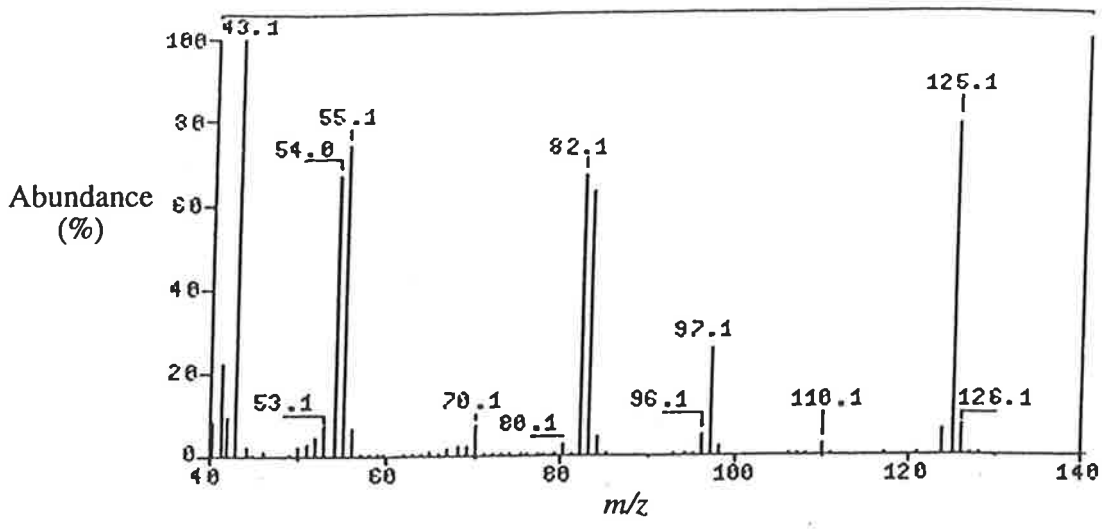


Figure 4.2 Mass spectrum of synthetic 2-ethyltetrahydropyridine (ETPY, *m/z* 111). Both tautomers exhibited almost identical mass spectra

Tautomer 1



Tautomer 2

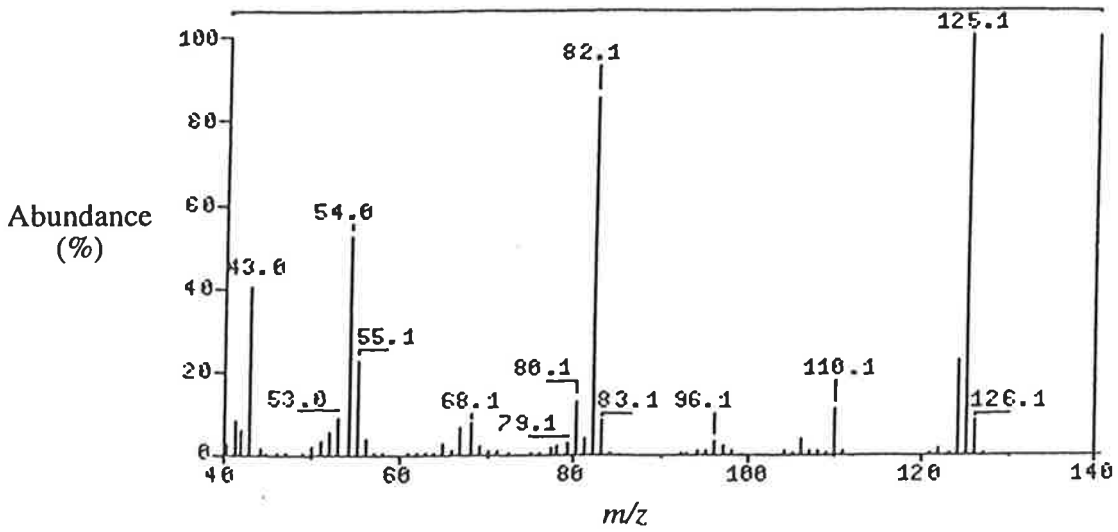
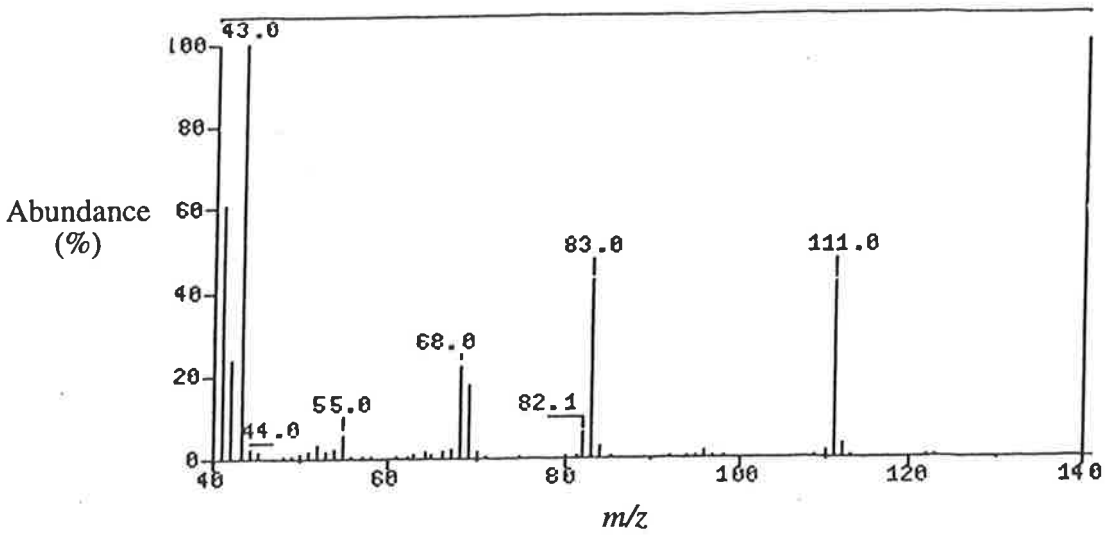


Figure 4.3 Mass spectra of tautomers of synthetic 2-acetyltetrahydropyridine (ACTPY, *m/z* 125)

Tautomer 1



Tautomer 2

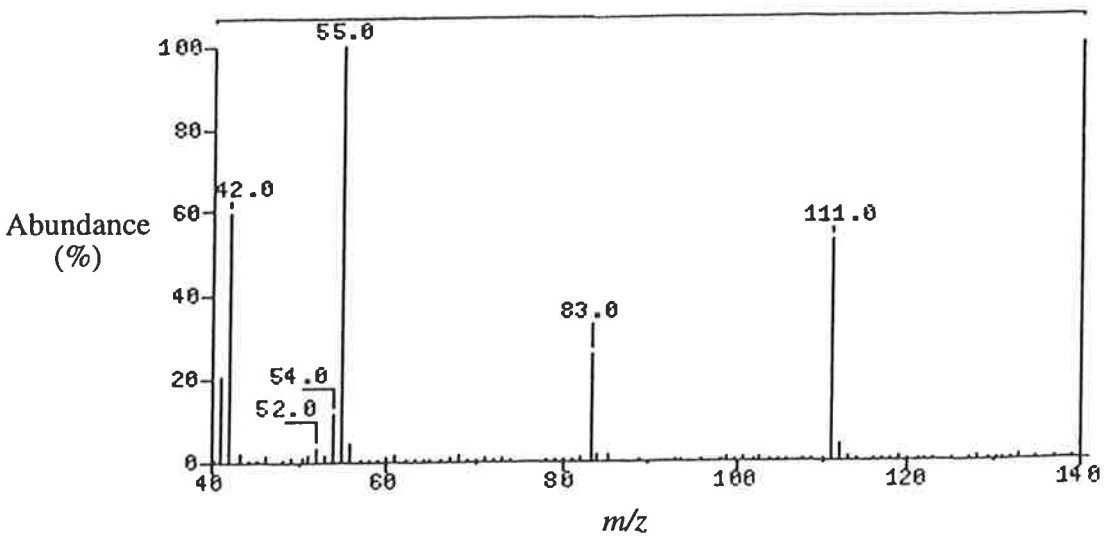


Figure 4. 4 Mass spectra of tautomers of synthetic 2-acetylpyrroline (ACPY, *m/z* 111)

Table 4.3 Comparison of mass spectra, retention indices and aroma descriptions of reference mousy compounds

Compound*	Mass spectrum (EI) *m/z* (%)	Retention index††	Aroma description†††
ETPY I			
Current study	*111* (60)†, 110 (100), 96 (15), 82 (25), 56 (60), 55 (25), 54 (18), 41 (20)	1217	mousy #
Other reports:	*111* (42), 110 (62), 96 (15), 82 (43), 56 (100), 55 (50), 41 (61) [e]	N.A.##	mousy [e]
ETPY II			
Current study	*111* (60), 110 (100), 96 (15), 82 (25), 56 (60), 55 (25), 54 (18), 41 (20)	N.D.###	N.D.
ACPY I			
Current study	*111* (45), 83 (45), 69 (20), 68 (25), 43 (100), 42 (25), 41 (60)	1328	mousy
Other reports	*111* (5), 69 (11), 67 (0.5), 55 (2), 54 (0.5), 52 (0.9), 43 (100),42 (24), 41 (50) [b]	1320**, [b,a]	mousy [q] cooked rice [b]
	111 (5), 83 (11), 69 (11), 68 (8), 67 (0.2), 54 (0.2), 55(2), 52 (0.9), 43 (100), 42 (24), 41 (50) [a]	1306 [o]	roasty, popcorn-like [k] roasty [o, p]
	111 (35), 83 (60), 69 (36), 68 (39), 43 (100), 41 (100) [k]		roasty, sweet [f, n] popcorn [a, d] cracker-like [j, d]
ACPY II			
Current study	*111* (55), 83 (25), 55(100), 54 (10), 42 (60), 41 (20)	1427	N.D.
ACTPY I			
Current study	*125* (80), 83 (65), 82 (70), 55 (75), 54 (68), 43 (100)	1456	mousy, cracker-like#
Other reports	*125* (62), 124 (12), 92 (10), 83 (40), 82 (76), 55 (64), 54 (72), 53 (10), 43 (100), 42 (8), 41 (22), 40 (32) [i]	1433 [k(***), o]	mousy [r] mousy; tending bread-like at lower intensity [g]
	43, 55, 82, *125*, 97, 70 [c]		bready, cracker-like [h, t] cracker-like [m, j, s]
	126 (6), *125* (47), 121 (4), 110 (3), 97 (6), 83 (34), 82 (64), 55 (50), 54 (70), 43 (100), 41 (21) [r]		roasty (savoury cracker) [l] roasted [n] roasty, popcorn-like [k]
ACTPY II			
Current study	*125* (100), 83 (9), 82 (88), 55 (28), 54 (55), 43 (40)	1675	mousy, cracker-like#
Other reports	82, *125*, 54, 43, 110, 68 [c]	1655 [k(***), o]	mousy [g, r] roasty, popcorn-like [k]

† Italicized figure denotes molecular ion

†† Retention data calculated from the retention times of alkanes. Other literature data reported using similar GC conditions.

††† Aroma description assigned at sniffing port of GC unless otherwise indicated

Aroma description assigned to diluted or neat reference compound.

N.A. Comparative literature data not available

N.D., Not determined

* The first and second tautomers of 2-ethyltetrahydropyridine (ETPY I & II), 2-acetylpyrroline (ACPY I & II) and 2-acetyltetrahydropyridine (ACTPY I & II) assigned according to GC elution order.

** Retention index reported as Kovats Index

*** Retention index calculated using a program for cubic spline interpolation.

[a] Buttery *etal.* (1983b); [b] Buttery *et al.* (1982);

[c] Buttery and Ling (1995): data reported for 2-acetyl-1,4,5,6-tetrahydropyridine and 2-acetyl-3,4,5,6-tetrahydropyridine for ACTPY I and ACTPY II respectively: MS data reported with most intense ions first.

[d] Buttery *et al.* (1994); [e] Craig and Heresztyn (1984); [f] Gasser and Grosch (1988); [g] Heresztyn (1986); [h] Hunter *et al.* (1969)

[i] De Kimpe and Stevens (1993): MS data only reported for 6-acetyl-2,3,4,5-tetrahydropyridine (2-acetyl-3,4,5,6-tetrahydropyridine)

[j] Schieberle (1990a); [k] Schieberle (1991); [l] Schieberle and Grosch (1983); [m] Schieberle and Grosch (1985); [n] Schieberle and Grosch (1987b); [o] Schieberle and Grosch (1991)

[p] Schieberle and Grosch (1992); [q] Seitz *et al.* (1993); [r] Strauss and Heresztyn (1984): both tautomers of ACTPY reported to have similar mass spectra; [s] Teranashi *et al.* (1975); [t] Tressl *et al.* (1981b)

4.3.2 Development of a procedure for the quantitative extraction of mousy compounds.

Extensive investigations were undertaken to develop a sufficiently reliable procedure for the artefact-free extraction and quantitative recovery of mousy compounds from aqueous media and wine. Two methods initially investigated for the extraction of ACTPY were ion exchange chromatography and simultaneous steam distillation and extraction. Two other methods, continuous liquid-liquid extraction with subsequent simultaneous steam distillation and extraction, and finally an optimized continuous liquid-liquid extraction method were also assessed.

4.3.2.1 Ion exchange chromatography and simultaneous steam distillation and extraction

Table 4.4 shows that simultaneous steam distillation and extraction (SDE) provided greater recovery of ACTPY (80.5%) from aqueous buffer than that obtained by ion exchange chromatography (46.8%). Another advantage of the SDE method was its relative simplicity, thus indicating the potential of this technique for its use throughout this study. Further validation of this method was therefore undertaken by surveying a range of full-bodied red and white table wines for the presence of ACPY and ACTPY after extraction by SDE. Analysis of the wine extracts by GC-MS (Table 4.5) indicated that ACTPY was detected in all five white wines in the range 29.1–353.1 μg/L, while ACPY was detected in two of the white wines in the range 5.2–11.1 μg/L. The two red wines tested, however, did not contain any detectable levels of either ACPY or ACTPY. Somewhat surprisingly, however, sensory assessment revealed that none of these wines exhibited mousy off-flavour.

An experiment was thus undertaken to determine whether the SDE process could generate mousy compounds as artefacts. Such thermal generation of ACTPY and ACPY was demonstrated by subjecting a model solution containing proline and fructose to boiling and extraction by SDE (Table 4.6). The high concentration of ACTPY (1,226 μg/L) as well as the significant amount of ACPY (31 μg/L) generated by this system suggested that the SDE method could also cause false positive results in the analysis of wine and fermentation media. The SDE method was therefore considered unsuitable for reliable analysis of mousy compounds in this study.

Table 4.4 Comparison of cation exchange and simultaneous steam distillation and extraction (SDE) methods for the recovery of 2-acetyltetrahydropyridine (ACTPY) from spiked aqueous media

Extraction method	Recovery of ACTPY (%)††
SDE†	80.5
Ion exchange†	46.8

† See Methods section for respective methodologies

†† Recoveries determined from the relative concentration of ACTPY in spike solution before and after extraction

Table 4.5 Determination of 2-acetyltetrahydropyridine (ACTPY) and 2-acetylpyrroline (ACPY) in commercial table wines by GC-MS analysis† of extracts obtained by simultaneous steam distillation and extraction (SDE).

Wine sample	ACTPY (µg/L)	ACPY (µg/L)	Sensory detection of mousy off-flavour††
White wines			
Chardonnay (92A)	353.1[a]	5.2[b]	-
Chardonnay (92B)	131.9	11.1	-
Chardonnay (90C)	82.1	nd	-
Chardonnay (91D)	44.9	nd	-
Semillon (91A)	29.1	nd	-
Red wines			
Cabernet sauvignon (89A)	nd	nd	-
Shiraz (91A)	nd	nd	-

† GC-MS analysis performed using Carbowax 20 CAM column. Concentrations of ACTPY and ACPY calculated from the ratio of total ion responses of target compounds to internal standard (2-acetylpyridine; 51.2 µg/L).

†† Sensory (taste) assessment of mousy off-flavour performed by a panel of 5 experienced wine tasters. Relative off-flavour level: -, no off-flavour; +, weak; ++, moderate; +++, strong.

[a] average of duplicate extractions

[b] nd, not determined

Table 4.6 Generation of 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by simultaneous distillation / extraction (SDE) of model wine solutions containing proline and fructose†

N-heterocycle	Concentration (µg/L)
ACPY††	31 (+/- 4)
ACTPY††	1,226 (+/- 103)

† Proline (4.6 g/L) and fructose (3.6 g/L) were boiled in a phosphate buffer (pH 8.0) and continuously steam distilled and extracted (see Materials and Methods for details).

†† Concentrations of ACPY and ACTPY calculated from the ratio of total ion responses of target compounds to internal standard (2-acetylpyridine; 51.2 µg/L). GC-MS analyses undertaken with Carbowax 20 CAM column. Values presented are average of duplicate assays, figures in brackets denote range.

4.3.2.2 Continuous liquid-liquid extraction with subsequent simultaneous steam distillation and extraction

A more complex approach to the extraction of off-flavour compounds, ie. continuous liquid-liquid extraction with subsequent simultaneous steam distillation and extraction (CLLE-SDE), was examined in an endeavour to avoid the formation of artefacts from SDE alone. It was also anticipated that the final stage of SDE would provide a cleaner extract of basic volatile compounds than that of liquid-liquid extraction, hence prolonging the life of the sensitive Carbowax 20 CAM column.

Assessment of this technique by GC-MS analysis of extracts from a Rhine Riesling wine revealed that it did not produce ACTPY as an artefact (Table 4.7). However, the recoveries of ACTPY and first internal standard (3-acetylpyridine) spiked into the same wine were only 34% and 18.9% respectively (Table 4.7), which thus rendered the technique unsuitable.

4.3.2.3 Optimized continuous liquid-liquid extraction method

A modified continuous liquid-liquid extraction (CLLE) method was also assessed for the extraction of mousy compounds. Apart from exclusion of the SDE stage, other modifications introduced with this technique included saturation of samples with NaCl to aid extraction of organic compounds, use of a water - jacketed Vigreux column to provide greater control over the rate of reflux of Freon 11 during concentration of extracts, and final ten-fold concentration of the extract into *iso*-octane prior to GC-MS analysis. As shown in Table 4.8, the optimized CLLE method provided a satisfactory and artefact - free extraction of ACPY and ACTPY from a spiked Rhine Riesling wine. Slight variations in the quantification and recoveries of target compounds occurred depending upon the source of GC-MS data used, ie. peak area or height from total ion response or molecular ion chromatograms. In addition, the recovery of ACTPY from the spiked wine (87.8% by peak area of the molecular ion) was efficient while that of ACPY, however, was elevated (152.1 % by peak area of the molecular ion). Moreover, the recovery of the first internal standard (4-acetylpyridine: 73.2% by peak area of the molecular ion) demonstrated that the CLLE procedure provided a sufficiently acceptable rate of extraction for quantitative analysis.

Table 4.7 Recovery of 2-acetyltetrahydropyridine (ACTPY) from white wine using continuous liquid - liquid extraction with subsequent simultaneous distillation / extraction (CLLE-SDE).

Sample	ACTPY (µg/L)	Recovery of ACTPY (%)	Recovery of 3-acetylpyridine (%)†††
Control wine	nd**	-	nd
Wine plus ACTPY†,*	9.6	34.0	18.9

† Wine spiked with ACTPY (28.2 µg/L) and IS (3-acetylpyridine, 33.1 µg/L) from an ethanolic stock solution. Concentration of ACTPY in stock solution determined by GC analysis at time of addition to wine and calculated with respect to 3-acetylpyridine

* Results from single analysis only

** nd, not detected

†† Concentrations of ACPY and ACTPY calculated from the ratio of total ion responses of target compounds to internal standard

††† Recovery of 3-acetylpyridine calculated with respect to second internal standard (4-acetylpyridine) added after SDE

Table 4.8 Quantification† and recovery†† of 2-acetyl-1-pyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY), and recovery of 4-acetylpyridine from duplicate samples of spiked and non-spiked Rhine Riesling wine* after extraction using an optimized continuous liquid - liquid extraction (CL-LE) technique.

Sample Type	Data Source	ACPY			ACTPY			Recovery of 4-acetylpyridine†††	
		µg/L	Range (%)	Recovery †† (%)	µg/L	Range (%)	Recovery †† (%)	%	Range (%)
Spiked Wine	*Peak area*:								
	RIC	29.6	17.2	120.2	37.7	9.8	90.0	74.0	7.0
	Molecular ion	38.3	17.5	152.1	37.3	11.5	87.8	73.2	10.6
	Peak height:								
	RIC	32.1	29.7	115.8	42.2	1.8	96.6	77.7	7.0
	Molecular ion	37.5	15.2	136.3	39.0	10.1	89.2	76.7	9.8
Non-spiked Control Wine	*Peak area*:								
	RIC	nd**	-	-	nd	-	-	nd	-
	Molecular ion	nd	-	-	nd	-	-	nd	-
	Peak height:								
	RIC	nd	-	-	nd	-	-	nd	-
	Molecular ion	nd	-	-	nd	-	-	nd	-

* Duplicate lots of spiked and non-spiked wine samples were extracted and analysed

† Concentrations of ACPY and ACTPY in wine extracts were determined by GC-MS (Carbowax 20 CAM column) analysis and calculated from the ratio of total (RIC) or molecular ion responses to respective responses of internal standard (4-acetylpyridine). Calculations involving molecular ion data utilized appropriate relative response factors (RRf's). ACPY and ACTPY concentrations expressed as the sum of tautomers. Final data were averaged from analysis of duplicate assays

†† Recovery of ACPY and ACTPY calculated with respect to concentration of each compound in spike solution predetermined by GC analysis.

††† Recovery of 4-acetylpyridine determined with respect to second internal standard (3-acetylpyridine).

** nd, not detected

4.3.3 Quantitative analysis of mousy off-flavour compounds in mousy wines

The latter optimized CLLE method was used for the extraction of off-flavour compounds from five mousy wines (four red wines and one white wine) (Table 4.9). Interestingly, ACTPY was detected in each wine; the concentration ranged from 0.7 µg/L to 39.0 µg/L for four wines while that of a fifth wine (1R) was significantly higher at 106 µg/L. On the other hand, ACPY was only detected in three red wines; 1T (7.8 µg/L), 1R (7.1 µg/L) and 1O (trace amount). 2-Ethyltetrahydropyridine was also detected in three wines (two red wines (1O and 1I) and the white wine (1S)), and occurred over a concentration range similar to that of ACPY (trace amounts to 4.5 µg/L).

Table 4.9 Quantification of 2-acetylpyrroline (ACPY), 2-acetyltetrahydropyridine (ACTPY) and 2-ethyltetrahydropyridine (ETPY) in mousy and non-mousy wines[a] by GC-MS after continuous liquid-liquid extraction.

Wine type	Concentration (µg/L)		
	ACPY	ACTPY	ETPY
Mousy wines			
Red wine (1T)†	7.8	4.8	nd
Red wine (1R)†	7.1	106.0	nd
Red wine (1O)	tr††	39.0	1.9
Red wine (1I)	nd†††	14.7	4.5
White wine (1S)	nd	0.7	tr
Non-mousy wines			
White wine (1T)	nd	nd	ND

[a] Wines determined as mousy or non-mousy by experienced wine tasters familiar with the off-flavour

† Wine samples extracted without NaCl addition.

†† tr, trace amount

††† nd, not detected (< 0.1 µg/L)

* ND, not determined

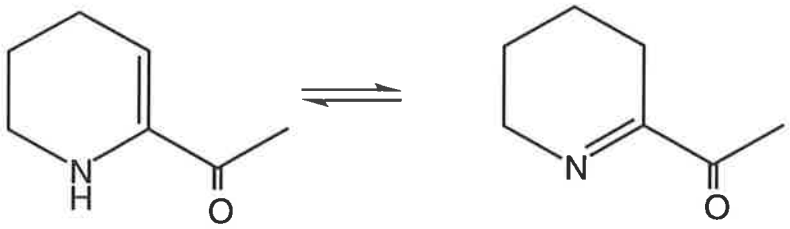
4.4 DISCUSSION

While the experiments of this chapter have focused upon the development of a procedure for quantitative analysis of mousy off-flavour compounds, some important chromatographic and chemical features of these compounds have also been revealed. First, poor resolution and instability of ACTPY encountered during initial GC analysis using the 1701 column was similarly described by Schieberle and Grosch (1991). Further, both the current study and that of Schieberle and Grosch (1991) also found that separation of ACTPY tautomers was enhanced with amine specific fused silica HRGC columns (Carbowax 20 CAM (Carbowax Amine Modified) and CP-Wax deactivated for amines, respectively). In contrast to the difficulties associated with the chromatography of ACTPY, Schieberle and Grosch (1991) found that ACPY was suitably chromatographed on several columns. An earlier report by Buttery *et al.* (1983b), however, points out that ACPY displayed considerable instability to general GC conditions and that better stability was obtained using an Amine 220 packed column or an all-glass capillary GLC system. This apparent conflict relating to the chromatography of ACPY may reflect the generally superior performance of current HRGC columns compared to those available a decade or so ago, as more recent studies by Buttery and co-workers in fact utilised a fused silica DB-1 capillary column for separating ACPY (Buttery *et al.* 1994, Buttery and Ling, 1995). It is worthy to note that the latter DB-1 column also separated both tautomers of ACTPY (Buttery and Ling, 1995). The importance of using an appropriate GC column for the analysis of such unstable compounds was highlighted by Buttery *et al.* (1983b), who suggested that the inability to detect ACPY in his previous studies of rice volatiles may have been caused by use of columns that were unable to chromatograph the compound. It could be further speculated that this latter reasoning may also explain why Tucknott (1977) was unable to detect ACTPY in earlier studies of mousiness in alcoholic cider (see later section of Discussion).

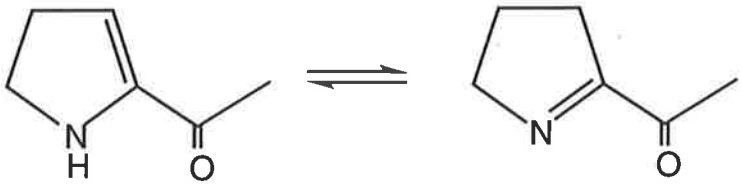
An interesting finding from the GC-MS analysis of ACPY in this study has been the consistent appearance of a possible second, albeit minor, tautomer or other isomer of ACPY. This second peak eluted soon after the main ACPY peak and exhibited the same molecular ion (*m/z* 111) and similar MS to that of reference ACPY (Table 4.3). The suggestion of a second isomeric form of ACPY, however, is in direct contrast with most other reports concerning the analysis of this compound, which generally concur that ACPY, unlike ACTPY, chromatographs as one peak (Buttery *et al.* 1983b, Schieberle 1991, Schieberle and Grosch 1991, Buttery and Ling 1995). While the appearance of a second ACPY peak in this study is not fully explainable, it may be possible that the Carbowax 20M CAM column used here has provided superior chromatography and resolution of ACPY compared to columns used by others. Such a view is further supported by the tentative identification of a second minor peak of ETPY, which also has not been reported by other investigators (Craig and Heresztyn 1984). Moreover, it is interesting that two recent studies of alternative syntheses of ACPY

provide conflicting views as to the formation of a second isomer of ACPY. In one study, De Kimpe and Keppens (1996) revealed that the enamino tautomer of ACPY, ie. 2-acetyl-2-pyrroline was not a product of their synthesis procedure, and contested the results of a similar synthesis by Duby and Huynh (1993), in which the formation of a minor amount of 2-acetyl-2-pyrroline (3%) was reported to occur. Further chemical studies are required to confirm or otherwise the existence of separable second isomers of ACPY and also of ETPY as depicted in Figure 4.5.

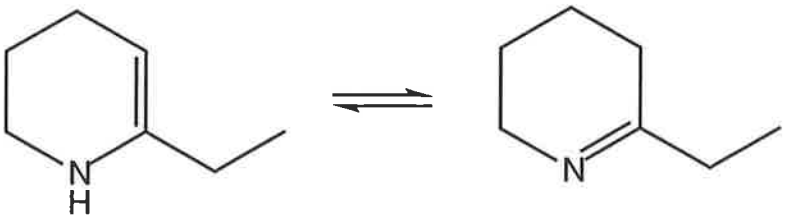
In addition to the difficulties associated with the GC analysis of these compounds, their highly labile nature and rapid degradation also posed considerable problems with purification, quantitation and storage. This was highlighted by the several unsuccessful attempts at purifying ACTPY (Table 4.1) in which it was found that the purified material rapidly darkened after concentration and exposure to air and also during storage at -20 °C. Such instability, particularly in concentrated form, has previously been documented for ACPY and ACTPY (Büchi and Wüest 1971, Buttery *et al.* 1982, Buttery *et al.* 1983b, De Kimpe and Keppens 1996). The instability of ACPY was speculated by Buttery *et al.* (1983b) to be caused by formation of a conjugated pyrroline polymer. Similar polymerization may also occur with ACTPY. Further, the ring structures 1-pyrroline and 1-piperideine are also known to be very reactive and to rapidly trimerize (Bock and Dammel 1987). In contrast to these reports, Craig and Heresztyn (1984) found that although ETPY darkened after six weeks at 5 °C, no changes were observed to occur in its purity. The current study has shown, however, that the degradation of ACTPY during storage at -20 °C could be limited by diluting the freshly vacuum distilled compound in ethanol (VDF 2.2, Tables 4.1 and 4.2). This approach was also recommended by Buttery *et al.* (1983b) and De Kimpe *et al.* (1993) for restricting the instability of ACPY, and presumably prevents rapid degradation by restricting the rate of polymerization. In addition to polymerization, the ring structure of off-flavour compounds may also undergo oxidative transformation. This was evidenced by the appearance of 2-acetylpyridine in older and highly degraded reference solutions of ACTPY (data not shown), which negated its use as an internal standard. It follows, therefore, that similar oxidative transformation of ACPY and ETPY could occur, yielding the respective end-products of 2-acetyl-pyrrollidine and 2-ethylpyridine.



2-acetyl-1,4,5,6-tetrahydropyridine 2-acetyl-3,4,5,6-tetrahydropyridine



2-acetyl-2-pyrroline 2-acetyl-1-pyrroline



2-ethyl-1,4,5,6-tetrahydropyridine 2-ethyl-3,4,5,6-tetrahydropyridine

Figure 4.5 Isomeric forms of 2-acetyltetrahydropyridine, and those proposed for 2-acetylpyrroline and 2-ethyltetrahydropyridine

In view of the above mentioned problems of degradation of the purified reference compounds, it is worthy to note that a highly effective means of stabilizing ACPY and ACTPY for extended periods is via formation of a bisulfite or hydrochloride salt (Büchi and Wüest 1971, Hunter and Walden 1971, 1973, De Kimpe and Stevens 1993). Further, although the relative amount of ACTPY in the bisulfite adduct produced in this study was low (9%, Table 4.1), due possibly to the presence of inorganic material, this procedure can provide further purification by separation of other degradation products. Such purified, stable forms of the acetylated N-heterocycles would provide a convenient source of starting material for future investigations including studies of sensory thresholds in wine.

The propensity for bisulfite adduct formation by ACTPY, as supported by the results of FAB/MS analysis (Figure 4.3), strongly suggests that ACTPY, and possibly ACPY, could also occur in the adduct form in wine. Such adduct formation would be plausible, particularly in wine containing free bisulfite, as other carbonyl compounds such as acetaldehyde undergo similar complexation.

Further to the chemical and chromatographic instability of the off-flavour compounds, considerable difficulty was encountered in the development of a suitable quantitative and artefact-free extraction procedure. It is worthy to emphasize here the critical role of GC-MS as an essential analytical tool for the analysis of off-flavour compounds, particularly of extracts from complex media such as wine. Even though extraction procedures may remove much interfering material, the vast array of compounds extracted from wine necessitates the selectivity and sensitivity of analysis by GC-MS.

One of the initial methods tested, ie. cation exchange, was previously described by Heresztyn (1986) for the extraction of mousy off-flavour compounds, yet was found here to give inefficient recovery of ACTPY from aqueous media (46.8 %, Table 4.4). The SDE method, on the other hand, which had previously been used extensively for the extraction of flavour compounds including ACPY and ACTPY from a variety of foods (Buttery *et al.* 1983b, 1986, Schieberle and Grosch 1987a, Gasser and Grosch 1988, Schieberle 1995), gave a more favourable recovery of ACTPY (80.5%, Table 4.4) and was also a relatively quick (2h per sample) and straightforward procedure. Despite these attributes, however, the validity of the SDE method applied to wine became questionable after ACTPY and ACPY were extracted, in some cases at high concentration, from a number of non-mousy (white) wines. That these compounds could be thermally generated during SDE was confirmed in a short experiment based upon that described by Schieberle (1990c), whereby a large quantity of ACTPY (1,226 μg/L) and minor amount of ACPY (31 μg/L) were produced after SDE of an aqueous model wine mixture containing proline and fructose (Table 4.6). Schieberle's (1990c) study also found that ACPY was a major end product of the heating of an aqueous solution of ornithine and fructose. It is well known that these and other important flavour

compounds can be thermally generated through the Maillard reaction between amino acids, sugars and other intermediates, and is the subject of considerable research by such authors as Tressl *et al.* (1981a, 1981b, 1986) and Schieberle (1990a,b,c, 1995). Residual levels of sugars and amino acids or other wine components could thus serve as reactants for Maillard type formation of ACPY and ACTPY as artefacts during SDE, thus rendering the method invalid for extraction of wine. Interestingly, the relatively high concentration of ACTPY generated by the proline / fructose model system suggests that this procedure has potential for the rapid synthesis of reference off-flavour compounds.

In order to prevent artefact formation from SDE, several experiments were undertaken to investigate the efficacy of continuous liquid-liquid extraction (CLLE) for the extraction of mousy compounds. Preliminary work showed that methylene chloride was an unsuitable solvent for CLLE of wine samples due to the extraction of large quantities of organic material (data not shown), which could potentially overload the sensitive Carbowax 20 CAM column. Further testing with CLLE utilised the non-polar, low boiling point solvent Freon 11. The third extraction method tested in this chapter, CLLE-SDE, combined prior liquid-liquid extraction of basic compounds with a final SDE stage. Although this combination of procedures was successful in preventing artefact formation (as ACTPY) in an unspiked control wine, it yielded unacceptably poor recoveries of ACTPY and was also highly labour intensive and tedious to perform.

Despite the shortcomings of the previous extraction methods, efficient and artefact-free extraction of off-flavour compounds from wine was achieved with an optimized continuous liquid-liquid extraction (CLLE) procedure similar to that described by Heresztyn (1984) for the extraction of amino acid esters in wine. Modifications of this method which aided in its efficacy included the use of a water-jacketed Vigreux column. This apparatus was essential in our laboratory for maintaining sufficient reflux during the concentration of Freon 11 extracts, which thus also minimized losses of the highly volatile off-flavour compounds. Another feature was the final ten-fold concentration of the extract into 10 µL of *iso*-octane, which increased the final sensitivity of subsequent GC-MS analyses (0.1 µg/L).

The accuracy and precision of the recoveries of ACPY and ACTPY in spiked and non-spiked Riesling wine using the CLLE procedure was considered satisfactory for the purposes of this study. However, despite the excellent recovery of ACTPY (approximately 90%), the elevated recovery observed for ACPY (approximately 150%) suggests that further testing is required to more fully validate this procedure. Alternatively, the inaccurate recovery of ACPY could also be a result of the inherent instability of this compound, particularly in concentrated form, although it is uncertain whether ACPY is relatively more unstable than ACTPY. Moreover, another drawback of the CLLE method was that it was relatively tedious to undertake, with a

batch of four to six samples taking two to three days to extract and concentrate prior to GC-MS analysis.

Other procedures for the extraction of mousy compounds were also investigated, including headspace analysis and solid phase (C-18) extraction, although they were not further utilised in this study due to poor recovery of target compounds (data not shown). Nevertheless, greater accuracy and precision of the quantitative analysis of mousy compounds could be achieved through the use of a stable isotope dilution assay (SIDA). Using stable (deuterated) isotopes of respective target compounds for internal standards, this elegant technique was previously described by Schieberle and Grosch (1987b) and Schieberle (1995) for the quantification of ACPY, ACTPY and other odorants from bread crust and popcorn. A major advantage of SIDA was highlighted in the studies of Schieberle and Grosch (1987b), who demonstrated excellent recoveries (around 100%) of target compounds in spite of poor yields from the extraction process, eg. 12% and 32% for ACPY by vacuum sublimation and SDE respectively. Potential protium / deuterium exchanges during the analytical procedure were reported by the above authors to proceed very slowly, if at all, and were not considered to hinder the overall quantitation. However, the necessity to synthesize stable isotopes of the target compounds was a major drawback to the use of SIDA in the current study. Nevertheless, this technique would be advantageous for future studies and would allow the use of more rapid extraction techniques including reverse phase (C-18) or head space techniques, which were briefly examined here (data not shown) but gave less efficient recovery than CLLE.

In the final experiment of this chapter, the CLLE procedure has enabled the first quantitative study of off-flavour compounds in mousy and non-mousy wines to be undertaken. In addition, use of this procedure has revealed several important features of the chemical basis of mousy off-flavour. Foremost, mousy off-flavour may be due to at least three different structurally related nitrogenous heterocyclic bases, ie. ACPY, ACTPY and ETPY, which extends earlier findings attributing off-flavour to either ETPY (Tucknott 1977, 1978) or ACTPY (Strauss and Heresztyn 1984, Heresztyn 1986). It is of major significance that the present study has discovered, for the first time, the occurrence of ACPY in wine and, specifically, wine spoiled by mousy off-flavour. That ACPY can be identified as another key off-flavour compound was further evidenced by the mousy aroma property ascribed to it through GC-sniff assessment (Table 4.3). These data support recent work by Seitz *et al.* (1993), who similarly attributed the formation of undesirable mousy aroma in wetted ground pearl millet to the presence of ACPY. Although the concentration of ACPY in mousy wine was generally lower than that of ACTPY (maxima of 7.8 µg/L and 106 µg/L respectively), its higher potency renders its occurrence in wine of major significance; the aroma threshold of ACPY in water (0.1 µg/L) is over ten times lower that of ACTPY (1.6 µg/L) (see Literature Review).

From the survey of wines, the off-flavour compound most commonly detected and which occurred over the broadest concentration range was ACTPY. This supports the previous work of Strauss and Heresztyn (1984) and Heresztyn (1986) who regarded ACTPY as the major cause of mousy off-flavour. Interestingly, the current survey also showed the occurrence of ETPY, albeit at low concentration (< 5 µg/L), in two mousy wines. Moreover, despite the taste threshold of ETPY (150 µg/L in wine) indicating that it would have little sensory impact, the detection of other off-flavour compounds in association with ETPY suggests that it could contribute to the overall mousy character. While lending weight to the earlier claim of Tucknott (1977) that ETPY caused mousy off-flavour in alcoholic cider, the current findings also support the conclusions drawn by Craig and Heresztyn (1984) that mousy off-flavour was possibly due to several compounds, either individually or in combination, and ETPY may be involved in some instances. The irregular occurrence and low concentration of ETPY from the current survey suggests that the inability of Craig and Heresztyn (1984) to detect ETPY in several mousy wines may have been due to insufficient sensitivity of their analytical technique (< 20 µg/L) or, alternatively, that it was absent from the samples analysed.

It should be noted that the divergence in sensory data now reported for ACPY, and also for ACTPY, ie. mousy as well as cracker-like, roasty etc, may arise from differences in relative concentration. This was intimated from GC-sniff assessment of ACPY, whereby the main peak was mousy, yet regions both prior to and after exhibited cracker and crust-like aromas. Heresztyn (1986) made similar observations during GC-sniff assessment of ACTPY, and experiments from the previous chapter showed that LAB cultures often exhibited cracker-like and bready aromas, particularly when mousy aroma was negligible or did not occur. Other explanations for the divergence in aroma could relate to matrix effects in different foods and beverages, or the wide variation in sensitivity towards mousy aroma (from ACTPY) noted between individuals (data not shown). Further sensory studies are thus required to more fully elucidate the reasons for the apparent dichotomy of aroma descriptions given to the off-flavour compounds.

Finally, the variation in type and concentration of mousy compounds observed in the survey of wines from this study could be a result of the proliferation of different species and strains of off-flavour-producing microorganisms, such as LAB, yeast (*Brettanomyces* spp.) or other microflora. To this end, the quantification method developed in this chapter will be subsequently utilised to study more fully the range and concentrations of off-flavour compounds that are produced by wine bacteria, in particular LAB.

4.5 SUMMARY

Optimal GC separation and resolution of the two tautomers of ACTPY was achieved with a Carbowax 20 CAM column. Use of this column in conjunction with GC-MS revealed that two other structurally related mousy compounds, ETPY and ACPY, may also display more limited tautomerization.

Rapid degradation of reference ACTPY occurred following purification and exposure to air, rendering quantification extremely difficult. This degradation could be restricted by diluting ACTPY in ethanol and storing at -20 °C. Another convenient method for stabilizing reference ACTPY for long term storage was preparation of the bisulfite adduct.

A continuous liquid-liquid extraction procedure was developed and used in conjunction with GC-MS to enable efficient, reproducible and artefact-free quantification of low concentrations (µg/L level) of off-flavour compounds from wine. Using this procedure, a survey of wines was undertaken revealing that three structurally related N-heterocycles, ACTPY, ETPY and a newly discovered and highly potent off-flavour compound, ACPY, occurred in mousy wines, either singularly or in combination. Of these, ACTPY was the most common and occurred at the highest concentration (106 µg/L). Both ACPY and ETPY occurred less frequently and at lower concentration (tr -7.8 µg/L and tr-4.5 µg/L). The mousy aroma characteristic of ACPY was confirmed by GC-sniff assessment.

5. PRODUCTION OF MOUSY OFF-FLAVOUR COMPOUNDS BY WINE BACTERIA

5.1 INTRODUCTION

Previous studies of the bacterial formation of mousy off-flavour (Tucknott 1977, Heresztyn 1986) identified certain *Lactobacillus* spp. as key off-flavour-producing microorganisms, while Heresztyn (1986) further identified the important mousy compound, ACTPY, that was produced by a strain of *L. brevis*. Significantly, the results of the present study have indicated that a range of other LAB strains including *O. oeni* and, to a lesser extent, *Pediococcus* spp., were also capable of off-flavour formation and, furthermore, that two other N-heterocyclic bases in addition to ACTPY, ie. ACPY and ETPY, may also be produced and could contribute to this spoilage. Despite these important findings, quantitative studies of the abilities of wine bacteria to produce mousy compounds are lacking. Moreover, the need to investigate the production of mousy off-flavour compounds other than ACTPY by wine bacteria is highlighted by the first report of the bacterial (*Bacillus cereus*) formation of ACPY (Romanczyk *et al.* 1995).

In the initial experiment of this chapter, a range of wine bacteria were surveyed for the ability to produce ACTPY as well as ACPY and ETPY in a complex (Carr-MEt) medium using the quantitative GC-MS procedure developed in Chapter 4. A second experiment tested the abilities of four selected LAB strains to produce off-flavour compounds in a chemically defined medium.

5.2 MATERIALS AND METHODS

5.2.1 Species and strains of wine bacteria

Ten different wine bacteria listed in Table 5.1 comprising nine representative strains and species of LAB genera commonly associated with wine and an AAB strain were screened for the production for mousy compounds. Of the LAB tested, four were used as reference mousy-producing strains. These included *O. oeni* Lc5b and *L. hilgardii* L21c, which displayed strong mousy off-flavour producing ability in Chapter 3; *Lactobacillus brevis* IV-1 which had been previously reported by Heresztyn (1986) to produce mousiness and ACTPY in a grape juice medium, and the type strain of *Lactobacillus hilgardii* (DSM 20176), originally considered to cause spoilage and mousy off-flavour in Californian red wines (Douglas and Cruess 1936), was also obtained. In addition, four other strains of *O. oeni* were tested including three isolates of commercially produced MLF strains; *O. oeni* Lo-42, *O. oeni* MCW and *O. oeni* Viniflora, as well as *O. oeni* Lc5c from the AWRI culture collection. Another LAB strain, tentatively identified as *Pediococcus* spp. HV, was isolated from a mousy red wine (1R) which was described in Chapter 4. Methods for the isolation and tentative identification of LAB are given in Appendix 5.1, and those for the isolation of *O. oeni* strains from commercial MLF preparations are given in Appendix 5.2. The tenth bacterium to be tested in this chapter was an AAB strain, *Gluconobacter oxydans* Gb-86 (refer to Appendix 5.3 for method of culture for this strain).

In the first experiment of this chapter, each of the above 10 bacteria strains was tested for mousy compound formation in Carr-MEt medium. In a second experiment, four of these strains, *O. oeni* Lc5b, *O. oeni* MCW, *L. hilgardii* DSM 20176 and *L. hilgardii* IV-1, were further tested for taint compound formation in a chemically defined test medium.

Table 5.1 Species and strains of wine bacteria screened for the production of mousy off-flavour compounds.

Genus / species	Strain reference	Source / manufacturer
Oenococcus oeni	Lc5b	AWRI[1]
" "	Lc5c	AWRI
" "	Lo-42	Condimenta Pty Ltd[2]
" "	MCW	Lalvin (Vinquiry)[3]
" "	Viniflora	Chr. Hansen[4]
Lactobacillus hilgardii	DSM 20176	DSM[5]
" "	L21c	AWRI
Lactobacillus brevis	IV-1	AWRI
Pediococcus sp.	HV	Isolated from mousy red wine (1R, see Chapter 4)
Gluconobacter oxydans	Gb-86	AWRI

[1] AWRI, The Australian Wine Research Institute, PMB Glen Osmond, South Australia

[2] Obtained as a commercial freeze-dried preparation through Monbat Pty Ltd, McLaren Vale, South Australia, 5171

[3] Obtained as a commercial freeze-dried preparation through Lallemand Australia Pty Ltd, Norwood, South Australia, 5067

[4] Christian Hansen Pty Ltd, PO Box 591, Bayswater, Victoria, Australia

[5] DSM, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, D-3300

5.2.2 Media for testing the production of mousy compounds

Experiments investigating the production of mousy compounds by wine bacteria were conducted in Carr-MEt medium and a chemically defined (S1) medium, the formulations of which are given in Chapter 3 and Table 5.2, respectively. Carr-MEt medium was utilised as it generally support strong mousy off-flavour formation by LAB (see Chapter 3). In a second experiment, a chemically defined (S1) medium was utilised and was based upon that described by Liu *et al.* (1994) for studying the metabolism of arginine by LAB. This medium was modified by using higher concentrations of glucose (10 g/L) and fructose (20 g/L) to stimulate the rate of growth and biomass of cells of LAB, and the concentration of citric acid was also increased (2 g/L). Further, ethanol (5% v/v) was added and pH adjusted to pH 4.5.

5.2.3 Preculture of bacteria

For the initial experiment using Carr-MEt test medium, LAB strains and the AAB strain were precultured in 10 mL of MRSA medium for 7 days at 27 °C. Aliquots (approximately 0.2 mL) of this culture were subcultured in a further 30 mL of MRSA medium. Cells for experimental use were obtained from this second culture after 7 days at 27 °C.

Strains of LAB for the second experiment using chemically defined (S1) medium were initially precultured in 10 mL of MRSA medium for 7 days at 27 °C. This culture was then inoculated (3% v/v) into 50 mL of S1 medium (without ethanol). Cells for experimental use were obtained from S1 medium (without ethanol) after 5 days at 27 °C.

Table 5.2. Composition of chemically defined (S1) medium* for growth and induction of mousy off-flavour by lactic acid bacteria (adapted from Liu *et al.* 1994)

Compound	Concentration	Stock solution
Sugars	**(g/L)**	
Glucose	10	
Fructose	20	
Organic acids	**(g/L)**	
Tartaric	3	} 10X H_2O
L-Malic	3	}
Citric	2	}
Mineral salts	**(g/L)**	
A.		
KH_2PO_4	1	} 10X H_2O
$K_2HPO_4.3H_2O$	1.31	}
B.		
$MgSO_4.7H_2O$	0.2	} 10X, H_2O
$MnSO_4.H_2O$	0.05	}
$FeSO_4.7H_2O$	0.05	}
Vitamins	**(mg/L)**	
Thimain-HCl	1.0	} 50X H_2O
Riboflavin	1.0	}
Pyridoxine-HCl	1.5	}
Pantothenic acid calcium salt	5.0	}
Nicotinic acid	2.0	}
myo-Inositol	20.0	}
p-Aminobenzoic acid	0.05	}
Biotin	0.05	}
Cobalamine (Vitamin B12)	0.07	}
Folic acid	0.05	}
Purines and Pyrimidines		
Guanine	10	)100X 0.1N NaOH
Adenine sulfate	10	)
Uracil	10	)
Thymine	10	)
Xanthine	5	)

Compound	Concentration	Stock solution
Amino acids	**(mg/L)**	
A.		
L-Alanine	75	} 10X H_2O
L-Arginine	200	}
L-Aspartic acid	50	}
L-Cysteine	500	}
L-Glycine	20	}
L-Histidine	20	}
L-Isoleucine	30	}
L-Leucine	30	}
L-Lysine.HCl	250	}
L-Methionine	25	}
L-Phenylalanine	20	}
L-Proline	100	}
L-Serine	40	}
L-Threonine	100	}
L-Tryptophan	10	}
L-Valine	20	}
B.		
L-Cystine	50	10X, 1N HCl
C.		
Tyrosine	20	10X 1N KOH
D.		
L-Glutamic acid	200	10X, H_2O
Other components		
Tween 80	0.1 g/L	
Ethanol	5% v/v	

* A starter culture formulation of S1 medium was prepared by omission of ethanol.

5.2.4 Inoculation and incubation of test media

Prior to inoculation, aliquots of sterile Carr-MEt (750 mL) and S1 (400 mL) test media were dispensed into 1 L and 500 mL sterile culture bottles (Schott), respectively. For the initial experiment using Carr-MEt test medium, cells were harvested from preculture in MRSA by centrifugation (6,800 x *g*, 10 min, 20°C) and washed in 30 mL of sterile Carr-MEt medium. The process was repeated and cells were washed for a second time. The washed cells were finally inoculated into duplicate 750 mL volumes of Carr-MEt test medium at a standardised absorbance of 0.02 a.u. (650 nm; multiwell plate method). The latter method was also used for the second series of experiments using S1 test media. In this case, however, cells precultured in S1 medium (without ethanol) were washed and also inoculated into duplicate 400 mL volumes of S1 test media.

After inoculation, test media were incubated statically and aerobically at 23 °C. Samples (1 - 2 mL) were taken periodically to test for cell growth (see below) and in some cases subjective assessment of mousy off-flavour by alkaline test strip. Uninoculated test media served as controls. Respective test cultures were removed from incubation approximately 1-2 days into stationary phase of growth, and then immediately centrifuged (9,950 x *g*, 10 min, 2 °C) to remove cells. The clarified supernatant from each culture and also control media was stored at 2 °C (ca. 2 weeks) prior to extraction and analysis of mousy compounds.

5.2.5 Microbiological analyses and sensory assessment of mousy off-flavour

In the experiments of this chapter, cell density was determined spectrophotometrically by measurement of absorbance (650 nm) using the multiwell plate procedure described in Chapter 3. Mousy off-flavour was determined qualitatively at periodic intervals during growth of bacteria in Carr-MEt and S1 test media by the alkaline test strip procedure also described in Chapter 3.

5.2.6 Quantitative analysis of mousy compounds

The concentrations of ETPY, ACTPY and ACPY were determined in 250 - 300 mL of clarified culture medium using the optimised quantitative extraction (CLLE) and GC-MS procedures described in Chapter 4. Mousy compounds were quantified from molecular ion GC-MS traces with 4- and 3-acetylpyridine as first and second internal standards, respectively.

5.3 RESULTS

5.3.1 Production of mousy off-flavour by wine bacteria in chemically undefined (Carr-MEt) and defined (S1) media.

5.3.1.1 Chemically undefined (Carr-MEt) medium

The abilities of the 10 wine bacteria strains to produce mousy off-flavour (as detected by the alkaline test strip) during growth in Carr-MEt medium are shown in Figures 5.1 and 5.2. These data, together with descriptions of other aroma characteristics in addition to mousiness, are also presented in Table 5.3. As observed in Chapter 3, considerable variation in the growth of bacteria strains occurred during culture in Carr-MEt medium. Despite the variations in cell density, *O. oeni* strains were capable of producing moderate to high levels of mousy off-flavour levels during the incubation period. The other bacteria tested in Carr-MEt medium (*L. hilgardii* L21c and DSM 20176, *Ped.* sp. HV and *Gluconobacter* sp. Gb-86) exhibited large variation in off-flavour formation, ranging from no off-flavour by *Ped.* sp. HV, weak off-flavour by *Gluconobacter* sp. Gb-86 and moderate to strong levels of off-flavour by the three *Lactobacillus* spp. Furthermore, bready and cracker aromas were also commonly produced by most bacteria strains in association with mousy off-flavour.

5.3.1.2 Chemically defined (S1) medium

Four strains of LAB comprising two representative strains of *O. oeni* (Lc5b and MCW) and two *Lactobacillus* spp. (*L. hilgardii* DSM 20176 and *L. brevis* IV-1) were selected for testing in chemically defined (S1) medium on the basis of their ability to produce moderate to high levels of mousy aroma in Carr-MEt medium. The growth of these strains in chemically defined (S1) medium (Table 5.4) was generally similar to their respective growth response in Carr-MEt medium (Figs. 5.1 and 5.2). The production of mousy off-flavour (as detected by alkaline test strip) and other aromas by *O. oeni* Lc5b and *L. hilgardii* DSM 20176 during growth in S1 medium is also indicated in Figure 5.3. Moderate levels of off-flavour were exhibited by both strains, ie. at days 6 and 10 with *O. oeni* Lc5b, and at day 10 with *L. hilgardii* DSM20176. In addition, bready aroma was also produced by both strains in association with mousiness on these occasions (Table 5.4).

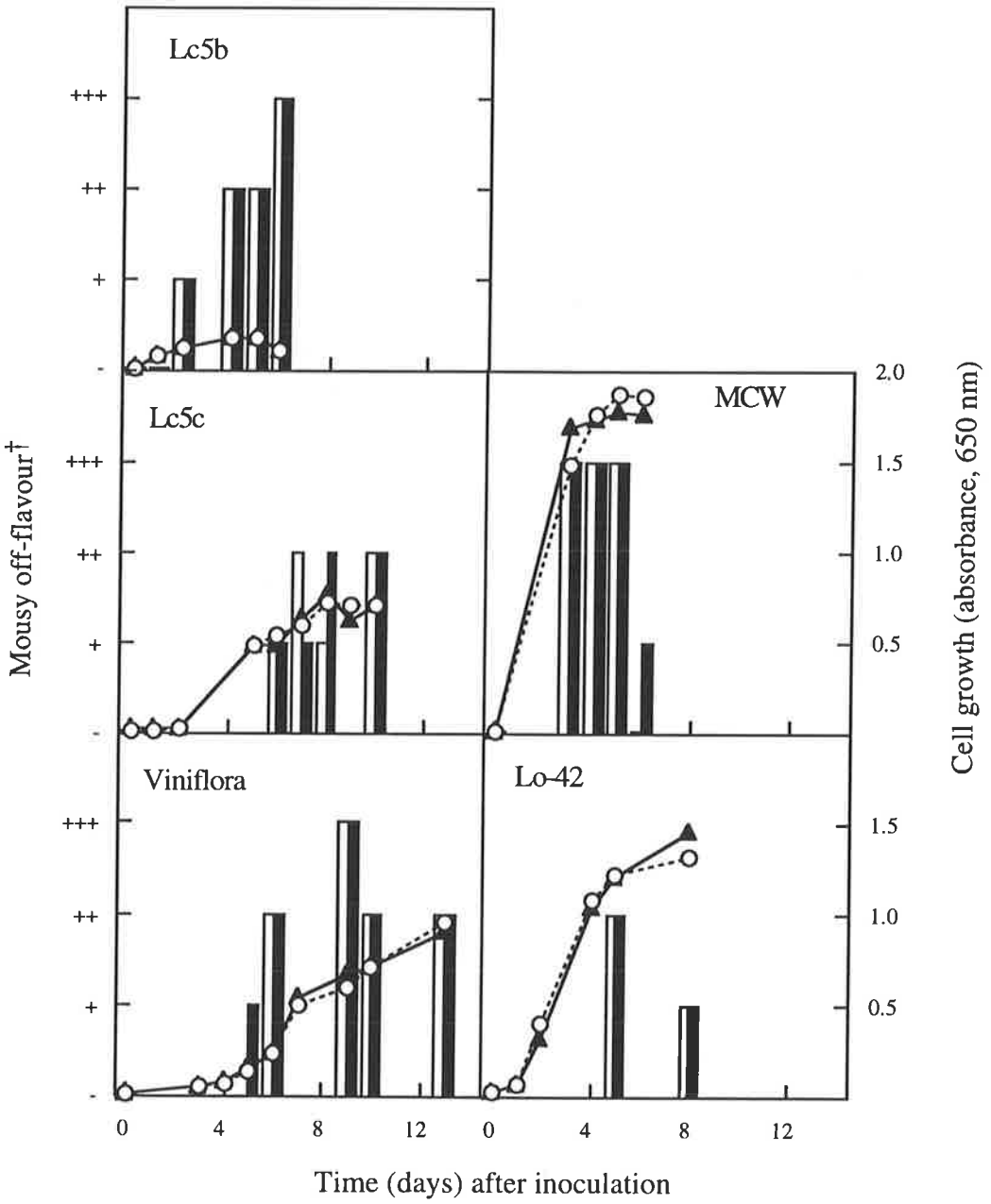


Figure 5.1 Growth (lines) and production of mousy off-odour(bars) by strains of *Oenococcus oeni* in Carr-MEt medium. Data from duplicate fermentations are presented: growth and off-flavour for replicate #1 (open circles, open bars), and replicate #2 (closed triangles, closed bars), respectively.

†Mousy off-flavour was determined as mousy odour detected using the alkaline test strip method. Level of mousy off-flavour: - no off-flavour; +, weak; ++ moderate; +++, strong.

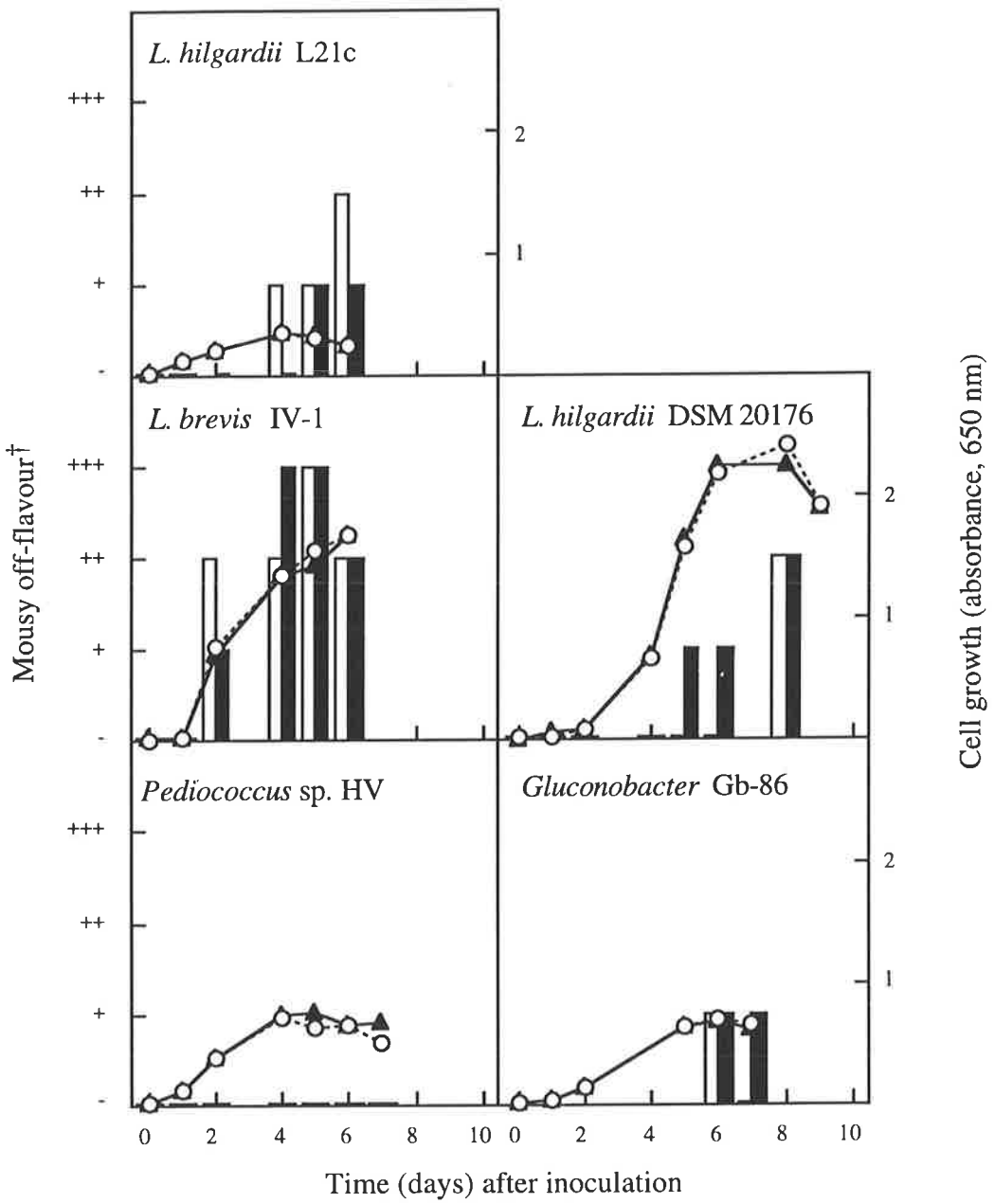


Figure 5.2 Growth (lines) and production of mousy off-flavour (bars) by strains of *Lactobacillus* , *Pediococcus* and *Gluconobacter* in Carr-MEt medium. Data from duplicate fermentations are presented: growth and off-flavour for replicate #1 (open circles, open bars), and replicate #2 (closed triangles, closed bars), respectively.

†Mousy off-flavour was determined as mousy odour detected using the alkaline test strip method. Level of mousy off-flavour: - no off-flavour; +, weak; ++ moderate; +++, strong.

Table 5.3 Production of mousy off-flavour‡ and other aroma characteristics by lactic acid bacteria and an acetic acid bacterium during growth in Carr-MEt medium (p. 1 of 2)

Bacteria strain			Time (days) after inoculation											
			0		1		2		3		4		5		6	
Genus / species	Strain Reference	Replicate No.	Growth* (a.u.)	Mousy taint**	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour
Oenococcus oeni	Lc5b	#1	0.02	- cr, br†	0.08	- cr, br	0.12	+, cr	nd	nd	0.18	+ cr	0.18	++ cr	0.18	+++ cr, br
		#2	0.02	- cr, br	0.08	- cr, br	0.12	+, cr	nd	nd	0.18	+ cr	0.15	++ cr	0.15	+++ cr, br
Oenococcus oeni	Lc5c	#1	0.02	nd††	0.02	nd	0.03	nd	nd	nd	nd	nd	0.48	nd	0.54	+ cr, br
		#2	0.02	nd	0.02	nd	0.03	nd	nd	nd	nd	nd	0.48	nd	0.48	+ cr, br
Oenococcus oeni	Viniflora	#1	0.02	-	nd	nd	nd	nd	0.06	- dia	0.07	- dia	0.14	- cr	0.23	++ cr
		#2	0.02	-	nd	nd	nd	nd	0.06	- dia	0.08	- dia	0.15	- cr, dia	0.24	++ cr
Oenococcus oeni	Lo-42	#1	0.02	nd	0.07	- dia	0.40	nd	nd	nd	1.08	-	1.22	++ br	nd	nd
		#2	0.02	nd	0.07	- dia	0.32	nd	nd	nd	1.04	-	1.20	++ br,	nd	nd
Oenococcus oeni	MCW	#1	0.02	-	nd	nd	nd	nd	1.48	++ cr	1.76	++ cr	1.88	++ cr	1.86	- cr
		#2	0.02	-	nd	nd	nd	nd	1.70	++ cr	1.74	++ cr	1.78	++ cr	1.76	+ cr
Lactobacillus hilgardii	L21c	#1	0.02	-	0.12	-	0.20	-	nd	nd	0.34	+ cr	0.31	+ cr,	0.24	+++ cr, br
		#2	0.02	-	0.12	-	0.24	-	nd	nd	0.34	-	0.32	+ cr,	0.24	+++ cr, br
Lactobacillus hilgardii	DSM 20176	#1	0.02	-	0.02	-	0.08	-	nd	nd	0.66	-	1.59	- acet	0.65	- acet
		#2	0.01	-	0.06	-	0.08	-	nd	nd	0.68	-	1.64	+ acet	0.66	+ acet
Lactobacillus brevis	IV-1	#1	0.01	-	0.02	-	0.78	++ cr	nd	nd	1.36	++ cr, acet	1.57	+++ cr, acet	1.69	++ cr, acet
		#2	0.02	-	0.02	-	0.78	+ cr	nd	nd	1.36	+++ cr, acet	1.44	+++ cr	1.69	++ cr
Pediococcus sp.	HV	#1	0.01	-	0.12	-	0.38	-	nd	nd	0.72	-	0.64	- dia	0.65	- che
		#2	0.01	-	0.12	-	0.38	-	nd	nd	0.74	-	0.76	- dia	0.66	- che
Gluconobacter	Gb-86	#1	0.02	nd	0.04	nd	0.14	nd	nd	nd	nd	nd	0.64	nd	0.70	+ cr, br
		#2	0.02	nd	0.04	nd	0.14	nd	nd	nd	nd	nd	0.64	nd	0.68	+ cr, br
Uninoculated control		#1	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-	0.00	- meaty
		#2	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-	0.00	- meaty

‡ Mousy off-flavour determined as mousy odour detected using the alkaline test strip method.

* Cell growth measured by absorbance at 650 nm using a microplate reader (sample volume 300 µL). a.u. = absorbance units.

** Level of mousy off-flavour: -, no off-flavour; +, weak, ++ moderate; +++, strong

† Abbreviations refer to other aroma charcteristics observed during assessment of alkaline test strip: cr, cracker; br, bready; dia, diacetyl; acet, acetic; che, cheesy.

†† nd = not determined

Table 5.3 Production of mousy off-flavour[‡] and other aroma characteristics by lactic acid bacteria and an acetic acid bacterium during growth in Carr-MEt medium (p. 2 of 2)

Bacteria strain			Time (days) after inoculation									
			7		8		9		10		13	
Genus / species	Strain Reference	Replicate No.	Growth* (a.u.)	Mousy off-flavour**	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour
Oenococcus oeni	Lc5b	#1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		#2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Oenococcus.oeni	Lc5c	#1	0.60	++ cr[†]	0.72	+ cr	0.70	nd	0.70	++ br	nd	nd
		#2	0.64	+ br, cr	0.76	++ cr	0.62	nd	0.70	++ br	nd	nd
Oenococcus.oeni	Viniflora	#1	nd	nd[††]	nd	nd	0.60	-	0.70	+++ cr	0.96	++ cr
		#2	nd	nd	nd	nd	0.66	-	0.70	+++ cr	0.90	++ cr
Oenococcus oeni	Lo-42	#1	nd	nd	1.32	+ dia, acet	nd	nd	nd	nd	nd	nd
		#2	nd	nd	1.46	+ dia, acet	nd	nd	nd	nd	nd	nd
Oenococcus.oeni	MCW	#1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		#2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Lactobacillus hilgardii	L21c	#1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		#2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Lactobacillus hilgardii	DSM 20176	#1	nd	nd	2.42	++ cr	1.92	nd	nd	nd	nd	nd
		#2	nd	nd	2.24	++ cr	1.90	nd	nd	nd	nd	nd
Lactobacillus brevis	IV-1	#1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		#2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Pediococcus sp.	HV	#1	nd	nd	0.52	nd	nd	nd	nd	nd	nd	nd
		#2	nd	nd	0.68	nd	nd	nd	nd	nd	nd	nd
Gluconobacter	Gb-86	#1	0.66	- br	nd	nd	nd	nd	nd	nd	nd	nd
		#2	0.62	+ cr, br	nd	nd	nd	nd	nd	nd	nd	nd
Uninoculated control		#1	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		#2	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-

[‡] Mousy off-flavour determined as mousy odour detected using the alkaline test strip method.

* Cell growth measured by absorbance at 650 nm using a microplate reader (sample volume 300 µL). a.u. = absorbance units.

** Level of mousy off-flavour: -, no off-flavour; +, weak, ++ moderate; +++, strong

[†] Abbreviations refer to other aroma charcteristics observed during assessment of alkaline test strip: cr, cracker; br; bready; dia, diacetyl; acet, acetic; che, cheesy.

[††] nd = not determined

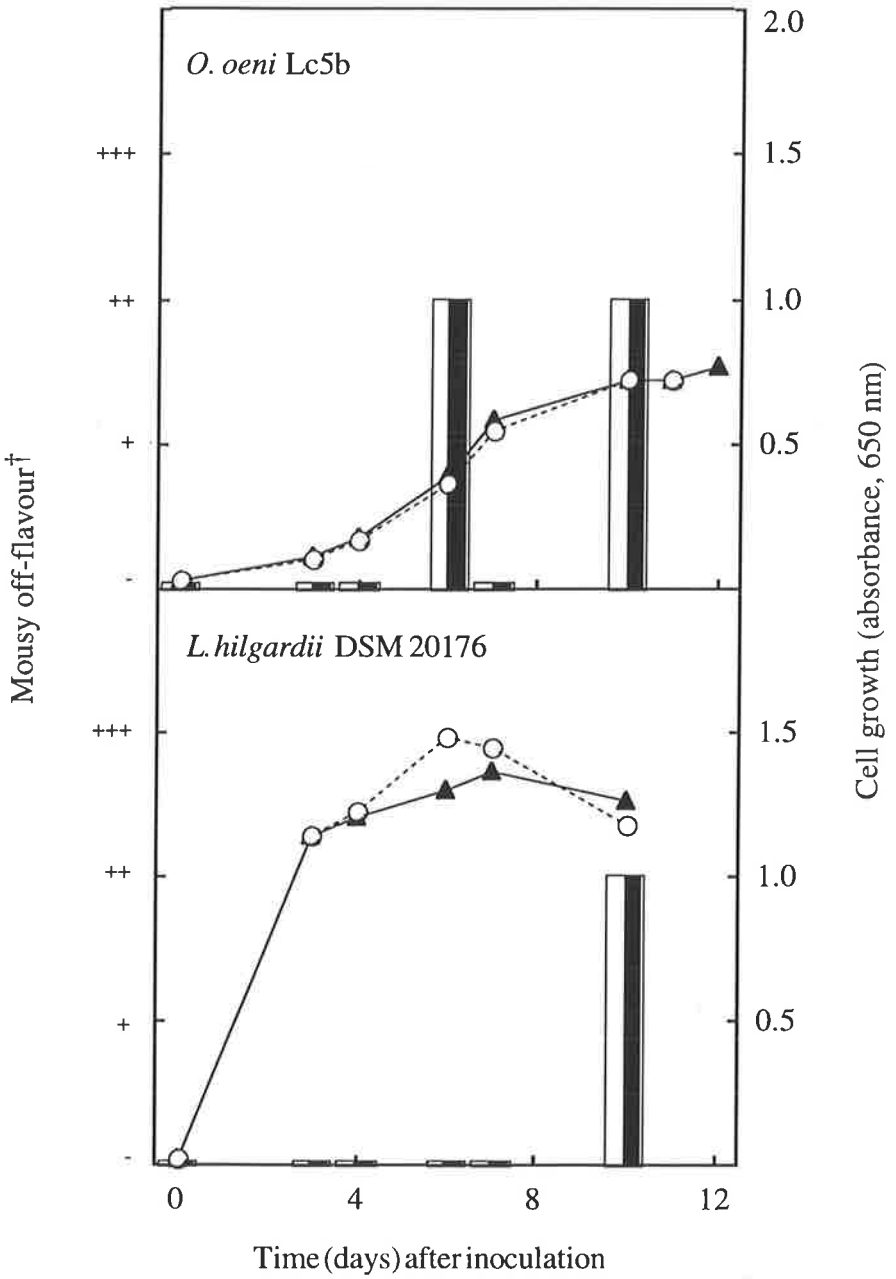


Figure 5.3 Growth (lines) and production of mousy off-flavour (bars) by *O. oeni* Lc5b and *L. hilgardii* DSM 20176 in chemically defined (S1) medium. Data from duplicate fermentations are presented: growth and off-flavour for replicate #1 (open circles, open bars), and replicate #2 (closed triangles, closed bars), respectively.

† Mousy off-flavour was determined as mousy odour detected using the alkaline test strip method. Level of mousy off-flavour: - no off-flavour; +, weak; ++ moderate; +++, strong.

Table 5.4 Production of mousy off-flavour[‡] and other aroma characteristics by lactic acid bacteria during growth in chemically defined (S1) medium (page 1 of 2)

Bacteria strain			Time (days) after inoculation									
			0		2		3		4		6	
Genus / species	Strain Reference	Replicate No.	Growth* (a.u.)	Mousy off-flavour**	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour
Oenococcus oeni	Lc5b	#1	0.02	-	nd	nd	0.10	-	0.16	-	0.36	++, br[†]
		#2	0.02	-	nd	nd	0.11	-	0.17	-	0.38	++, br
Oenococcus oeni	MCW	#1	0.02	nd	0.20	nd	0.66	nd	nd	nd	1.04	nd
		#2	0.02	nd	0.18	nd	0.76	nd	nd	nd	1.00	nd
Lactobacillus hilgardii	DSM 20176	#1	0.02	nd	nd	nd	1.14	-	1.22	-	1.48	-
		#2	0.02	nd	nd	nd	1.14	-	1.20	-	1.30	-
Lactobacillus brevis	IV-1	#1	0.02	nd	0.92	nd	1.60	nd	nd	nd	1.58	nd
		#2	0.02	nd	1.00	nd	1.66	nd	nd	nd	1.72	nd
Uninoculated control		#1	0.00	-	0.00	nd	0.00	-	0.00	-	0.00	-
		#2	0.00	-	0.00	nd	0.00	-	0.00	-	0.00	-

[‡] Mousy off-flavour determined as mousy odour detected using the alkaline test strip method

* Cell growth measured by absorbance at 650 nm using a microplate reader (sample volume 300 µL). a.u. = absorbance units.

** Level of mousy off-flavour: -, no off-flavour; +, weak, ++ moderate; +++, strong

[†] Abbreviations refer to other aroma charcteristics observed during assessment of alkaline test strip: cr, cracker; br; bready

[††] nd = Not determined

Table 5.4 (continued) Production of mousy off-flavour[‡] and other aroma characteristics by lactic acid bacteria during growth in chemically defined (S1) medium (page 2 of 2)

Bacteria strain			Time (days) after inoculation									
			7		8		10		11		12	
Genus / species	Strain Reference	Replicate No.	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour	Growth (a.u.)	Mousy off-flavour
Oenococcus oeni	Lc5b	#1	0.54	-	nd	nd	0.72	++, br	0.72	nd	nd	nd
		#2	0.58	-	nd	nd	0.72	++, br	0.76	nd	0.76	nd
Oenococcus oeni	MCW	#1	nd	nd	1.04	nd	nd	nd	nd	nd	nd	nd
		#2	nd	nd	1.10	nd	nd	nd	1.00	nd	nd	nd
Lactobacillus hilgardii	DSM 20176	#1	1.44	-	nd	nd	1.18	++, br	nd	nd	nd	nd
		#2	1.36	-	nd	nd	1.26	++, br	nd	nd	nd	nd
Lactobacillus brevis	IV-1	#1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		#2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Uninoculated control		#1	0.00	-	0.00	nd	0.00	-	0.00	-	0.00	-
		#2	0.00	-	0.00	nd	0.00	-	0.00	-	0.00	-

‡ Mousy off-flavour was determined as mousy odour detected using the alkaline test strip method.

* Cell growth measured by absorbance at 650 nm using a microplate reader (sample volume 300 µL). a.u. = absorbance units.

** Level of mousy off-flavour: -, no off-flavour; +, weak, ++ moderate; +++, strong

† Abbreviations refer to other aroma charcteristics observed during assessment of alkaline test strip: cr, cracker; br; bready

†† nd = Not determined

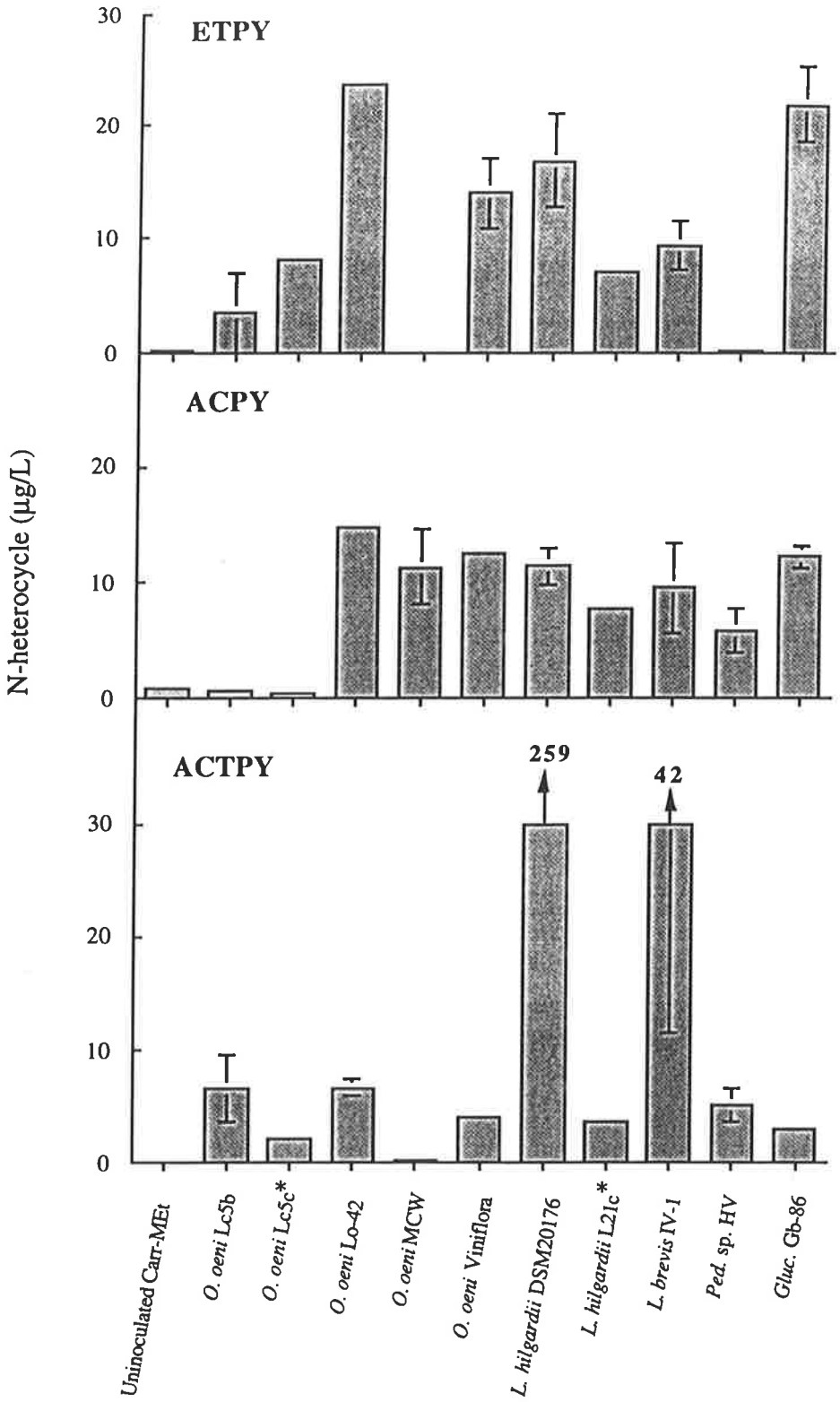
5.3.2 Production of mousy compounds by wine bacteria in chemically undefined (Carr-MEt) and chemically defined (S1) media

5.3.2.1 Chemically undefined (Carr-MEt) medium

The formation of ETPY, ACPY and ACTPY by each bacteria strain in Carr-MEt medium was quantified by GC-MS (Figure 5.4 and Table 5.5). These results reveal that low concentrations of ACTPY (0.1 µg/L), ETPY (0.25 µg/L) and ACPY (0.8 µg/L) were present in the uninoculated Carr-MEt medium. The survey revealed that the majority of bacteria strains were capable of producing each of the three mousy compounds. Formation of individual mousy compounds was not detected, however, with certain strains, ie. ACPY formation by *O. oeni* Lc5b and Lc5c, ETPY formation by *Ped.* sp. HV, and ACTPY and ETPY formation by *O. oeni* MCW was not detected. The concentrations of mousy compounds produced by most bacteria was generally in the range <0.1 - 25 µg/L for ETPY, <0.1 - 15 µg/L for ACPY and <0.1 - 10 µg/L for ACTPY. Two notable exceptions to this were the very high concentrations of ACTPY produced by *L. hilgardii* DSM20176 and *L. brevis* I.V.-1, being 259.3 µg/L and 42.1 µg/L, respectively. In contrast to *L. hilgardii* DSM20176, however, *L. hilgardii* L21c only produced the relatively low concentration of 3.6 µg/L of ACTPY.

Strains of *O. oeni* exhibited variation in the ability to produce mousy compounds in Carr-MEt medium. *Oenococcus oeni* Lo-42 produced the highest concentrations of ACTPY (6.7 µg/L), ACPY (14.7 µg/L) and also ETPY (23.6 µg/L), while *O. oeni* Viniflora produced slightly lower concentrations of each compound (ACTPY, 4.0 µg/L; ACPY, 12.6 µg/L and ETPY, 13.9 µg/L). *Oenococcus oeni* Lc5b also produced a relatively high concentration of ACTPY (6.6 µg/L), yet only produced a lower concentration of ETPY (3.5 µg/L) and essentially no ACPY. As previously noted, the formation of ACTPY and ETPY by *O. oeni* MCW was not detected, yet this strain produced a relatively high concentration of ACPY (11.3 g/L). In addition to the nine LAB surveyed, *Gluconobacter oxydans* Gb-86 also produced ACTPY (3.0 µg/L), and relatively high concentrations of ACPY (12.2 µg/L) and ETPY (21.8 µg/L) in Carr-MEt medium.

The variability in the concentration of each mousy compound between replicate fermentations in Carr-MET media was generally low and for most strains was less than +/- 3 µg/L (Fig. 5.4, Table 5.5). The variability in ACTPY production between replicate fermentations of *L. hilgardii* strains IV-1 and DSM 20176, however, was much greater (+/- 28.3 µg/L and +/- 30.7 µg/L, respectively). Further studies using a greater number of replicates would be required to verify and also identify the source this apparent variation.



Figue 5.4 Production of ethyltetrahydropyridine (ETPY), 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by strains of lactic acid bacteria and a *Gluconobacter* in Carr-MEt medium. Data presented are mean concentrations of duplicate assays; error bars indicate concentration range of replicates. Single assays were performed on strains indicated with an asterisk (*)

Table 5.5 Concentrations of 2-ethyltetrahydropyridine (ETPY), 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) produced by wine bacteria and a *Gluconobacter* sp. in Carr-MEt medium, and by lactic acid bacteria in chemically defined (S1) medium[a].

Test medium	Bacteria strain		Mousy N-heterocycle (µg/L)		
	Genus / species	Strain reference	ETPY	ACPY	ACTPY
Carr-MEt	*Oenococcus oeni*	Lc5b	3.5 (< 0.1, 7.0)[†]	0.6 (0.6, 0.6)	6.6 (3.6, 9.5)
	" "	Lc5c	8.1[††]	0.5[††]	2.1[††]
	" "	Lo-42	23.6 (23.5, 23.7)	14.7[††]	6.7 (6.0, 7.4)
	" "	MCW	<0.1[b] (<0.1, Tr[c])	11.3 (8.0, 14.5)	0.3 (Tr, 0.6)
	" "	Viniflora	13.9 (10.9, 16.9)	12.6 (12.4, 12.7)	4.0 (3.8, 4.1)
	Lactobacillus hilgardii	DSM 20176	16.8 (12.7, 20.8)	11.4 (9.8, 12.9)	259.3 (231.1, 287.5)
	Lactobacillus hilgardii	L21c	7.2[††]	7.8	3.6[††]
	Lactobacillus brevis	IV-1	9.4 (7.4, 11.4)	9.5 (5.6, 13.3)	42.1 (11.4, 72.7)
	Pediococcus sp.	HV-1	0.3 (0.2, 0.3)	5.8 (3.9, 7.7)	5.2 (3.7, 6.7)
	Gluconobacter sp.	Gb-86	21.8 (18.6, 25.0)	12.2 (11.2, 13.1)	3.1 (2.9, 3.2)
	Uninoculated control		0.3 (0.1, 0.4)	0.8 (0.3, 1.3)	0.1 (<0.1, 0.1)
Chemically defined (S1)	*Oenococcus oeni*	Lc5b	<0.1 (<0.1, <0.1)	<0.1 (<0.1, <0.1)	4.4 (3.6, 5.2)
	" "	MCW	<0.1[††]	1.8[††]	0.4[††]
	Lactobacillus hilgardii	DSM20176	1.1 (1.0, 1.1)	1.3 (1.3, 1.3)	4.6 (3.2, 6.0)
	Lactobacillus brevis	IV-1	0.4 (0.2, 0.6)	1.3 (0.9, 1.6)	6.6 (2.3, 10.9)
	Uninoculated control		<0.1 (<0.1, <0.1)	<0.1 (<0.1, <0.1)	<0.1 (<0.1, <0.1)

[a] Mousy N-heterocycles quantified by GC-MS analysis of Freon extracts obtained using the optimised CLLE extraction method described in Chapter 4

[†] Mean value of analysis of duplicate cultures; individual values shown in braces

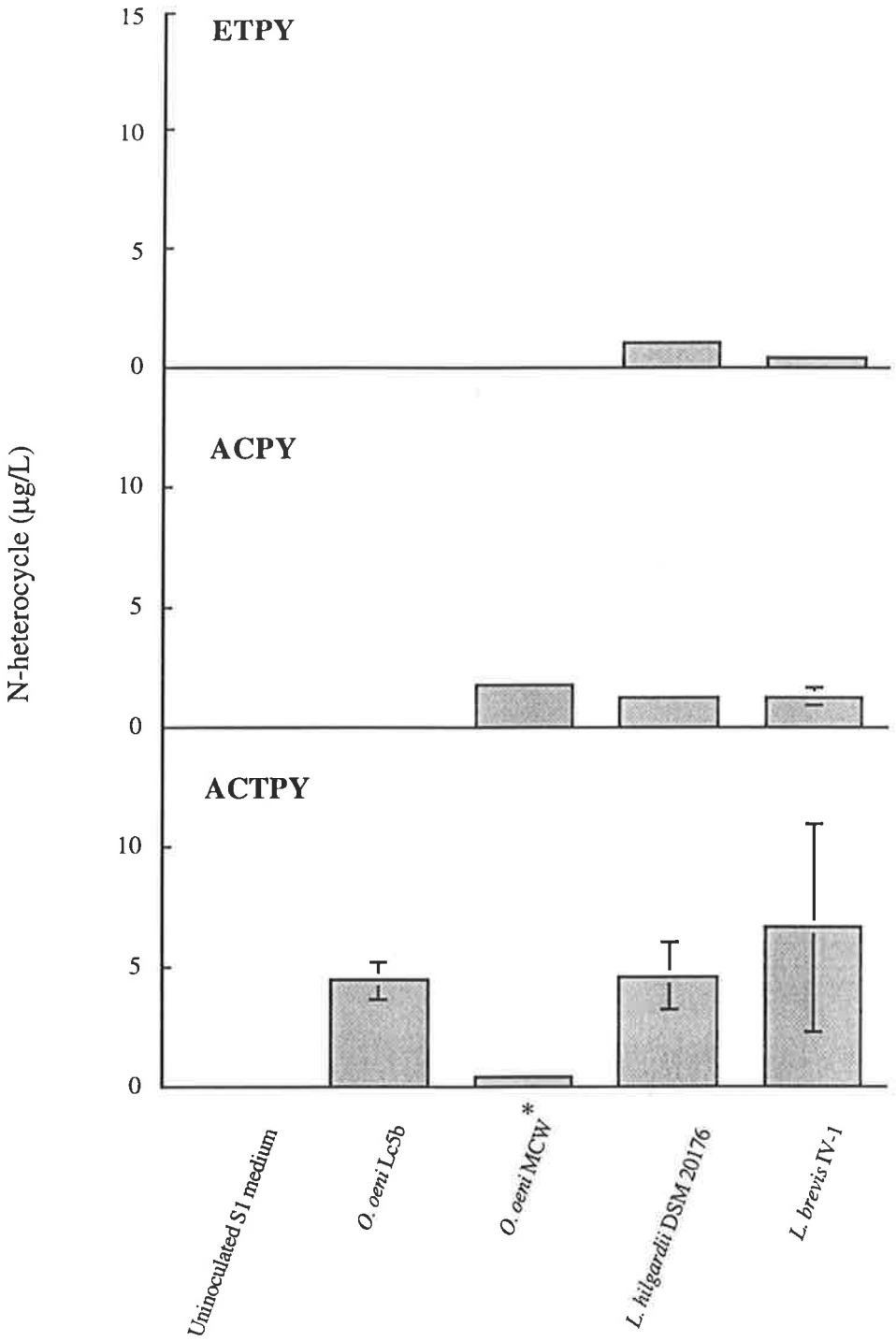
[††] Value from analysis of a single culture

[b] Detection limit = 0.1 µg/L

[c] Tr = trace amount

5.3.2.2 Chemically defined (S1) medium

The concentrations of mousy compounds produced by LAB in S1 medium are shown in Figure 5.5 and Table 5.5. Unlike Carr-MEt medium, mousy off-flavour compounds were not detected in the uninoculated S1 medium. Moreover, the LAB strains were generally capable of producing one or more mousy compounds in S1 medium. The concentrations of the latter compounds, however, were generally much less than those produced in Carr-MEt medium. This was particularly evident with *L. hilgardii* DSM 20176 and *L. brevis* IV-1, which respectively produced only 4.6 µg/L and 6.6 µg/L of ACTPY in S1 medium (Fig 5.5), compared to almost 60-times and 10-times, respectively, higher amounts in Carr-MEt medium. Additionally, the concentrations of ETPY produced in chemically defined (S1) medium by *L. hilgardii* DSM20176 (0.1 µg/L) and *L. brevis* IV-1 (0.3 µg/L) was 168 - times and 39 - times, respectively, lower in content than in Carr-MEt medium (Fig. 5.4). Similarly, the concentrations of ACPY produced in S1 medium by *O. oeni* MCW (1.8 µg/L), *L. hilgardii* DSM20176 (1.3 µg/L) and *L. brevis* IV-1 (1.3 µg/L) were, on average, approximately 7.5 - fold lower than the concentrations of ACPY produced by these strains in Carr-MEt medium. On the other hand, *O. oeni* strains Lc5b and MCW produced similar concentrations of ACTPY in S1 medium (4.4 µg/L and 0.4 µg/L, respectively) to that in Carr-MEt medium (6.6 µg/L and 0.3 µg/L, respectively).



Figue 5.5 Production of ethyltetrahydropyridine (ETPY), 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by strains of lactic acid bacteria in a chemically defined (S1) medium. Data presented are mean concentrations of duplicate assays; error bars indicate concentration range of replicates. Single assays were performed on strains indicated with an asterisk (*)

5.4 DISCUSSION

The studies of this chapter not only highlight the diversity of wine bacteria that are capable of producing mousy compounds, but also reveal the types and concentrations of mousy N-heterocycle compounds that are formed. Foremost, the earlier report of Heresztyn (1986) relating the formation of ACTPY to the metabolism of certain *Lactobacillus* spp. (*L. brevis*) has been extended to encompass a wide range of other oenologically important bacteria with this capability. These include the spoilage bacteria *L. hilgardii* , *Pediococcus* spp. and a *Gluconobacter* spp., as well as strains of the more desirable malolactic bacterium, *O. oeni*. In addition to the formation of ACTPY, the current study has also demonstrated for the first time that different strains of the latter wine bacterium were also capable of producing two other mousy compounds, ETPY and the highly potent ACPY.

As noted in the Literature Review, other workers have attributed the formation of ACPY and ETPY to the metabolic activity of certain microorganisms. Tucknott (1977) was the first to demonstrate the microbial formation of ETPY by the spoilage yeast, *Brettanomyces anomalus*. Moreover, Romanczyk *et al.* (1995) recently described the first case of the bacterial formation of ACPY by *Bacillus cereus* strains isolated from cocoa fermentation. In contrast to the current study, however, in which a diversity of wine bacteria were shown to be capable of producing ACPY, it is interesting to note from the report of Romanczyk *et al.* (1995) that ACPY production did not occur with other *Bacillus* spp. and was restricted to *B. cereus*. Moreover, the latter authors were unable to detect the formation of other cracker-like aroma compounds (including ACTPY) by this species, which again contrasts with the heterogeneity of mousy N-heterocyclic compounds shown to be produced by wine bacteria.

In addition to the production of ACPY and ETPY, the production of extremely high concentrations of ACTPY by *L. hilgardii* DSM20176 and *L. brevis* IV-1 in Carr-MEt medium (Fig 5.4) concurs with the common association of *Lactobacillus* spp. with the incidence of mousy off-flavour (see Literature Review). Specifically, the propensity for *L. hilgardii* DSM 20176 to produce ACTPY supports the original description of this type strain as a major cause of spoilage (including mousiness) in Californian wine (Douglas and Cruess, 1936). Similarly, the production ACTPY by *L. brevis* IV-1 supports the findings of Heresztyn (1986), who originally isolated this strain from mousy Australian wine and with which the production of ACTPY was first demonstrated. Moreover, although all three *Lactobacillus* spp. tested in Carr-MEt medium were capable of producing each of the three mousy compounds, strain variation in the ability to produce ACTPY was particularly evident between *L. hilgardii* DSM 20176 and *L. hilgardii* L21c, whereby DSM 20176 produced over 70 times more ACTPY than L21c (259.3 and 3.6 µg/L, respectively). The reasons for such strain variation in ACTPY formation are as yet unknown.

The finding that strains of *O. oeni* were capable of producing one or more mousy N-heterocycles substantiates the conclusions drawn from Chapter 3 that this oenologically important species may exhibit the ability to produce mousy off-flavour under certain conditions. Moreover, since this species is predominantly responsible for the induction of MLF in red and white wines (Davis *et al.* 1985, Henick-Kling 1993), these results are of major consequence to both the wine industry and producers of malolactic starter culture preparations. Such findings, however, appear to conflict with the widely accepted view that 'clean' and desirable flavour characteristics are generally obtained through MLF when the fermentation is carried out with *O. oeni* , rather than with *Lactobacillus* spp. or *Pediococcus* spp. which may cause spoilage reactions (Davis *et al.* 1985, Henick-Kling 1993). Henderson and McDaniel (1987) and Henick-Kling (1993) also show that mousiness is not included among the numerous sensory descriptors attributed to MLF, eg. buttery, nutty, yeasty, earthy, fruity and spicy. A recent study by Sauvageot and Vivier (1997), however, found that Chardonnay and, to a lesser extent, Pinot Noir wines which had undergone MLF with a commercial *O. oeni* strain (Micro oenos B1) were perceived higher in a number of characters including that of fresh bread. Bready and cracker aromas are well known aroma characters of ACPY and ACTPY (see Chapter 4 and Literature Review) and in the current study have been commonly detected in association with mousy off-flavour (see Table 5.3 and results of Chapter 3). Similarly, some tasters are suggested to perceive the mousy off-flavour in wine as 'cracker biscuit' (Bartowsky and Henschke 1995). It is possible, therefore, that the fresh bread character described by Sauvageot and Vivier (1997) could be due to *O. oeni* producing low concentrations (near sensory detection levels) of mousy compounds. Furthermore, it is interesting to note that anecdotal evidence from some winemakers in Australia (Costello *et al.* 1993) indicates that mousy characters may transiently occur in some wines during the course of MLF, which subsequently dissipate after the addition of sulfite. Further study is thus required to ascertain the abilities of *O. oeni* strains (as well as other LAB) to produce mousy compounds in wine during MLF, and to further determine the impact of such mousy off-flavour compounds on the sensory properties of wine.

The demonstration for the first time that a *Pediococcus* spp. was capable of producing both ACTPY (5.2 µg/L) and ACPY (5.8 µg/L) supports the earlier view of Vaughn (1955) that this species (then classified as *Micrococcus* spp.) could cause mousiness in wine. Furthermore, the formation of these compounds by *Ped.* sp. HV indicates that this strain may have been a cause of mousiness in the wine from which it was originally isolated. It is somewhat surprising, however, that unlike most other bacteria strains which produced mousy N-heterocycles in association with mousy off-flavour [as detected by alkaline test strip (Figs. 5.1 - 5.3)], *Ped.* sp. HV did not produce detectable mousy aroma during culture in Carr-MEt medium. This apparent anomaly could be caused, at least in part, by masking with other volatile compounds such as diacetyl (Table 5.3). Homofermentative LAB are recognised for their ability to produce diacetyl (Mayer 1974, Henick-Kling 1993, Sponholz 1993) and, since

this feature was evident with *Pediococcus* spp. surveyed in Chapter 3, mousy off-flavour production could in fact be a general characteristic of *Pediococcus* spp. Further screening of other species and strains of *Pediococcus* spp. is needed, however, to verify the extent of mousy compound production within this genus. It is further recommended that the alkaline test strip method should only be used as a preliminary guide for assessment of mousy off-flavour, and that quantitative (GC-MS) analysis is required to confirm the presence and the concentrations of mousy off-flavour compounds in fermentation media.

The ability of *Gluconobacter oxydans* Gb-86 to produce relatively high concentrations of ACPY and ETPY, as well as ACTPY in Carr-MEt medium confirms an earlier association in AAB (Vaughn 1938, 1955). That AAB can produce mousy off-flavour compounds extends the range of bacterial genera that are known to be capable of producing the N-heterocyclic mousy compounds. Other *Acetobacter* spp. were initially investigated for mousy off-flavour compound formation in this study (data not shown), yet considerable difficulty was experienced in attempting to culture these strains in the liquid Carr-MEt test medium, thus preventing subsequent testing of these bacteria. Further studies are therefore required to determine the extent of mousy compound formation amongst this bacterial group.

Four LAB strains were tested in a second test medium, chemically defined (S1) medium, in order to study the effects of medium composition on the production of mousy compounds. Despite comparatively similar growth of LAB in the two different test media (Figs. 5.1 - 5.3), the concentrations of mousy off-flavour compounds produced in chemically defined (S1) medium were less than those produced in Carr-MEt media. This was particularly evident with *L. hilgardii* DSM 20176 and *L. brevis* IV-1 which exhibited 60- and 10-fold reductions, respectively, in the production of ACTPY, and 168- and 39-fold reductions, respectively, in ETPY formation in chemically defined (S1) medium compared with Carr-MEt medium. Tucknott (1977) similarly described a lack of mousy producing ability with *Lactobacillus* spp. in a chemically defined test medium and found it necessary to incorporate yeast extract for mousy off-flavour to develop. Romanczyk *et al.* (1995) also noted that medium composition had a profound effect on the formation of ACPY by *B. cereus*. These workers found that such metabolism was only supported on Plate Count Agar (PCA), but not with Tryptone Soy Agar or liquid plate count medium. Furthermore, Romanczyk *et al.* (1995) found that glucose, as well as proline and glutamic acid supplements, but not yeast extract, played an integral role in the formation of ACPY by *B. cereus* in PCA (these aspects are discussed in more detail in the following chapter).

The results of this study suggest that in order to carry out further study of precursors involved in mousy off-flavour formation by LAB, other synthetic media formulations require evaluation. Alternatively, other techniques such as high cell density incubation could be examined, which would have the additional advantage of providing standardised cell density.

Finally, further verification of the findings of this chapter require experiments to be carried out using wine as a substrate. Such experiments, however, are beyond the scope of this study.

Overall, the results of this chapter are novel and imply that the ability of wine bacteria to produce one or more of three different mousy N-heterocyclic compounds could be a characteristic feature of their metabolism at least under the culture conditions described here. The results also clearly show that the type(s) and concentration(s) of mousy off-flavour compounds produced were dependent upon the species and strains of bacteria involved, as well as upon medium composition.

5.5 SUMMARY

Each of ten wine bacteria comprising strains of *Lactobacillus* spp., *O. oeni*, *Pediococcus* spp. and a *Gluconobacter* was capable of producing one or more of the mousy compounds ACTPY, ACPY and ETPY in a chemically undefined test medium. Three *Lactobacillus* spp. and *Gluconobacter* Gb-86 produced all three mousy compounds while *O. oeni* exhibited considerable strain variation in this ability. The concentrations of ACTPY, ACPY and of ETPY produced by most strains in the undefined medium respectively ranged up to 10 µg/L, 15 µg/L and 30 µg/L, although much higher concentrations of ACTPY were produced by *L. hilgardii* DSM 20176 (259 µg/L) and *L. brevis* IV-1 (42 µg/L).

Four LAB strains produced detectable levels of two or more of ACTPY, ACPY and ETPY in a chemically defined test medium. The concentrations of mousy compounds produced by LAB in synthetic test medium were generally much lower than those produced in the chemically undefined test medium.

6. HIGH CELL DENSITY INCUBATION STUDIES ON THE FORMATION OF MOUSY N-HETEROCYCLES

6.1 INTRODUCTION

Comprehensive study of the metabolism of mousy N-heterocycles by LAB requires the use of a chemically defined test medium. However, the production of mousy compounds by LAB in chemically defined (S1) medium in chapter 5 was relatively poor. While the reasons for this phenomenon are not known, the poor results necessitated the use of different methodology to enable further study of mousy compound precursors. One such approach involves the use of whole or resting cell suspensions. In this method, cells are precultured, washed and finally resuspended in an assay medium of defined composition. Following incubation, the cells are removed and the supernatant is assayed for metabolic end products. The use of resting cells has several advantages over conventional cell culture, including standardised cell density and separation of metabolic function from the requirements of cell growth. The application of this technique to the study of mousy N-heterocycle formation by LAB appears to be novel.

In this chapter, the metabolism of mousy N-heterocycles by LAB was studied using a high cell density incubation technique which exploited the biocatalytic properties of a high concentration of LAB cells. A chemically defined assay medium was initially developed to facilitate mousy off-flavour formation by high cell density incubation. Using this method, LAB strains were screened for the ability to produce mousy N-heterocycles. In a final series of experiments, major substrates and precursors of mousy N-heterocycle formation were studied with a test strain, *L. hilgardii* DSM 20176.

6.2 MATERIALS AND METHODS

6.2.1 Bacteria strains

Eleven different strains of LAB listed in Table 6.1 were used for the work of this chapter, ten of which were selected from the representative genera and species studied in Chapter 3. The 11 LAB tested included 5 strains of *O. oeni*, comprising the 3 commercial strains (MCW, Lo-42 and Viniflora), *O. oeni* Lc5b, and the type *O. oeni* strain DSM 20252, which had not been tested in previous experiments. Four *Lactobacillus* species, *L. hilgardii* DSM 20176 (type strain), *L. hilgardii* L21c, *L. brevis* L17a and *L. plantarum* L11a and two *Pediococcus* species, *P. damnosus* P1a and *P. parvulus* P6b were also tested. These 11 strains were screened for the production of mousy compounds, and the strong mousy producing strain *L. hilgardii* DSM 20176 was utilised as a test bacterium for the majority of experiments.

Table 6.1 Species and strains of wine bacteria tested for the production of mousy compounds by high cell density incubation

Genus / species	**Strain reference**	**Source / manufacturer†**
Oenococcus oeni	Lc5b	AWRI
" "	Lo-42	Condimenta Pty Ltd
" "	MCW	Lalvin (Vinquiry)
" "	Viniflora	Christian Hansen
" "	DSM 20252	DSM
Lactobacillus hilgardii	DSM 20176	DSM
" "	L21c	AWRI
Lactobacillus brevis	L17a	AWRI
Lactobacillus plantarum	L11a	AWRI
Pediococcus damnosus	P1a	AWRI
Pediococcus parvulus	P6b	AWRI

† Refer to Chapter 3 for further details of the origin of strains

6.2.2 Media for lactic acid bacteria preculture

The following media were used for the culture of LAB for the high cell density incubation studies. Strains were initially precultured in MRSA medium, then transferred to final preculture in either Modified Carr-MEt (Carr-M) medium, or Modified de Man, Rogosa and Sharpe (MRS-CT) medium described below.

(i) Modified Carr-MEt medium

The composition of Carr-M medium was the same as Carr-MEt medium described in Chapter 3 with the exception that ethanol was omitted.

(ii) Modified de Man, Rogosa and Sharpe medium.

Modified de Man, Rogosa and Sharpe (MRS-CT) medium was a modification of de Man, Rogosa and Sharpe medium in which the peptone and beef extract were replaced with Casamino Acids (Difco) and Trypticase peptone (BBL), and to which fructose (10 g/L) and L-malic acid (3 g/L) were also added (Table 6.2).

6.2.3 Preculture of lactic acid bacteria

Strains of LAB were initially precultured in 10 mL of MRSA medium for 5 - 7 days at 25 °C. Aliquots of this culture were then inoculated (2 % v/v) into 250 mL - 3L of Carr-M medium or MRS-CT medium for final preculture at 25 °C. Samples (1 mL) were periodically removed from final preculture media to monitor cell growth (section 6.2.8). Cells for experimental use were obtained from final preculture at stationary phase of growth. Initial development of an assay medium for mousy off-flavour formation by high cell density incubation utilised cells precultured in either Carr-M or MRS-CT media, while remaining experiments only used cells that were precultured in MRS-CT medium.

Table 6.2 Composition of modified de Man, Rogosa and Sharpe (MRS-CT) medium.

Component	Amount (per litre)
Yeast extract (Oxoid)	5 g
Casamino acids (Difco)	5 g
Trypticase peptone (BBL)	5 g
D-Glucose	20 g
D-Fructose	10 g
L-Malic acid	3g
Sodium acetate.$3H_2O$	5.0 g
Tri-ammonium citrate	2 g
K_2HPO_4	2 g
$MgSO_4.7H_2O$	0.2 g
$MnSO_4.4H_2O$	0.05 g
Tween 80	1 mL
pH 4.5	

6.2.4 Assay media for high cell density incubation studies

Assay media used for high cell density incubation (HCDI) studies were based upon a synthetic formulation of Carr-MEt (Carr-MEt-Synth) medium described in Table 6.3. In the complete Carr-MEt-Synth medium, yeast extract and casamino acids were omitted and replaced by individual amino acids, vitamins and purines and pyrimidines. The concentrations of amino acids were determined from the approximate amino acid compositions of yeast extract and casein hydrolysate described in The Oxoid Manual (Bridson, 1990), while the concentrations of vitamins and purines and pyrimidines were the same as those used in synthetic (S1) medium described in Chapter 3. Stock solutions of Carr-MEt-Synth medium components were stored frozen at -20 °C prior to use.

Table 6.3 Composition of Carr-MEt-Synth medium used as an assay medium in initial high cell density incubation studies

Compound	Concentration	Stock solution
Sugars†	**(g/L)**	
D-Fructose	50	
Organic acids†	**(g/L)**	
Citric	2	
L-Malic	5	
Mineral salts†	**(g/L)**	
KH_2PO_4	5.5	
KCl	4.25	
$MgSO_4.7H_2O$	1.25	
$MnSO_4.H_2O$	0.025	
$FeSO_4.7H_2O$	0.043	
$CaCl_2.2H_2O$	1.66	
Vitamins and co-factors††	**(mg/L)**	50x in H_2O
Thiamin-HCl	1.0	
Riboflavin	1.0	
Pyridoxine-HCl	1.5	
Pantothenic acid calcium salt	5.0	
Nicotinic acid	2.0	
myo-Inositol	20.0	
p-Aminobenzoic acid	0.05	
Biotin	0.05	
Cobalamine (Vitamin B12)	0.07	
Folic acid	0.05	
Pyridoxal phosphate	5.0	
Purines and Pyrimidines††		100x in
Guanine	10	0.1N NaOH
Adenine sulfate	10	
Uracil	10	
Thymine	10	
Xanthine	5	

Compound	Concentration	Stock solution
Amino acids†††	**(mg/L)**	
A.		5x in H_2O
L-Alanine	100	
L-Arginine	200	
L-Aspartic acid	500	
L-Glutamic acid	1200	
L-Cysteine	250	
L-Glycine	350	
L-Isoleucine	300	
L-Leucine	450	
L-Methionine	100	
L-Phenylalanine	300	
L-Proline	250	
L-Serine	200	
L-Threonine	200	
L-Tryptophan	50	
L-Valine	200	
B.		
L-Cystine	75	5x in 1N HCl
C.		
Tyrosine	300	5x in 1N KOH
D.		
L-Ornithine	450	5x in H_2O
E.		
L-Lysine	450	5x in H_2O
Other components		
Tween 80	1 mL/L	
Ethanol	5% v/v	
pH 4.5		

† Components based upon Carr-MEt medium formulation (see Chapter 3).
†† Vitamins, purines & pyrimidines based upon those used in chemically defined (S1) medium (see Chapter 3).
††† Amino acid components formulated from the amino acid composition of yeast extract and casamino acids (Bridson, 1990)

From initial studies investigating the effects of Carr-MEt-Synth medium composition on mousy off-flavour formation, a simpler basal assay (BA) medium (Table 6.4) was developed and utilised for subsequent HCDI studies of this chapter. The components of BA medium were individually prepared as filter sterilised (0.22 μm) stock solutions (Table 6.4) and were stored at 2 °C. Prior to HCDI experiments, assay media were prepared as 0.22 μm filter sterilised stock solutions and adjusted to pH 4.5.

Table 6.4 Composition of basal assay (BA) medium used for testing mousy off-flavour formation by high cell density incubation of lactic acid bacteria

Component	Amount (per litre)
D- Fructose	50g
L-Ornithine	5g
L-Lysine	5g
Citric acid	2g
L-Malic acid	5g
KH_2PO_4	5.5g
KCl	4.25
$MgSO_4.7H_2O$	0.025g
$MnSO_4.H_2O$	0.025g
$FeSO_4.7H_2O$	0.043g
$CaCl_2.2H_2O$	1.66g
Ethanol (redistilled, 96% v/v)	52 mL (5.0 % v/v ethanol)
Acetaldehyde	100 mg
pH 4.5	

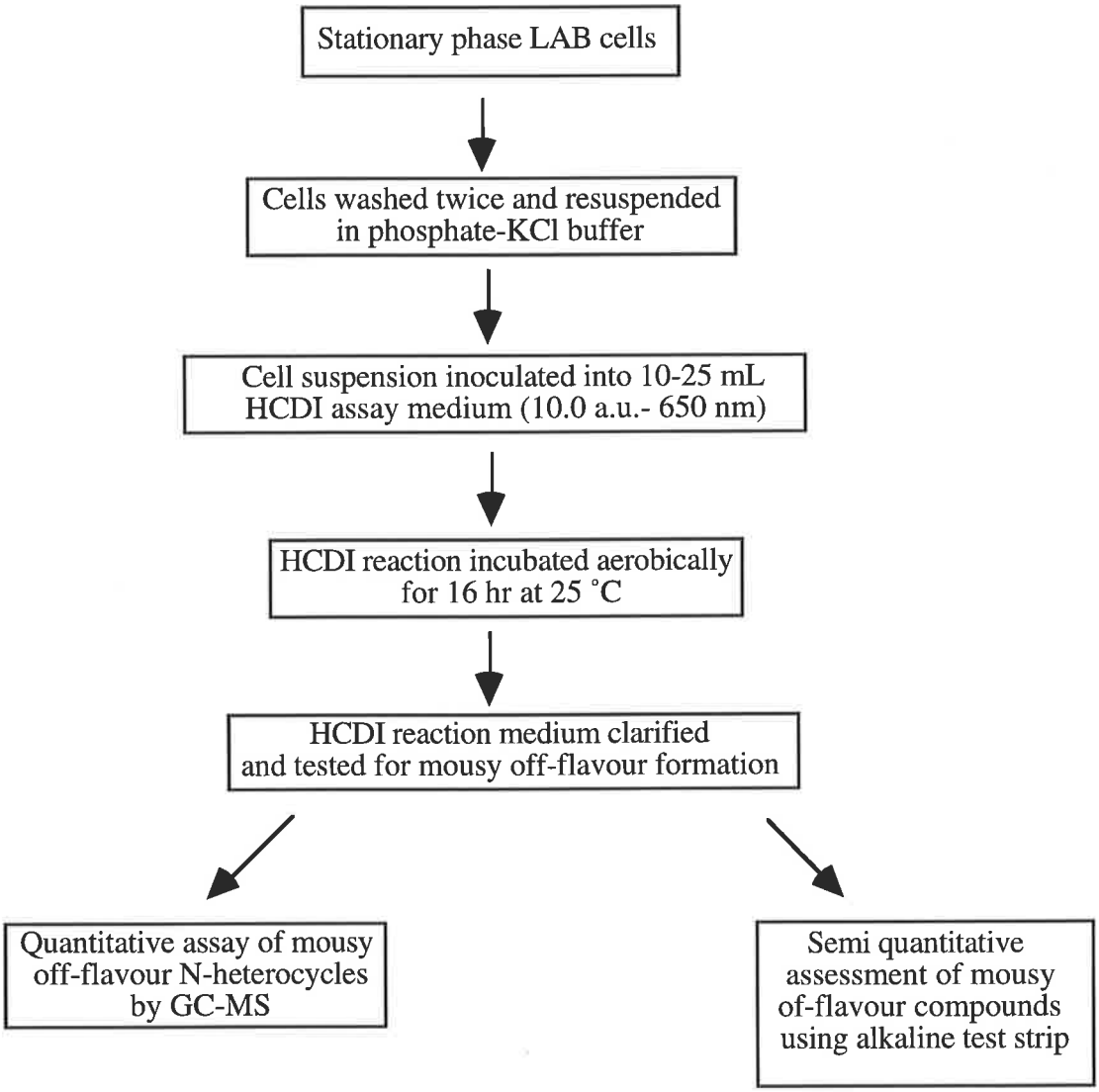
6.2.5 High cell density incubation of lactic acid bacteria for rapid determination of mousy off-flavour induction

Stationary phase cells of LAB were harvested from the final preculture medium by centrifugation (approximately 9,000 x *g*, 15 min, 20 °C) and washed twice with phosphate-KCl buffer (pH 4.5, 0.22 µm filter sterilised) (Table 6.5). The cell pellet was resuspended in a further 20 - 25 mL of phosphate-KCl buffer. The absorbance [650 nm, multiwell method (see Chapter 3)] of this suspension was then measured, and the volume required to achieve a final cell density of 10.0 a.u. in 10 - 25 mL of HCDI assay medium calculated. An appropriate volume of stock HCDI assay medium was then dispensed into 30 mL screw-capped glass bottles for final assay volumes of 10 - 25 mL. To initiate the HCDI reaction, the washed cell suspension, together with an appropriate quantity of phosphate-KCl buffer to make up final assay volume, were respectively added to the HCDI assay medium. Uninoculated HCDI assay media served as controls and were prepared by substituting the washed cell suspension with an equivalent volume of phosphate-KCl buffer. Inoculated and control assay media were incubated statically and aerobically at 25 °C for 16 h, after which time cells were removed by centrifugation (approximately 9,000 x *g*, 15 min, 4 °C). For initial studies of the development of an HCDI assay medium, the clarified supernatant was immediately tested for the production of mousy off-flavour using a modification of the alkaline test strip procedure (see section 6.2.9). For remaining HCDI experiments, supernatants were frozen (- 20 °C) for subsequent extraction and GC-MS quantification of mousy N-heterocycles (see section 6.2.9). A flow chart describing the HCDI procedure is given in Figure 6.1.

Table 6.5 Composition of phosphate-KCl buffer used for washing and concentrating bacteria cells prior to high cell density incubation

Component	Amount (per litre)
KH_2PO_4	5.5 g
KCl	4.25 g
pH 4.5	

Figure 6.1 Flow chart of the high cell density incubation procedure used for the rapid determination of mousy off-flavour formation by lactic acid bacteria



6.2.6 Screening of lactic acid bacteria for the formation of mousy off-flavour N-heterocycles

Each of the representative strains of LAB listed in Table 6.1 were tested by the HCDI procedure for the ability to produce the three N-heterocycles, ETPY, ACPY and ACTPY. In these experiments, BA medium (without acetaldehyde) was used as the HCDI assay medium.

6.2.7 Substrates, precursors and other factors of mousy compound formation

Substrates of mousy compound formation were studied by HCDI by omitting or changing the concentrations of individual components of BA medium, ie. D-fructose, ethanol, acetaldehyde, L-ornithine, L-lysine, citric acid, L-malic acid or metal ions. Using this approach, the effects of using different carbohydrates (glucose and xylose), nitrogen sources (DL-pipecolate, L-proline L-glutamate), alcohols (*n*-propanol, *iso*-propanol and *n*-butanol) and aldehydes (butyraldehyde and propionaldehyde) on mousy compound formation were also investigated. In addition, some of the precursors of mousy compounds were studied using BA medium in which ethanol, acetaldehyde and fructose were replaced by respective deuterated isotopes, ie. d_6-ethanol (99%,); d_4-acetaldehyde (99%) and 2-*d*-D-glucose (99%) (Cambridge Isotope Laboratories, Andover, Massachusetts, USA).

In one experiment, the effects of anaerobiosis on mousy compound production was investigated by incubation in an anaerobic jar containing an Anaerobic Gas Pak Generator (Oxoid).

6.2.8 Determination of cell growth and cell density

Cell growth and cell density of LAB strains were determined spectrophotometrically by measurement of absorbance (650 nm) using the multiwell plate procedure described in chapter 3.

6.2.9 Analysis of mousy off-flavour and mousy N-heterocycles

After HCDI, mousy of-flavour was qualitatively determined using a modification of the alkaline test strip procedure described in Chapter 3. Samples (1 mL) of BA medium supernatant were mixed with 2 drops of phenol red indicator solution (0.1g phenol red / 250 mL H_2O, basified with 2.82 mL of 0.1 N NaOH) in a 1.5 mL Eppendorf tube, and adjusted to pH 8.4 (red end-point) with 1 N NaOH. Three or more colleagues who were experienced in detecting the off-flavour then used the alkaline test strips to assess the level of mousy off-flavour in the samples by sniffing the strips immediately after they had been dipped into the basified samples.

In the experiments of this chapter, the N-heterocycles ETPY, ACPY and ACTPY were quantified in BA medium after HCDI using the optimised CLLE and GC-MS procedure described in Chapter 4, with the exception that smaller sample volumes (10 - 22 mL) were extracted, and the pre-extraction of acidic and neutral compounds (pH 2.5) with Freon 11 was not undertaken.

6.2.10 Other chemical analyses - D-fructose and acetic acid

The concentrations of D-fructose and acetic acid were determined enzymatically using the kit reagents for D-glucose and D-fructose, and acetic acid, respectively, and methods described by Boehringer Mannheim (Anon 1995).

Table 6.4 Composition of basal assay (BA) medium used for testing mousy off-flavour formation by high cell density incubation of lactic acid bacteria

Component	Amount (per litre)
D- Fructose	50g
L-Ornithine	5g
L-Lysine	5g
Citric acid	2g
L-Malic acid	5g
KH_2PO_4	5.5g
KCl	4.25g
$MgSO_4.7H_2O$	0.025g
$MnSO_4.H_2O$	0.025g
$FeSO_4.7H_2O$	0.043g
$CaCl_2.2H_2O$	1.66g
Ethanol (redistilled, 96% v/v)	52 mL (5.0 % v/v ethanol)
Acetaldehyde	100 mg
pH 4.5	

Table 6.5 Composition of phosphate-KCl buffer used for washing and concentrating bacteria cells prior to high cell density incubation

Component	Amount (per litre)
KH_2PO_4	5.5 g
KCl	4.25 g
pH 4.5	

6.3 RESULTS

6.3.1 Development of an assay medium for mousy off-flavour formation using high cell density incubation

Table 6.6 shows the results of three experiments designed to investigate the effects of medium composition on the ability of *L. hilgardii* DSM 20176 to produce mousy off-flavour under HCDI conditions. In the first experiment (I), only weak (+) mousy off-flavour was produced in the complete Carr-MEt-Synth assay medium. Varying levels of mousy off-flavour were produced, however, when several groups of components (including one or more of amino acids, vitamins, purines and pyrimidines and metal ions and organic acids) were omitted from the medium. Further investigations (experiments II and III, Table 6.6) revealed that moderate (++) levels of mousy off-flavour were produced after the following components were omitted from the complete Carr-MEt-Synth assay medium: amino acids (except L-ornithine and L-lysine), vitamins, Tween 80, purines and pyrimidines. Further testing with this latter medium, referred to as basal assay (BA) medium (Table 6.5), revealed that addition of vitamins had little effect on mousy off-flavour formation by *L. hilgardii* DSM 20176, while omission of D-fructose reduced the level of off-flavour production from moderate (++) to weak (+). Furthermore, substituting the test strain with *L. hilgardii* L21c also produced weak (+) off-flavour with BA medium (Table 6.7). The BA medium was subsequently used as the reference HCDI assay medium in the study of factors affecting mousy off-flavour formation.

Table 6.6 The effect of assay medium composition on the production of mousy off-flavour by high cell density incubation of *Lactobacillus hilgardii* DSM 20176

Experiment	Assay medium †	Components omitted or added from assay medium	Level of mousy off-flavour ††
I*	Carr-MEt-Synth	Complete medium (control)	+
	"	Amino acids omitted except L-ornithine & L-lysine	++
	"	Vitamins & Tween 80 omitted	++
	"	Purines & pyrimidines omitted	+
	"	Amino acids (except L-ornithine & L-lysine), vitamins, Tween 80, purines & pyrimidines, metal ions, L-malic acid and citric acid omitted	+
II**	BA medium	Complete medium (control)	++
	"	Vitamins added	++
III**	BA medium	Complete medium (control)	++
	"	D-Fructose omitted	+

† The composition and preparation of Carr-MEt-Synth and basal assay (BA) media are described in sections 6.2.4 and 6.2.5 respectively.

†† Mousy off-flavour determined as mousy odour detected using the alkaline test strip method. Level of mousy off-flavour: -, no off-flavour; +, weak; ++, moderate; +++, strong. Results averaged from 3 or more assessors

* Bacteria precultured in Carr-M medium

** Bacteria precultured in MRS-CT medium

Table 6.7 Production of mousy off-flavour in basal assay (BA) medium by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 and L21c*

Bacteria strain	Level of mousy off-flavour††
Lactobacillus hilgardii DSM 20176	++
Lactobacillus hilgardii L21c	+

* Bacteria precultured in MRS-CT medium

†† Mousy off-flavour determined as mousy odour detected using the alkaline test strip method. Level of mousy off-flavour: -, no off-flavour; +, weak; ++, moderate; +++ strong. (Results averaged from 3 or more assessors)

6.3.2 Survey of lactic acid bacteria for the production of mousy compounds using high cell density incubation

The concentrations of ETPY, ACPY and ACTPY produced by 11 strains and species of *Lactobacillus*, *Pediococcus* and *Oenococcus oeni* in basal assay medium (without acetaldehyde) using HCDI are presented in Figure 6.2 and Appendix 6.1. Most strains were capable of producing each of the three off-flavour compounds, with the exception of *P. damnosus* P1a which was unable to produce ACPY. The concentrations of ACPY produced by the remaining 10 LAB strains ranged from 8.5 µg/L (*O. oeni* MCW) to 54.7 µg/L (*O. oeni* Viniflora). In contrast, the production of ETPY and ACTPY varied considerably between the 11 different LAB strains tested. Three *O. oeni* strains (Lc5b, Lo-42 and Viniflora) produced notably high concentrations of ETPY (162 µg/L, 86.8 µg/L and 128.9 µg/L, respectively) compared with the much lower concentrations of this compound produced by the eight remaining LAB (0.2-8.1 µg/L). Further, the highest concentrations of ACTPY were produced by *L. hilgardii* DSM 20176 (579.9 µg/L), *L. hilgardii* L21c (403.9 µg/L) and also *L. brevis* L17a (328.1 µg/L), with relatively high amounts of this compound also produced by *O. oeni* strains Viniflora (103.8 µg/L) and Lc5b (47.9 µg/L). In contrast, low concentrations of ACTPY were produced by *O. oeni* strains MCW and DSM 20252 (11.9 µg/L and 4.5 µg/L, respectively) and *P. damnosus* P1a (0.7 µg/L), *P. parvulus* P6b (10.5 µg/L) and *L. plantarum* L11a (2.9 µg/L).

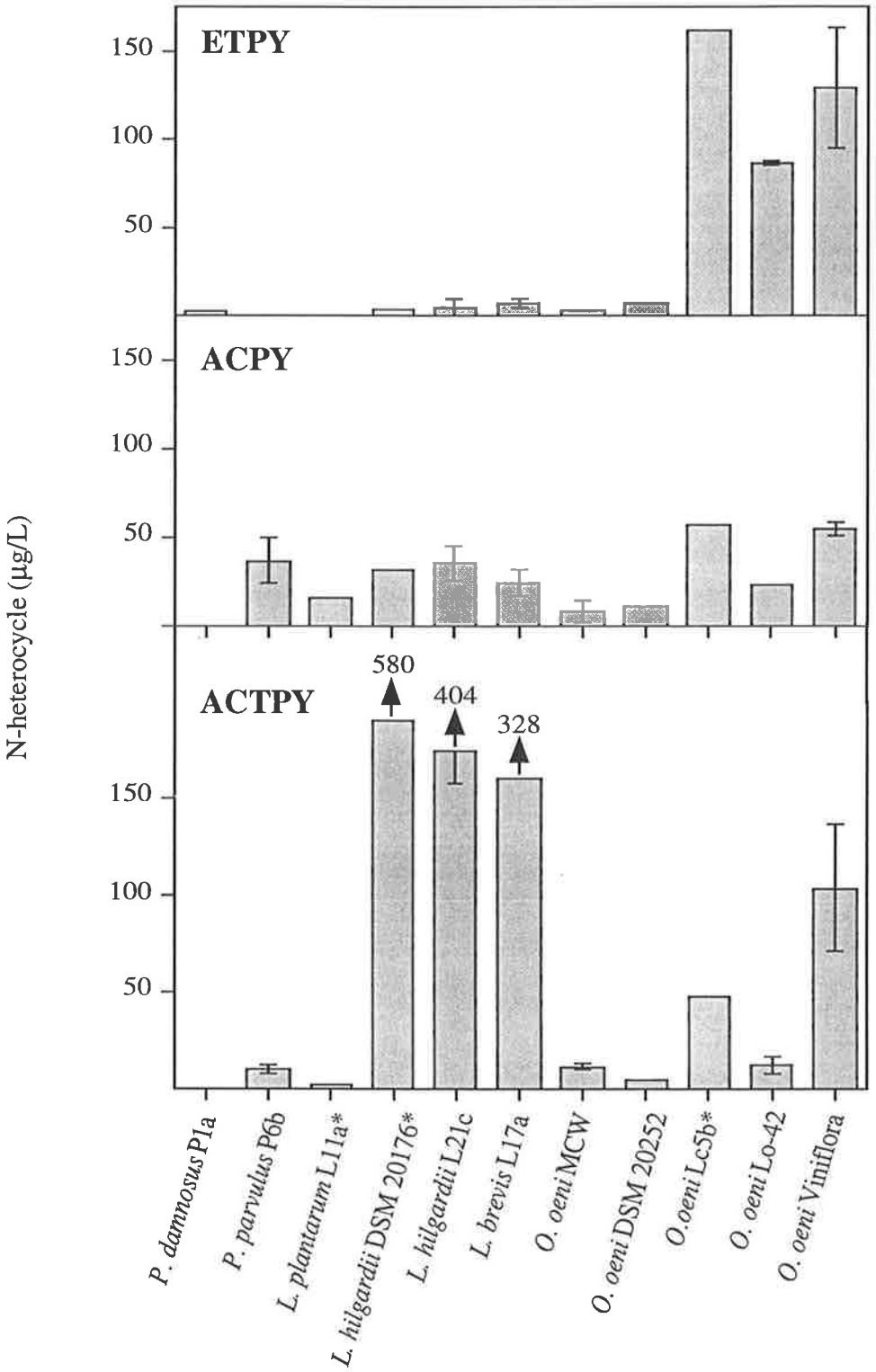


Figure 6.2 Production of 2-ethyltetrahydropyridine (ETPY), 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by high cell density incubation of strains of *Pediococcus* spp., *Lactobacillus* spp. and *Oenococcus oeni* in basal assay medium (without acetaldehyde). Data presented are mean concentrations of duplicate assays; error bars indicate concentration range, and data from strains indicated "*" were determined from a single assay

6.3.3 Factors affecting the production of mousy compounds by high cell density incubation of *Lactobacillus hilgardii* DSM 20176

6.3.3.1 Reproducibility of N-heterocycle production by high cell density incubation

The variation in the concentrations of ETPY, ACPY and ACTPY produced by *L. hilgardii* DSM 20176 over 7 individual HCDI experiments is shown in Appendix 6.2. While the variation in the concentrations of mousy compounds between replicate assays within experiments was relatively low, much greater variation was observed in the concentrations of N-heterocycles between separate experiments. This was particularly evident for the production of ACTPY and ACPY, the average concentrations of which varied approximately 4-fold (270.2-1040.5 µg/L) and 3-fold (34.3-89.3 µg/L), respectively, over the 7 experiments. Nevertheless, the relative proportions of ETPY, ACPY and ACTPY produced in these experiments remained relatively consistent: for the 7 experiments, the average ratio of ETPY: ACPY: ACTPY (expressed as a percentage of the sum concentration of the 3 N-heterocycles in each experiment) was 0.6 : 8.3 : 91.1, with respective standard deviations of 0.4, 2.2 and 2.0%.

6.3.3.2 Concentration of D-fructose

The concentration of fructose in BA medium had a significant effect on the production of ACPY and ACTPY by *L. hilgardii* DSM 20176 (Figure 6.3, Appendix 6.3). In the absence of D-fructose, comparatively low concentrations of ACPY (19.5 µg/L) and ACTPY (7.5 µg/L) were produced. At 10 g/L fructose, the concentration of ACTPY increased 28-fold to 211.9 µg/L, while that of ACPY remained at 19.5 µg/L. In comparison to the control (0 g/L D-fructose), the concentrations of ACPY respectively increased 4.6-fold (89.3 µg/L) and 4.0-fold (78.1 µg/L) at 50 g/L and 100 g/L fructose, respectively, while considerably greater increases in ACTPY content were exhibited in the latter samples [respectively 140 - fold (1040.5 µg/L) and 147-fold (1100.6 µg/L)]. As seen in Figure 6.3, the increased production of ACPY and ACTPY by *L. hilgardii* DSM 20176 was also associated with greater utilisation of D-fructose: at 10 g/L, 50 g/L and 100 g/L fructose, 100% (10.0 g/L), 97% (48.4 g/L) and 47.2% (47.2 g/L) of the carbohydrate was utilised, respectively. In contrast to the increased production of ACPY and ACTPY, however, the production of ETPY was generally unaffected by the concentration of D-fructose and remained in the range 1.8 to 4.7 µg/L.

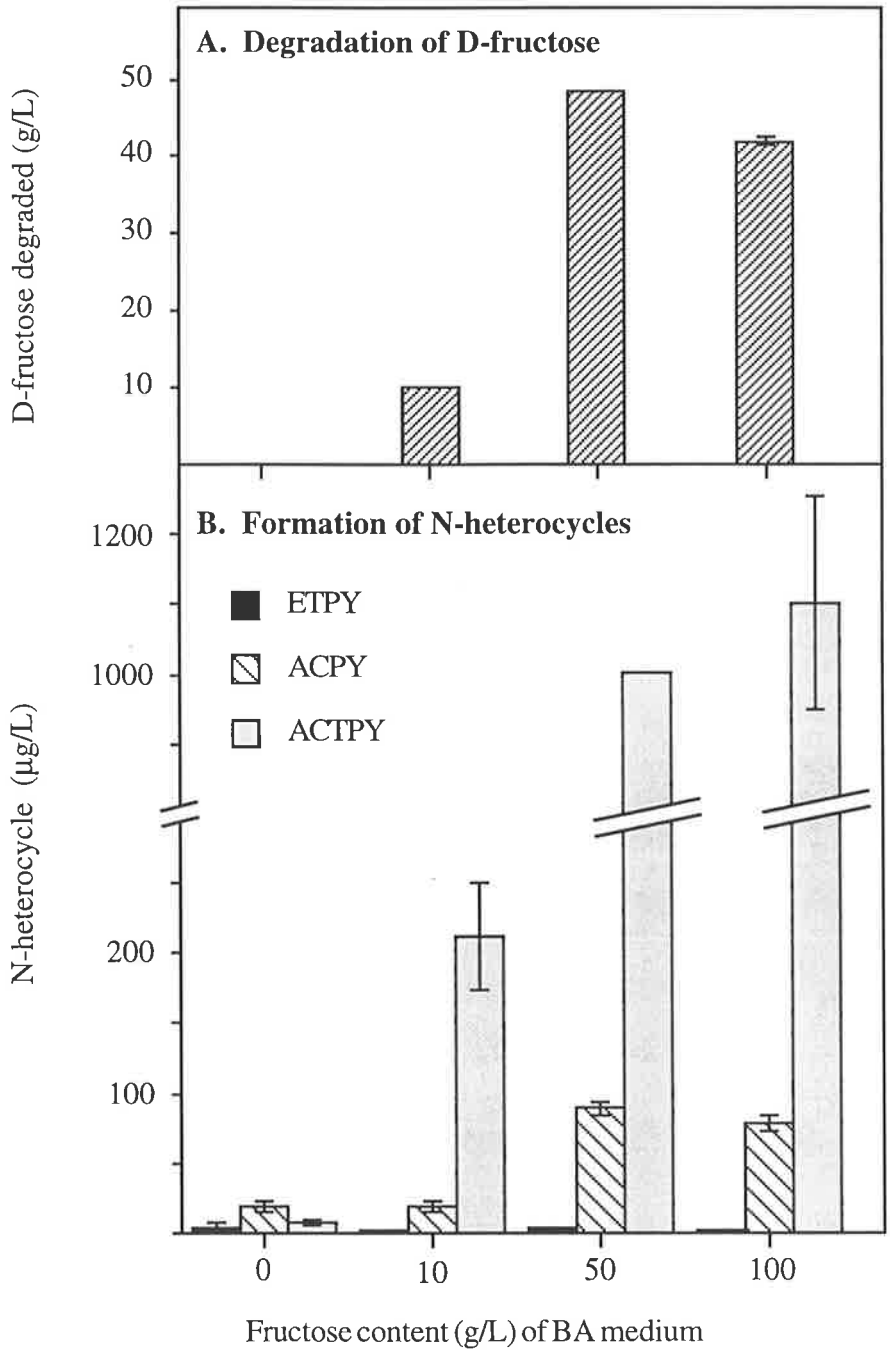


Figure 6.3 The effect of D-fructose content of basal assay (BA) medium on the formation of 2-ethyltetrahydropyridine (ETPY), 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 (caption B). Caption A shows the degradation of D-fructose in each assay. Data presented are mean concentrations of duplicate assays; error bars indicate concentration range of duplicate assays

6.3.3.3 Concentrations of L-ornithine and L-lysine

The concentrations of L-ornithine and L-lysine in basal assay medium had a considerable effect on the production ACPY and ACTPY by HCDI of *L. hilgardii* DSM 20176 (Figure 6.4, Appendix 6.4). In the absence of these amino acids, the concentrations of ACPY and ACTPY were respectively 24.5 µg/L and 894.9 µg/L. The addition of 1 g/L and 5 g/L L-ornithine respectively increased the concentration of ACPY 10.5-fold (258.4 µg/L) and 13.0-fold (319.6 µg/L), while ACTPY decreased by 40% (536.8 µg/L) and 21% (707.5 µg/L) respectively. In contrast, the addition of L-lysine, 1 g/L and 5 g/L, increased the concentration of ACTPY 1.6 - fold (1450.6 µg/L) and 1.7 - fold (1497.2 µg/L), respectively. Further, the formation of ACPY was reduced 24.5-fold (to 1.0 µg/L) with the addition of 1 g/L L-lysine, and was completely repressed by 5 g/L L-lysine. Compared to the production of mousy compounds in BA media containing (separately) 5 g/L L-ornithine and 5 g/L L-lysine, the combined addition of L-ornithine (5 g/L) and L-lysine (5 g/L) resulted in 70.1% and 30.5% reductions, respectively, in the formation of ACPY (89.3 µg/L) and ACTPY (1040.5 µg/L).

6.3.3.4 Other factors

In the following series of experiments, the effects of other factors including ethanol, acetaldehyde, metal ions, organic acids and other nitrogen and carbohydrate sources on the formation of mousy compounds were investigated by HCDI by omitting or varying the concentrations of these components in BA medium. The effects of incubation time and anaerobiosis on mousy compound formation by HCDI were also studied. Since the ability of the test strain *L. hilgardii* DSM 20176 to produce N-heterocycles (particularly of ACPY and ACTPY) in BA medium was variable between experiments (see section 6.3.3), comparative assessment of N-heterocycle production was accomplished by expressing the concentrations of N-heterocycles within each experiment as a relative percentage value of the concentrations of each compound produced in the control BA medium.

(i) Ethanol and acetaldehyde

The effects of ethanol and acetaldehyde on the formation of mousy compounds by HCDI of *L. hilgardii* DSM 20176 is shown in Figure 6.5 and Appendix 6.5. In this series of experiments, the formation of ETPY was not detected in either control or treated samples. Ethanol exerted a major influence on the production of ACPY and ACTPY, however, since the concentrations of these N-heterocycles were reduced by 87.5% and 88.9%, respectively, after the omission of ethanol from BA medium. In contrast, omission of acetaldehyde

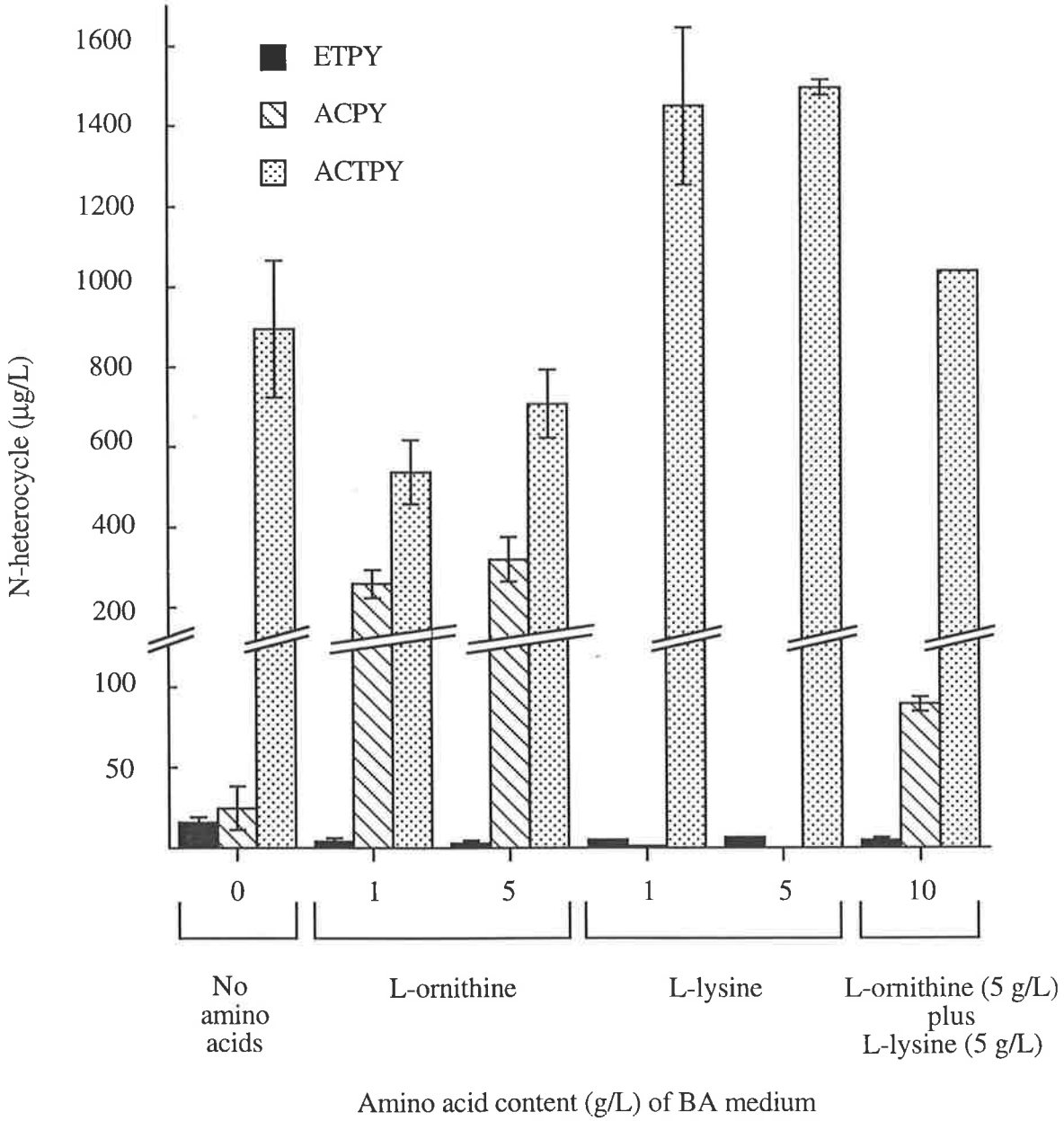


Figure 6.4 The effect of L-ornithine and L-lysine content of basal assay (BA) medium on the formation of 2-ethyl tetrahydropyridine (ETPY), 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by high cell density incubation of *Lactobacillus hilgardii* DSM 20176. Data presented are mean concentrations of duplicate assays; error bars indicate concentration range of replicates

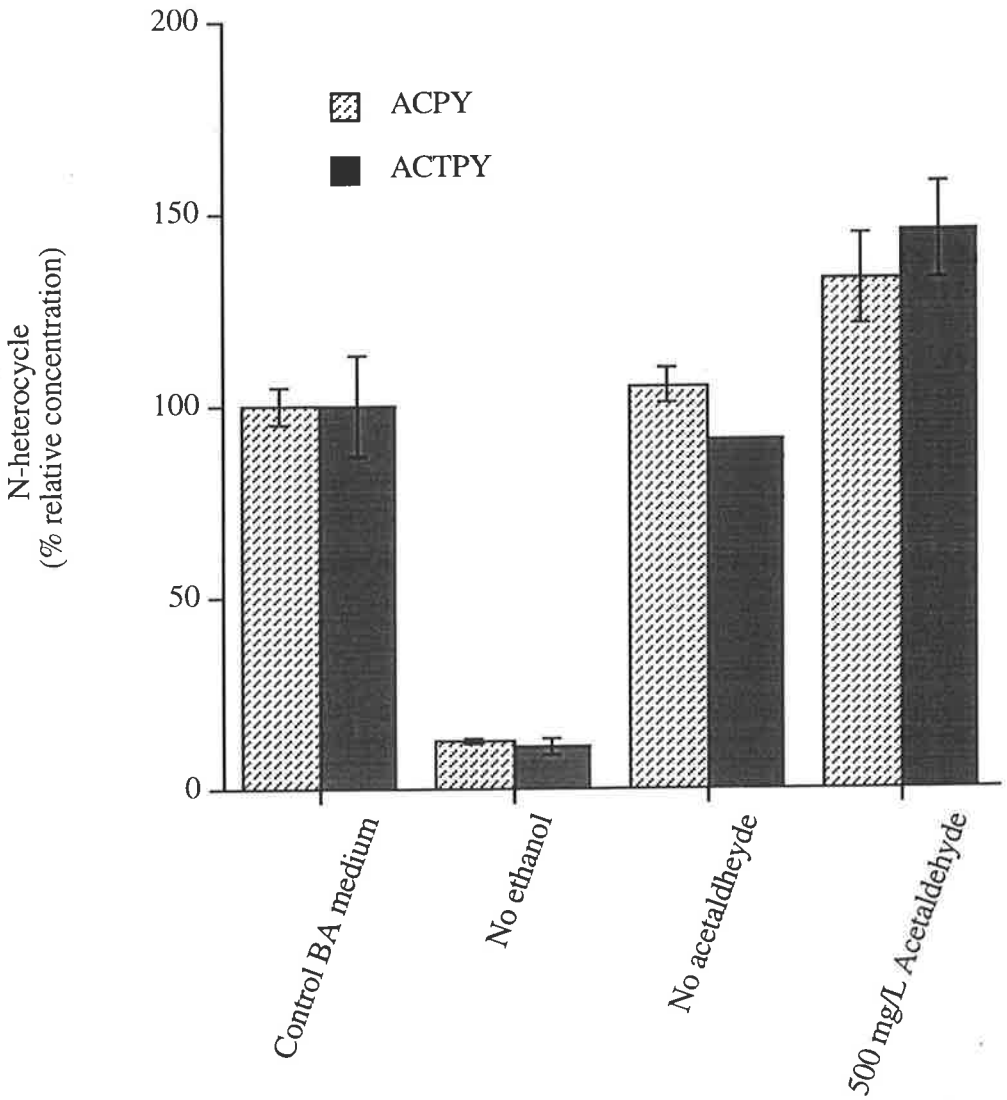


Figure 6.5 Effects of ethanol and acetaldehyde content of basal assay (BA) medium on the relative concentrations (%) of 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) produced by high cell density incubation of *Lactobacillus hilgardii* DSM 20176. Relative concentrations were calculated as the percentage ratio of the quantity of each compound of each sample relative to the control. Average data are presented; error bars indicate concentration range between replicates. Absolute concentrations (µg/L) of N-heterocycles are shown in Appendix 6.5

(100 mg/L) from the control BA medium had relatively small influence on the production of either ACPY (5% increase) or ACTPY (9% decrease) (Figure 6.5, Appendix 6.5). On the other hand, increasing the concentration of acetaldehyde in BA medium from 100 mg/L (control BA medium) to 500 mg/L caused relatively large increases in the formation of both ACPY (32.8%) and ACTPY (45.2%) compared to the control BA medium.

(ii) Metal ions

The effects of metal ion content on the production of mousy compounds by HCDI of *L. hilgardii* DSM 20176 are shown in Figure 6.6 and Appendix 6.6. Omission of all metal cations (Experiment I) from the BA medium reduced the production of ETPY from 6.8 µg/L to 2.7 µg/L. In the second set of treatments (Experiment II) in this series, the production of ETPY was not significantly affected by omission of either $MnSO_4$ or $MgSO_4$ and occurred in the range 1.4 - 2.5 µg/L. However, the production of this N-heterocycle was reduced by 90% (from 2.0 µg/L to 0.2 µg/L) by omission of $FeSO_4$, and increased over 3 - fold to 6.5 µg/L after omission of $CaCl_2$.

The formation of the other N-heterocycles, ACPY and ACTPY, was significantly affected by the omission of metal cations. Omission of all metal cations (Experiment I) completely prevented ACPY formation and reduced the formation of ACTPY by 96.3%. Similarly, omission of $FeSO_4$ from BA medium also gave large reductions in the formation of both ACPY (94.1% reduction) and ACTPY (93.9% reduction). In contrast, omission of either $MnSO_4$, $MgSO_4$ or $CaCl_2$ caused only slight reduction in the production of ACPY (reductions of 1.0%, 7.3% and 4.9%, respectively), and induced significant increases in the production of ACTPY (increases of 30.4%, 28.8% and 31.2%, respectively).

(iii) Citric and L-malic acids

The effects of omitting citric acid and L-malic acid from BA medium on the production of mousy compounds using HCDI with *L. hilgardii* DSM 20176 are shown in Figure 6.7 and Appendix 6.7. As noted previously, the production of ETPY was irregular, and was not detected in control BA medium of Experiment I, while 2.0 µg/L were produced in control BA medium of Experiment II. Moreover, omission of citric acid (Experiment I) or L-malic acid (Experiment II) had little impact on the formation of ETPY, and resulted in the formation of 2.5 µg/L and 2.6 µg/L of this N-heterocycle, respectively (Appendix 6.7). Furthermore, the formation of the remaining N-heterocycles was only slightly affected by the omission of citric acid, and resulted in 3.4% reduction in the production of ACPY and 8.4% increase in the production of ACTPY. On the other hand, omission of L-malic acid

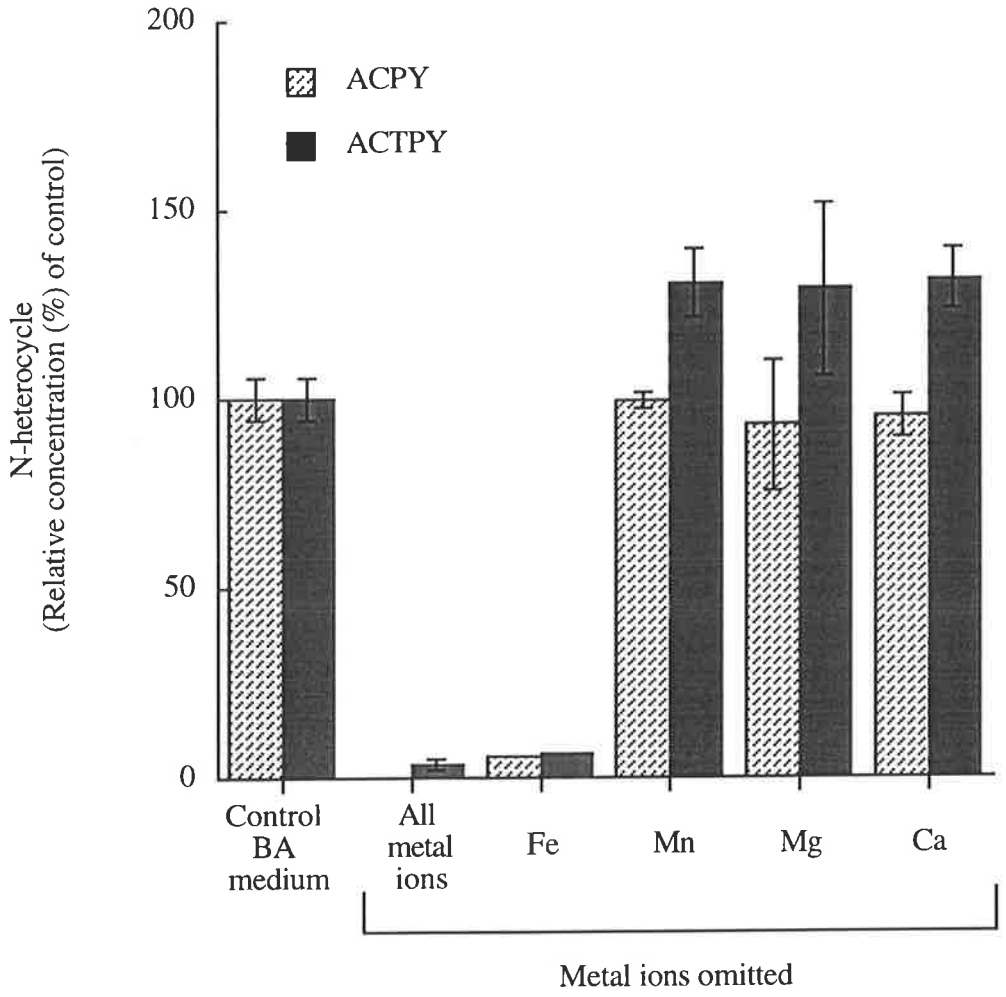


Figure 6.6 The effect of metal ion content of basal assay (BA) medium on the relative concentration (%) of 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) produced by high cell density incubation of *Lactobacillus hilgardii* DSM 20176. Relative concentrations were calculated as the percentage ratio of the concentration of each compound in each sample relative to the control. Average data from duplicate assays are presented; error bars indicate concentration range between replicates. Absolute concentrations (μg/L) of N-heterocycles are shown in Appendix 6.6

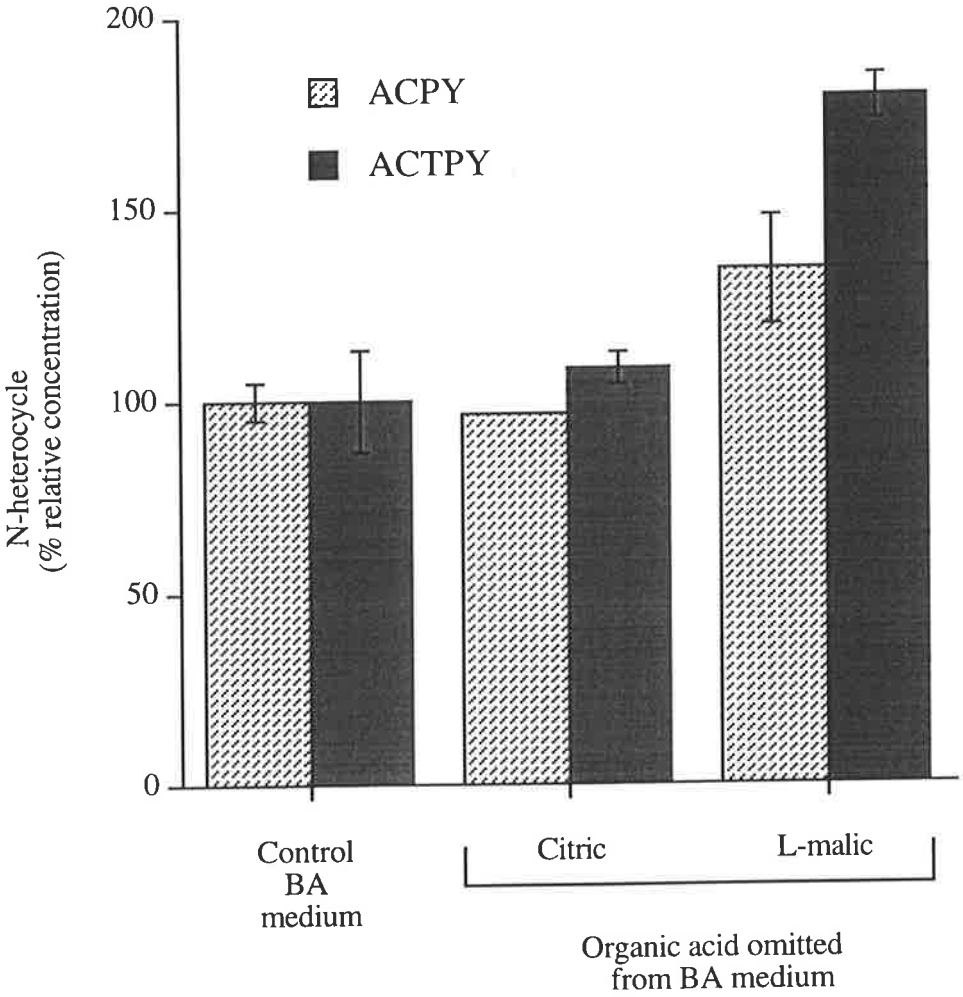


Figure 6.7 The effect of citric acid and L-malic acid content of basal assay (BA) medium on the relative concentrations (%) of 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) produced by high cell density incubation of *Lactobacillus hilgardii* DSM 20176. Relative concentrations were calculated as the percentage ratio of the concentration of each compound in each sample relative to the control. Average data presented; error bars indicate concentration range between replicates. Absolute concentrations (μg/L) of N-heterocycles are shown in Appendix 6.7

from BA medium resulted in relatively large increases in the relative concentrations of both ACPY (33.6% increase) and, in particular, of ACTPY (78.9% increase).

(iv) Other nitrogen sources

The effects of using either L-proline (5 g/L), L-glutamate (5 g/L) or DL-pipecolate (1 g/L) as nitrogen sources in BA medium on the formation of mousy compounds by HCDI with *L. hilgardii* DSM 20176 are shown in Figure 6.8 and Appendix 6.8. In these experiments, the formation of ETPY was not significantly influenced by the latter nitrogen sources and was produced in each sample in the concentration range 1.8 - 4.2 µg/L. In contrast, the use of either L-proline or L-glutamate as sole sources of nitrogen completely prevented ACPY formation and, furthermore, respectively reduced the production of ACTPY by 37.4% and 42.2% compared to the control (BA medium without amino acids). Similarly, use of DL-pipecolic acid as a sole nitrogen source significantly reduced the formation of both ACPY (62.1% decrease) and of ACTPY (53.5% decrease).

(iv) Other carbohydrate sources

The effects of using different carbohydrate sources, ie. D-fructose (50 g/L), D-glucose (50 g/L) or D-xylose (50 g/L), in BA medium on the formation of mousy compounds by HCDI of *L. hilgardii* DSM 20176 are shown in Figure 6.9 and Appendix 6.9. Replacement of D-fructose with either D-xylose or D-glucose caused slight reduction in the production of ETPY, from 5.5 µg/L to 3.1 µg/L and 2.2 µg/L, respectively. Moreover, replacing D-fructose with the latter carbohydrates caused greater than 90% reduction in the formation of both ACPY and ACTPY, ie. replacement with D-xylose yielded only 5.6% and 2.6% respectively of ACPY and ACTPY, while replacement with D-glucose respectively yielded only 6.4% and 4.2% of the two N-heterocycles.

(v) Incubation time and anaerobiosis

The effects of incubation time and anaerobiosis on mousy compound production by HCDI with *L. hilgardii* DSM 20176 are shown in Figure 6.10 and Appendix 6.10. Incubation for periods longer than 16h, ie. 72h and 104 h, had little effect on the production of ETPY and ACPY, yet increased the concentration of ACTPY by 11% and 41%, respectively. Compared to aerobic incubation, HCDI under anaerobic conditions had little effect on the production of mousy compounds, with the greatest change being that of ACTPY which decreased by 7.3%.

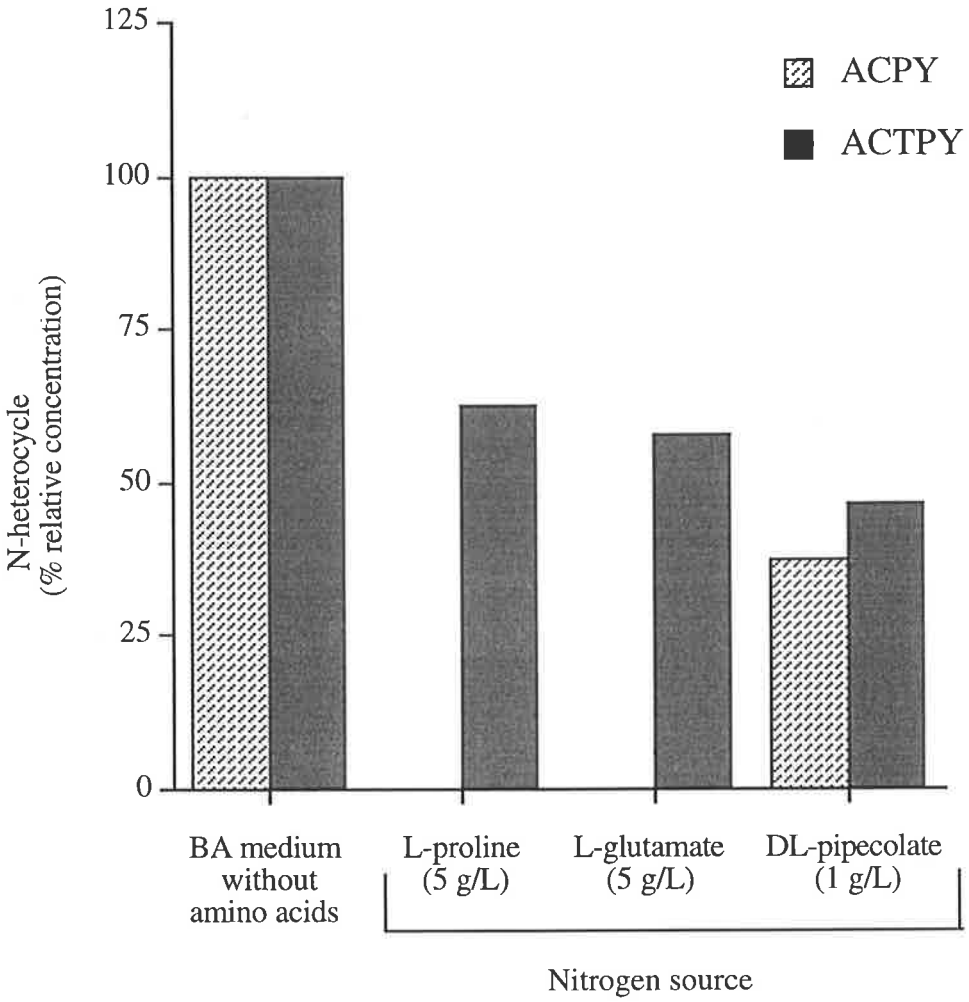


Figure 6.8 The effect of different nitrogen sources in the basal assay (BA) medium on the relative concentrations (%) of 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) produced by high cell density incubation of *Lactobacillus hilgardii* DSM 20176. Relative concentrations were calculated as the percentage ratio of the concentration of each compound in each sample relative to the control (BA medium without amino acids). Absolute concentrations (µg/L) of N-heterocycles are shown in Appendix 6.8

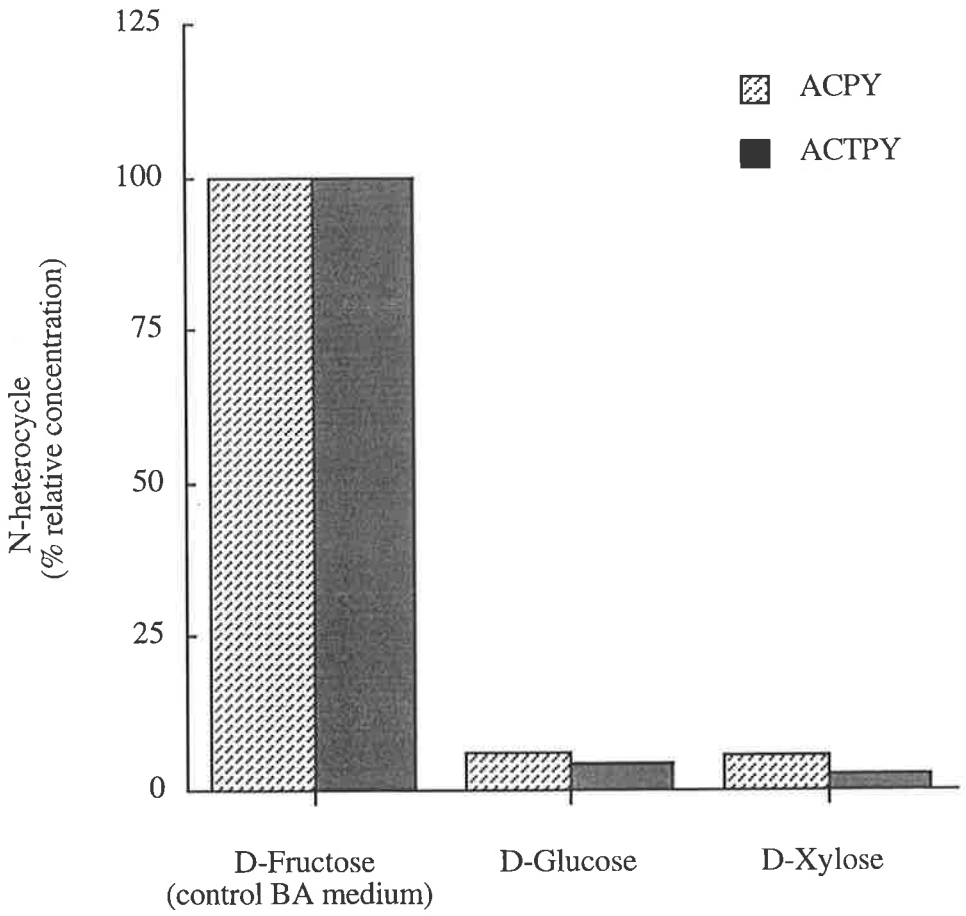


Figure 6.9 The effect of different carbohydrate sources (each 50 g/L) of basal assay (BA) medium on the relative concentrations (%) of 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) produced by high cell density incubation of *Lactobacillus hilgardii* DSM 20176. Relative concentrations were calculated as the percentage ratio of the concentration of each compound in each sample relative to the control. Absolute concentrations (µg/L) of N-heterocycles are shown in Appendix 6.9

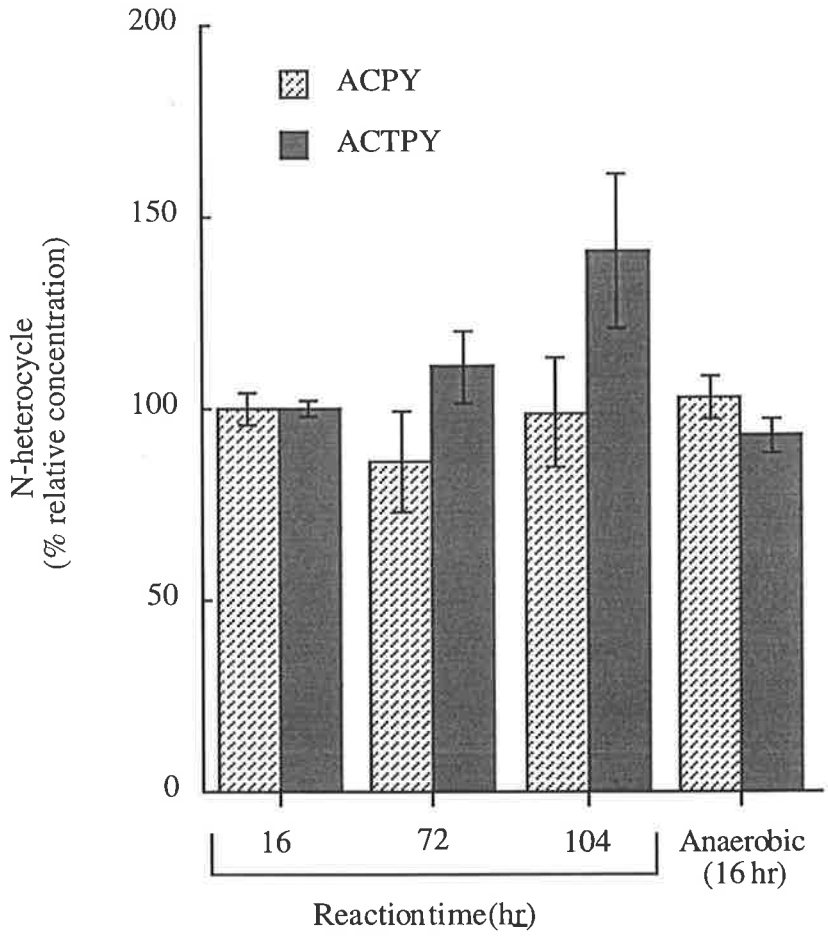


Figure 6.10 The effect of reaction time and anaerobic incubation of high cell density incubation of *Lactobacillus hilgardii* DSM 20176 on the formation of 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) in basal assay (BA) medium. Relative concentrations were calculated as the percentage ratio of the concentration of each compound in each sample relative to the control. Absolute concentrations (μg/L) of N-heterocycles are shown in Appendix 6.10

(vi) Production of mousy compounds in phosphate-KCl buffer

An experiment was performed to determine whether mousy compounds were produced by HCDI of *L. hilgardii* DSM 20176 in a phosphate-KCl buffer (pH 4.5) (Table 6.8). With the exception of a relatively insignificant quantity of ACTPY (2.1 µg/L) produced, no other N-heterocycles were detected after HCDI in phosphate-KCl buffer.

6.3.4 Substrates and precursors of mousy N-heterocycles produced by *Lactobacillus hilgardii* DSM 210176

6.3.4.1 Non-labelled substrates

(i) Alcohols

The effects of replacing ethanol with either *n*-propanol, *iso*-propanol or *n*-butanol (5% v/v) in BA medium on the formation of other homologues (C-3 and C-4 substituted) of ETPY, ACPY and ACTPY by HCDI with *L. hilgardii* DSM 20176 are shown in Table 6.9. The insolubility of *iso*-butanol, however, prevented its inclusion in this experiment. Substitution of ethanol with *n*-propanol produced two unique compounds having similar mass spectra and *m/z* M^+ 139, and which emerged from the GC column shortly after (approximately 120 scan units; 1.7 min) ACTPY I and ACTPY II, respectively. The mass spectral data of these two compounds (Table 6.9, Figure 6.11) were consistent with those reported for the tautomers of 2-propionyltetrahydropyridine (Heresztyn 1986), and hence were tentatively assigned the structures shown in Figure 6.12. The total concentration of both tautomers of 2-propionyltetrahydropyridine (PRTPY) in this sample (45.6 µg/L) was more than double that of ACTPY (18.4 µg/L). However, other C-3 substituted compounds including the propionyl homologue of ACPY, ie. 2-propionyl-1-pyrroline (*m/z* M^+ 125), and 2-propyltetrahydropyridine (*m/z* M^+ 125), as well as ETPY and ACPY, were screened for but not detected in this sample.

In contrast to the above findings, substitution of ethanol with *iso*-propanol in BA medium failed to yield PRTPY by HCDI with *L. hilgardii* DSM 20176. Another compound, however, occurred only in this extract and which chromatographed shortly after ETPY. The mass spectrum of this compound exhibited similarities to that of ETPY and, having an M+ *m/z* 125, was tentatively identified as 2-propyltetrahydropyridine (Figures 6.13 and 6.14). The concentration of the tentatively identified 2-propyltetrahydropyridine (0.7 µg/L) was only slightly less than that of ETPY (2.6 µg/L). Other C-3 substituted N-heterocycles were not detected in this sample. Furthermore, no substituted N-heterocycles were produced in

Table 6.9 The effect of different alcohol substrates on the production of substituted N-heterocycles† by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 in basal assay (BA) medium*

N-heterocycle	*m/z*	Concentration of N-heterocycle (μg/L) in response to alcohol substrate:		
		***n* - Propanol**	***iso* - Propanol**	***n* -Butanol**
C-2 substituted				
2-Ethyltetrahydropyridine	111	nd†	2.6	nd
2-Acetyl-1-pyrroline	111	nd	6.9	nd
2-Acetyltetrahydropyridine	125	18.4	96.1	nd
C-3 substituted:				
2-Propyltetrahydropyridine	125	nd	0.7	ND††
2-Propionyl-1-pyrroline	125	nd	nd	ND
2-Propionyltetrahydropyridine	139	45.6	nd	ND
C-4 substituted:				
2-Butyltetrahydropyridine	139	ND	ND	nd
2-Butyryl-1-pyrroline	139	ND	ND	nd
2-Butyryltetrahydropyridine	153	ND	ND	nd

* BA media prepared by substituting ethanol for either *n*-propanol, *iso*-propanol or *n*-butanol

† Alkyl-substituted pyrrolines, ie. 2-ethyl-1-pyrroline (m/z 97) and 2-propyl-1-pyrroline (m/z 111) were not detected in any of the sample extracts

nd = not detected

ND = not determined

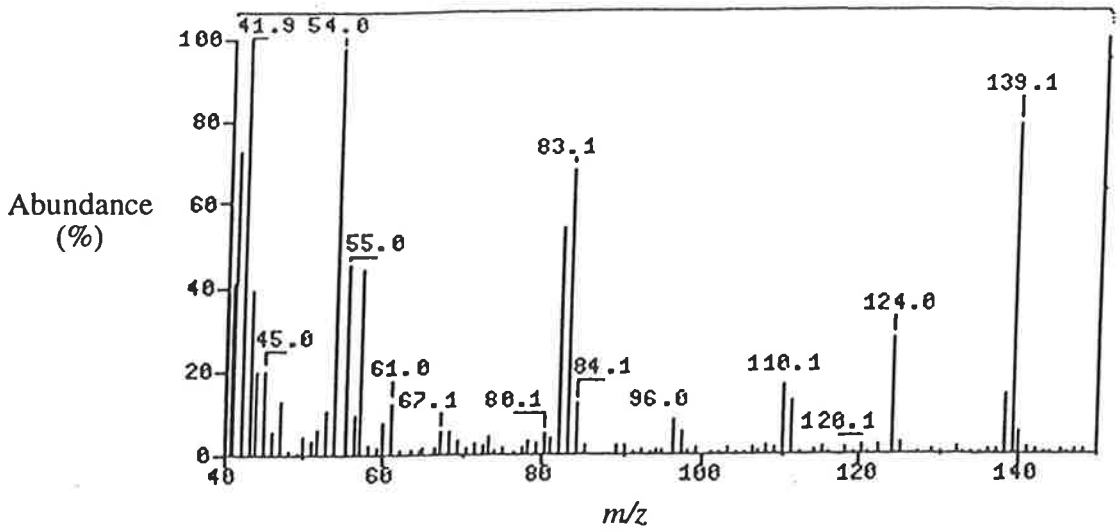
Table 6.8 Concentrations of 2-ethyltetrahydropyridine (ETPY), 2-acetyl-1-pyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) produced by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 in basal assay (BA) medium and a phosphate-KCl buffer

Assay medium	**N-heterocycle (μg/L)**		
	ETPY	**ACPY**	**ACTPY**
BA medium (control)	5.8 (nd, 11.5)†	39.1 (41.4, 36.9)	453.1 (422.7, 483.5)
Phosphate-KCl buffer	nd*	nd	2.1

† Data obtained from analysis of duplicate HCDI assays: first value denotes the mean concentration, and values in braces are the concentration of each replicate

* nd = not detected

Tautomer 1



Tautomer 2

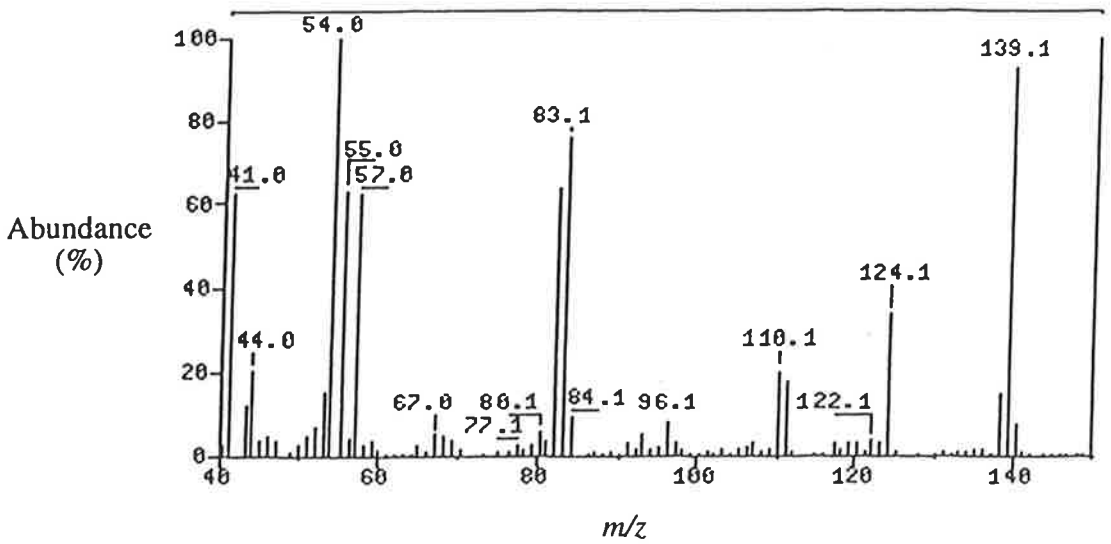


Figure 6.11 Mass spectra of tautomers of 2-propionyltetrahydropyridine (*m/z* 139) occurring in an extract of a high cell density incubation assay with *Lactobacillus hilgardii* DSM 20176. The source of alcohol in the assay medium was *n*-propanol

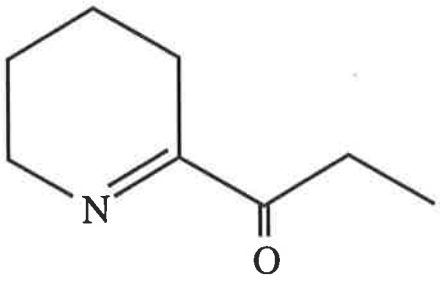


Figure 6.12 Structure assigned to 2-propionyltetrahydropyridine (*m/z* 139), produced by high cell density incubation of *Lactobacillus hilgardii* DSM 20176. The source of alcohol in the assay medium was *n*-propanol.

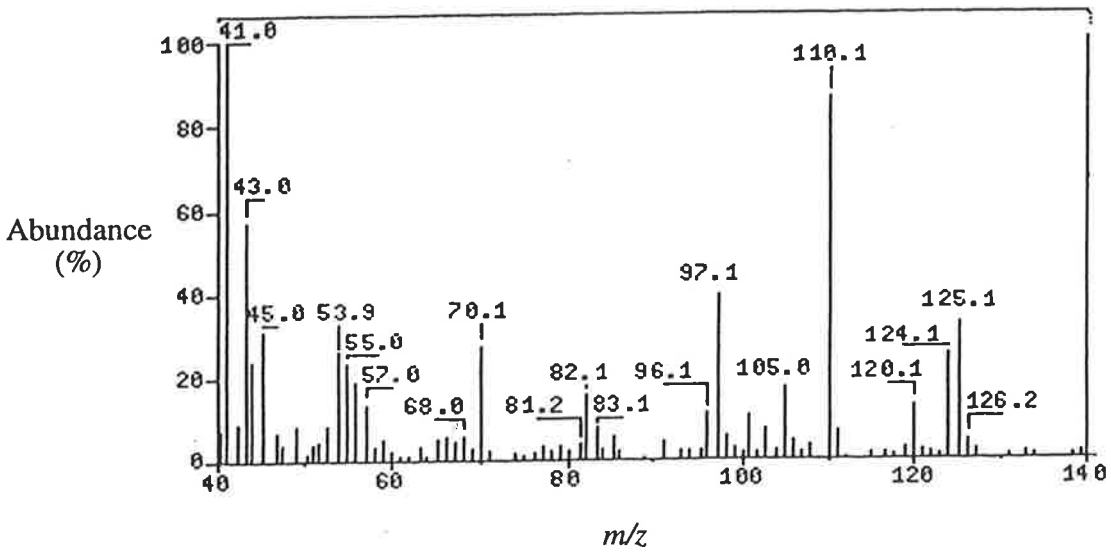


Figure 6.13 Mass spectrum of the tentatively identified 2-propyltetrahydropyridine (*m/z* 125) occurring in an extract of a high cell density incubation assay with *Lactobacillus hilgardii* DSM 20176. The source of alcohol in the assay medium was *iso*-propanol

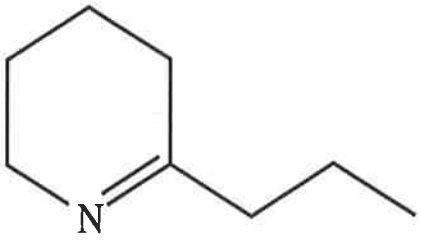


Figure 6.14 Structure assigned to the tentatively identified 2-propyltetrahydropyridine (*m/z* 125), produced by high cell density incubation of *Lactobacillus hilgardii* DSM 20176. The source of alcohol in the assay medium was *iso*-propanol. The position of the double bond is uncertain

BA medium by HCDI of *L. hilgardii* DSM 20176 when ethanol was substituted for *n*-butanol.

(ii) Aldehydes

Substitution of acetaldehyde for either propionaldehyde or butyraldehyde in BA medium did not result in the production of any C-3 or C-4 substituted homologues of ETPY, ACPY or ACTPY after HCDI with *L. hilgardii* DSM 20176 (Table 6.10).

6.3.4.2 Labelled substrates

(i) d_6-Ethanol

In addition to the production of ACPY and ACTPY, deuterated isotopes of both tautomers of ACTPY, (d_3-ACTPY, *m/z* 128) and of ACPY (d_3-ACPY, *m/z* 114) were produced from HCDI of *L. hilgardii* DSM 20176 when the ethanol component of BA medium was substituted with d_6-ethanol (Table 6.11, Figures 6.15, 6.17). The retention times of these isotopes were almost identical to the respective unlabelled N-heterocycles, and typically emerged approximately 6-7 scan units (0.05-0.06 min) earlier. Furthermore, the increase of 3 amu for the ion fragment *m/z* 43 $\rightarrow$ 46 of ACTPY I, ACTPY II and ACPY I, and of *m/z* 42 $\rightarrow$ 45 for ACPY II, indicated that three deuterium atoms from d_6-ethanol were incorporated into the acetyl side chain of each isotope. These data suggest the structures shown in Figures 6.16 and 6.18 for the isotopes d_3-ACTPY and d_3-ACPY. Moreover, the ratio of d_3-ACTPY to ACTPY (4.1:1.0) and of d_3-ACPY to ACPY (1.0:1.0) indicated similar proportions of both labelled and unlabelled N-heterocycles occurred in this sample. Further, while M+3 ions were the most predominant for each isotope, other M^+ isotopic peaks were also observed for ACTPY, eg. *m/z* 126 and *m/z* 127, and for ACPY, eg. *m/z* 113. No labelled isotopes were detected in extracts of samples that were prepared using unlabelled ethanol.

In contrast to the above formation of d_3-ACTPY and d_3-ACPY from HCDI of *L. hilgardii* DSM 20176 using d_6-ethanol, labelled isotopic peaks for ETPY (*m/z* 112-116) were not detected (Table 6.11).

Table 6.10 The effect of different aldehyde substrates on the production of substituted tetrahydropyridines and pyrrolines[†] by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 in basal assay medium*

N-heterocycle	*m/z*	Concentration of N-heterocycle (μg/L) in response to aldehyde substrate	
		Propionaldehyde	**Butyraldehyde**
C-2 substituted:			
2-Ethyltetrahydropyridine	111	nd[†]	6.8
2-Acetyl-1-pyrroline	111	54.5	65.6
2-Acetyltetrahydropyridine	125	520.0	673.7
C-3 substituted			
2-Propyltetrahydropyridine	125	nd	ND[††]
2-Propionyl-1-pyrroline	125	nd	ND
2-Propionyltetrahydropyridine	139	nd	ND
C-4 substituted			
2-Butyltetrahydropyridine	139	ND	nd
2-Butyryl-1-pyrroline	139	ND	nd
2-Butyryltetrahydropyridine	153	ND	nd

* BA medium prepared by substituting acetaldehyde for propionaldehyde or butyraldehyde

[†] Alkyl-substituted pyrrolines 2-ethyl-1-pyrroline (m/z 97) and 2-propyl-1-pyrroline (m/z 111) were not detected in any of the sample extracts

[†] nd = not detected

[††] ND = not determined

Table 6.11 Formation of deuterated mousy N-heterocycles (μg/L) by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 in basal assay (BA) medium containing d_6-ethanol and d_4-acetaldehyde*

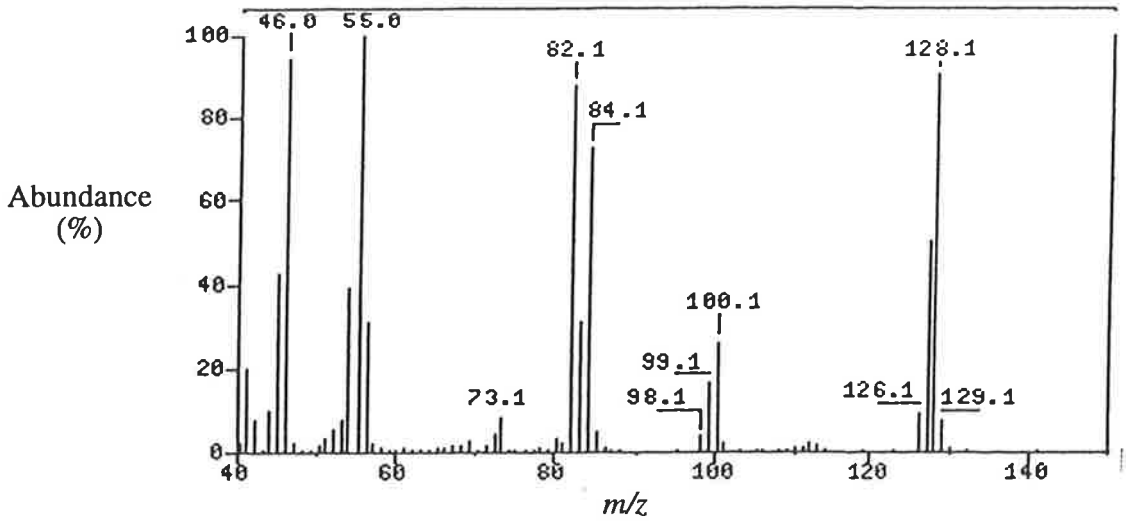
N-heterocycle	*m/z*	Concentration of N-heterocycle (μg/L) in response to labelled substrate		
		None (control BA medium)	d_6-Ethanol	d_4-Acetaldehyde
ETPY	111	1.5	Tr†	1.6
d_4-ETPY	115	nd	nd††	nd
d_5-ETPY	116	nd	nd	nd
ACPY	111	45.3	10.6	52.1
d_3-ACPY	114	nd	10.4	nd
ACTPY	125	469.3	59.4	479.2
d_3-ACTPY	128	nd	244.7	0.6

* BA media prepared by substituting ethanol for d_6-ethanol and acetaldehyde for d_4-acetaldehyde

† Tr = trace amount

†† nd = not detected

Tautomer 1



Tautomer 2

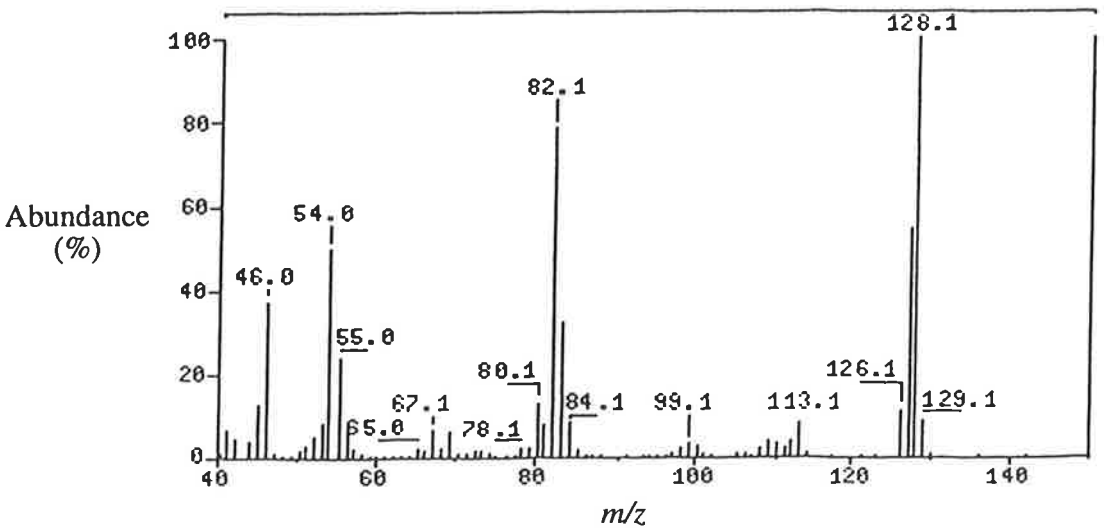


Figure 6.15 Mass spectra of tautomers of d_3-2-acetyltetrahydrahydropyridine (d_3-ACTPY, *m/z* 128), produced by high cell density incubation of *Lactobacillus hilgardii* DSM 20176. This deuterated compound was produced when the source of alcohol in the assay medium was d_6-ethanol, and also when acetaldehyde was replaced with d_4-acetaldehyde

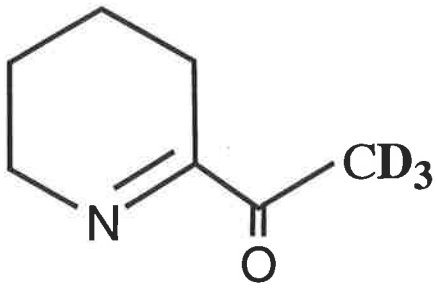
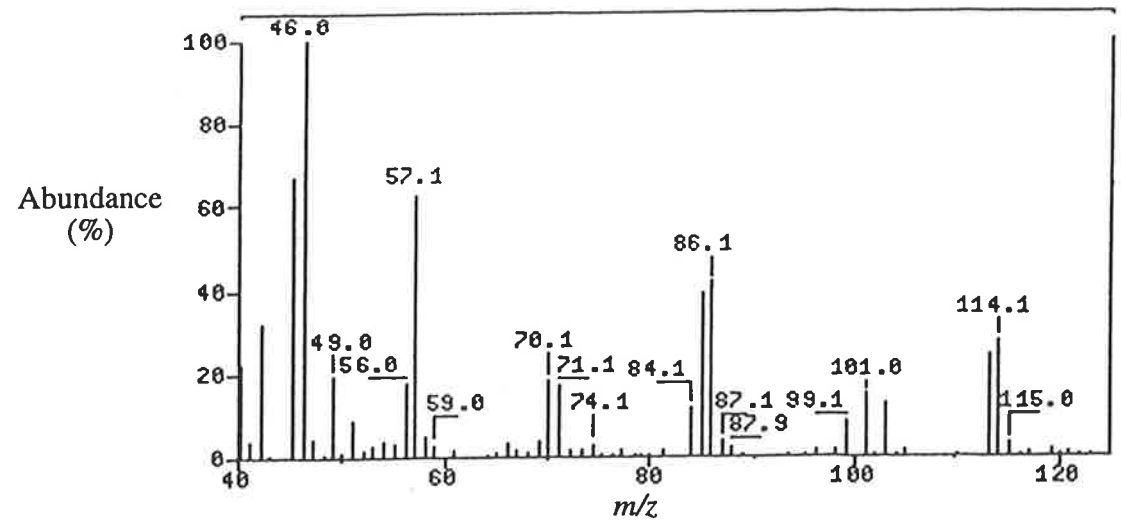


Figure 6.16 Structure assigned to d_3-2-acetyltetrahydrahydropyridine (d_3-ACTPY, *m/z* 128), produced by high cell density incubation of *Lactobacillus hilgardii* DSM 20176. This deuterated compound was produced when the source of alcohol in the assay medium was d_6-ethanol, and also when acetaldehyde was replaced with d_4-acetaldehyde. Note that labelling (3 deuterium atoms) occurs on the acetyl side chain. The position of the double bond is uncertain

Tautomer 1



Tautomer 2

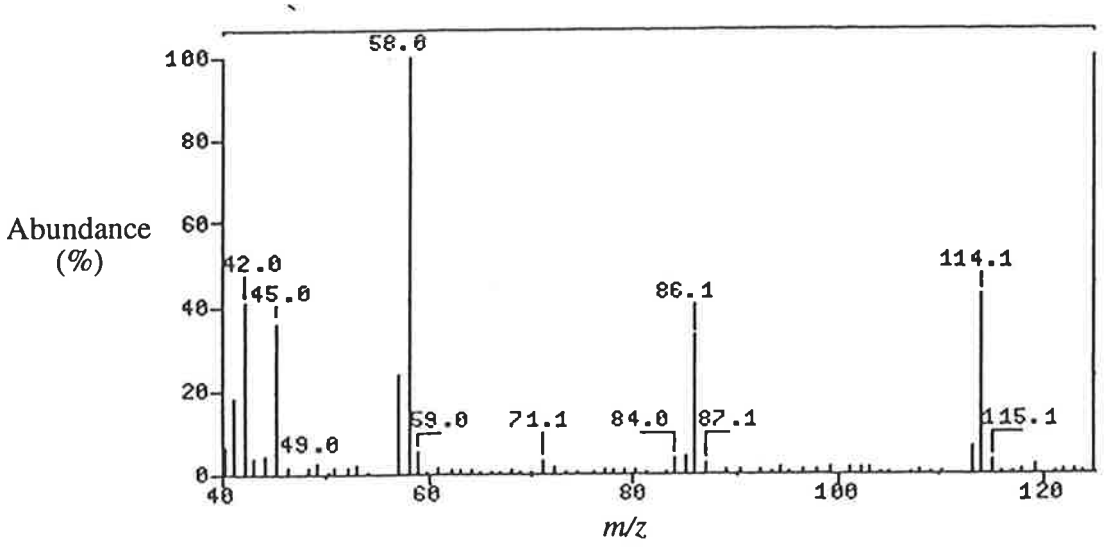


Figure 6.17 Mass spectra of tautomers of d_3-2-acetylpyrroline (d_3-ACPY, *m/z* 114), produced by high cell density incubation of *Lactobacillus hilgardii* DSM 20176. This deuterated compound was produced when the source of alcohol in the assay medium was d_6-ethanol

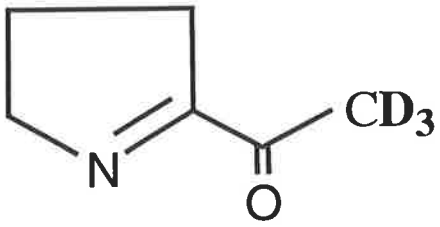


Figure 6.18 Structure assigned to d_3-2-acetylpyrroline (d_3-ACPY, *m/z* 114), produced by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 when the source of alcohol in the assay medium was d_6-ethanol. Note that labelling (3 deuterium atoms) occurs on the acetyl side chain. The position of the double bond is uncertain

(ii) *d*4-Acetaldehyde

Minor quantities of M+3 isotopes of both tautomers of ACTPY (*m/z* 128) (total concentration = 0.6 μg/L) were produced by HCDI of *L. hilgardii* DSM 20176 when the acetaldehyde component of BA medium was replaced with *d*4-acetaldehyde (Table 6.11, Figures 6.15-6.16). However, labelled isotopes of ACPY or ETPY were not detected after the substitution of acetaldehyde with *d*4-acetaldehyde (Table 6.11).

(iii) *2-d* -D-Glucose

Distinct chromatographic peaks of deuterated (M+1) ACTPY, ie. *d*-ACTPY, or other mousy compounds were not detected after HCDI of *L. hilgardii* DSM 20176 when D-fructose was replaced with 2-*d*-D-glucose. In this latter sample, however, the ratios of the peak areas of {M+1 (*m/z* 126)} versus {M+ (*m/z* 125)} ions for ACTPY I and ACTPY II were significantly greater than the ratios of the same ions produced in control BA medium, that is, when 2-*d*-D-glucose was substituted with D-fructose, the ratio of *m/z* 126 : *m/z* 125 ions for ACTPY I and ACTPY II increased by 13.6% and 54.2%, respectively (Table 6.12). This increase in the abundance of M+1 (*m/z* 126) ions suggests the possible formation of *d*-ACTPY tautomers from 2-*d*-D-glucose.

Table 6.12 Increase in the ratio of {M+1 (*m/z* 126) / M+ (*m/z* 125)} ions of 2-acetyltetrahydropyridine (ACTPY) produced by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 in basal assay (BA) medium containing 2-*d*-D-glucose*

Tautomer of ACTPY	Ratio (%) of {(*m/z* 126) / (*m/z* 125)} ions of ACTPY*		Increase (%) in ratio of {(*m/z* 126) / (*m/z* 125)} ions of ACTPY produced from 2-*d*-D-glucose
	Carbohydrate source		
	D-Fructose (unlabelled control)	$2d_1$-D-Glucose	
I	8.75	9.94	13.6
II	8.40	12.95	54.2

* Calculated from peak areas of respective ions

6. DISCUSSION

In the experiments of this chapter, the biocatalytic properties of a high concentration of resting cells of LAB were exploited to investigate the factors that affect the generation of mousy off-flavour compounds. The initial finding that mousy off-flavour could be generated by high cell density incubation of LAB is in itself significant, and demonstrates for the first time that this phenomenon is not a growth-dependent reaction.

Initial experiments involving the development of BA medium gave some indication of the components associated with the generation of mousy off-flavour by LAB. These included high sugar (D-fructose) content and the presence of metal ions, whereas vitamins, purines and pyrimidines and most amino acids could be omitted without apparent influence on mousy off-flavour formation.

Screening of lactic acid bacteria for the production of mousy N-heterocycles

The results of the screening of LAB for the production of mousy compounds using HCDI and by culture of cells in Carr-MEt medium (Chapter 4) have both demonstrated that a diversity of LAB strains were capable of producing each of the 3 mousy N-heterocycles. Furthermore, both testing procedures demonstrated that *L. hilgardii* DSM 20176 produced the highest concentration of any N-heterocycle, ie. 259 µg/L of ACTPY by culture in Carr-MEt medium and 580 µg/L of ACTPY by HCDI. However, of the 6 strains that were surveyed by both methods (*O. oeni* strains MCW, Lc5b, Lo-42, and Viniflora, and *L. hilgardii* strains DSM 20176 and L21c), the concentrations of N-heterocycles produced using HCDI were generally higher than those produced by cells cultured in Carr-MEt medium. For example, in addition to the aforementioned differences in ACTPY production by *L. hilgardii* DSM 20176, the concentrations of ETPY produced by *O. oeni* strains Lc5b, Lo-42 and Viniflora by HCDI were respectively 46 - fold, 4 - fold and 9 - fold higher than that produced by culture in Carr-MEt medium. The reasons for this result are unclear, but may relate, at least in part, to compositional differences between the synthetic BA medium and the undefined Carr-MEt medium, and also greater cell density and shorter period of incubation with the HCDI technique. Moreover, the results of the survey using HCDI provided relatively clear differentiation between genera and species of LAB in the ability to produce mousy compounds. The most potent producers of ACTPY were the heterofermentative *Lactobacilli*, in particular *L. hilgardii* and *L. brevis*, while the highest concentrations of ETPY (86.8 - 162.0 µg/L) were produced by 3 of 5 *O. oeni* strains. In contrast, with the exception of *P. damnosus* P1a, the production of ACPY was a characteristic of most LAB. Overall, the results of the survey of mousy N-heterocycle

formation by LAB using HCDI corroborate the findings of the survey conducted in Carr-MEt medium that the production of high concentrations of ACTPY was a characteristic of heterofermentative Lactobacilli (eg. *L. hilgardii* and *L. brevis*), while the homofermentative *Pediococci* and *L. plantarum* were generally lacking in this ability. The HCDI procedure has further provided new evidence to suggest that the formation of high concentrations of ETPY is a characteristic of the metabolism of *O. oeni*, but not all strains showed that characteristic

Metabolic studies using HCDI and the test strain *L. hilgardii* DSM 20176 demonstrated that the formation of the acetylated N-heterocycles, ACPY and ACTPY, involved the co-metabolism of several key substrates. Of the 13 components comprising BA medium, three were found to be essential for ACPY and ACTPY formation: (i) a fermentable carbohydrate (D-fructose), (ii) ethanol and (iii) the presence of Fe^{2+} ions. The importance of these compounds was highlighted by the large reductions in the production of ACPY and ACTPY (78% - 94% and 89%-99%, respectively) when either component was omitted from the BA medium. Significantly, these results suggest that the generation of acetylated N-heterocycles by LAB is concomitantly dependent upon the metabolic pathways involved in the lactic fermentation of sugars, and the metabolism of ethanol. Furthermore, stimulation of the formation of ACPY and ACTPY by *L. hilgardii* DSM 20176 in the presence of 500 mg/L acetaldehyde suggests the involvement of this C-2 carbonyl in the overall reaction process.

Origins of the side chain in acylated mousy N-heterocycles

(i) Alcohol and acetaldehyde

Further insight into the formation of mousy N-heterocycles, particularly that of the acetyl side chain, was obtained from HCDI experiments using various non-labelled and deuterium - labelled substrates. First, the demonstration that the propionyl analogues of the tautomers of ACTPY were produced when *n*-propanol was substituted for ethanol supported the earlier work of Heresztyn (1986), which demonstrated the important role of alcohol in the formation of the tetrahydropyridine side chain. Moreover, the occurrence of ACTPY in the *n*-propanol substituted fermentation, as reported by Heresztyn (1986), was also observed in this study. In contrast with results of Heresztyn (1986), however, the quantity of ACTPY produced in this sample was a significant proportion (40.4%) of the concentration of 2-propionyltetrahydropyridine. Heresztyn (1986) considered that the occurrence of ACTPY in the *n*-propanol fermentation was caused by traces of ethanol in the propanol, yet the use of redistilled *n*-propanol in the current work negates this possibility and, rather, indicates that the formation of the side chain of acylated N-heterocycles may utilise more than one precursor and / or pathway.

Further to the production of 2-propionyltetrahydropyridine in the *n*-propanol fermentations, it would be anticipated that propionyl analogues of ACPY may also be produced. However, neither of the propionyl- or acetyl- substituted pyrroline compounds were detected in the present studies. While the reason(s) for this is not known, it is possible that both substituted pyrrolines may have been produced at concentrations below the detection limits of the assay, ie. less than approximately 0.1 µg/L. This could also explain the absence of alkyl-substituted tetrahydropyridines in this extract. The inability of *L. hilgardii* DSM20176 to produce 2-propionyltetrahydropyridine (and 2-propionylpyrroline) with *iso*-propanol extends the findings of Heresztyn (1986) to suggest that the formation of acetylated N-heterocycles requires a primary alcohol, and not a secondary alcohol. Moreover, it appears that such a trend may be restricted to alcohols containing a maximum of three carbon atoms, since no N-heterocycles were detected when *n*-butanol was used as an alcohol source (Table 6.9).

A novel finding from the *iso*-propanol - containing assay was the tentative identification of 2-propyltetrahydropyridine. This is the first known report of the production of this compound by LAB. Moreover, while the role of *iso*-propanol in the formation of 2-propyltetrahydropyridine is not known, the latter finding further indicates the existence of separate pathways for the biosynthesis of alkyl - and acyl- substituted N-heterocycles.

Clear evidence that ethanol was a direct precursor in the biosynthesis of acetylated N-heterocycles was obtained from experiments using deuterium - labelled ethanol (*d* 6-ethanol) (Table 6.11). These results demonstrated, for the first time, that three deuterium atoms from *d* 6-ethanol were incorporated into the acetyl side chain of both ACPY and ACTPY by *L. hilgardii* DSM 20176. It is interesting to note, however, that the significant proportions of non-labelled ACPY (ACPY : *d* 3-ACPY = 1:1) and ACTPY (ACTPY : *d* 3-ACTPY = 1:4) in this assay did not appear to be a result of deuterium - proton exchange (data not shown). This further suggests that precursors other than ethanol are involved in the acetylation reaction.

Unlike ethanol, acetaldehyde was not essential for N-heterocycle formation, and a concentration of 100 mg/L acetaldehyde had little impact on mousy compound production. However, the stimulation of ACPY and ACTPY formation afforded by 500 mg/L acetaldehyde suggested that this carbonyl may be involved in the biosynthesis of mousy N-heterocycles (Figure 6.5 , Appendix 6.5). This was confirmed by the incorporation of three deuterium atoms from *d* 4-acetaldehyde(100 mg/L) into the acetyl side chain of ACTPY, producing *d* 3-ACTPY by *L. hilgardii* DSM 20176 (Table 6.11). Although the labelling pattern of *d* 3-ACTPY from *d* 4-acetaldehyde was essentially the same as that from *d* 6-ethanol, no labelling of ACPY was detected under these conditions. This may have been

due to the very low proportion of d_3-ACTPY : ACTPY (0.1%) produced from d_4-acetaldehyde; such a low proportion of d_3-ACPY would not be detectable in the current assay. It follows, therefore, that the likelihood of producing detectable amounts of labelled ACPY, or other substituted N-heterocycles derived from C-3 or C-4 aldehydes including propionaldehyde and butyraldehyde, could be increased, in future studies, by using higher concentrations of aldehyde substrate, ie. 500 mg/L rather than 100 mg/L.

In contrast to the labelling of acetylated N-heterocycles from d_6-ethanol and d_4-acetaldehyde, as described above, no such labelling of ETPY was observed. This may be a result of a lack of sensitivity as described above for ACPY, although the generally divergent results obtained for this compound compared to those of ACPY and ACTPY provides further evidence, as previously indicated, that the biosynthesis of alkyl- and acyl- substituted N-heterocycles involves the activities of separate pathways.

(ii) Carbohydrate source and the heterolactic fermentation

The significant dose-response relationship between D-fructose and the formation of both ACPY and ACTPY by *L. hilgardii* DSM20176 clearly demonstrated that the presence of a fermentable carbohydrate was a major nutritional factor involved in the formation of acetylated N-heterocycles. Furthermore, the indication, in this chapter, that 2-*d*-D-glucose was incorporated into ACTPY supports similar observations by Romanczyk *et al.* (1995), that *B. cereus* utilised ^{13}C- labelled D-glucose in the formation of ACPY. These authors also demonstrated that two carbons from ^{13}C labelled D-glucose were incorporated into ACPY as the acetyl group.

It is important to note from the current study that a carbohydrate source, together with ethanol and acetaldehyde, were collectively utilised as substrates of acetylated N-heterocycles. This association suggests, for the fist time, that the biosynthesis of acetylated N-heterocycles by LAB is linked with the heterolactic fermentation of sugars. In the heterolactic fermentation, hexoses are catabolised via the phosphoketolase (PK) pathway (or 6-phosphogluconate pathway), the main end-products of which are lactate, CO_2 and ethanol and / or acetate. Pyruvate and acetaldehyde, or more specifically the activated derivative acetyl-coenzyme A, also act as electron acceptors and are reduced to lactate and ethanol respectively in order to reoxidize NADH / NADPH (Cogan 1987, Axelsson 1993). Under the conditions of the HCDI assay, however, both the sugar substrate and C-2 end-products are supplied in excess, which may facilitate the accumulation of certain C-2 intermediates in the ethanol branch for other acetylation reactions.

In the latter connection, it is worthy to note the comparative affects of different carbohydrate sources on the formation of acetylated N-heterocycles. Clearly, the highest concentrations of ACPY and ACTPY were produced by the test strain in the presence of D-fructose, and these amounts decreased by more than 90% upon replacement with either D-glucose or the pentose D-xylose (Figure 6.9, Appendix 6.9). Interestingly, this response was also reflected in the much greater proportion of D-fructose degraded during the HCDI assay (average degradation of 87% over two experiments) than of D-glucose (27% degradation), and the production of more than double the amount of acetic acid from D-fructose (3.5 g/L) than from D-glucose (1.5 g/L) (data not shown). Other literature reports (Axelsson 1993) have also indicated that some heterofermentative LAB prefer D-fructose as a carbon source over other sugars, presumably since it can also serve as an external electron acceptor. In this case, D-fructose is reduced to mannitol by a NAD^+ - mannitol dehydrogenase which enables the cell to produce ATP through an acetate kinase reaction, thus sparing acetyl phosphate from the ethanol branch of the PK pathway (Cogan 1987, Axelsson 1993).

The preference of LAB for D-fructose compared to other sugars may provide further insight into a mechanism by which C-2 intermediates could accumulate under conditions of the HCDI assay. Compared with D-glucose, the more efficient utilisation of D-fructose by LAB would be expected to generate a greater flux of heterolactic intermediates. Coupled with this, augmented activity of the acetate kinase reaction would lessen the requirement for reduction of acetyl-phosphate to acetyl-coenzyme A in the ethanol leg. Therefore, the combination of a relatively high rate of D-fructose dissimilation, and a reversed activity of alcohol and acetaldehyde dehydrogenase enzymes from excess ethanol would force a greater accumulation of reactive C-2 intermediates such as acetyl-CoA within the cell than that anticipated from D-glucose metabolism. A scheme depicting the accumulation of C-2 intermediates in the heterolactic pathway under conditions of substrate and (C-2) end-product excess as described is shown in Figure 6.19.

The scheme suggests that a reservoir of C-2 intermediates could accumulate and become available for other, secondary acylation reactions, including those involved in the formation of acetylated N-heterocycles. The production of propionyl-tetrahydropyridine from *n*-propanol, as seen in this chapter, also fits into this scheme, whereby respective (reversed) alcohol and aldehyde dehydrogenase activities may generate the C-3 carrier, propionyl-CoA. Moreover, the considerable proportions of native ACTPY produced in the *n*-propanol and also d_6-ethanol fermentations strongly suggests that the acetyl group is derived, at least in part, from the carbohydrate source as well as from ethanol. Indeed, the indication that 2-*d*-D-glucose was incorporated into ACTPY supports this view. Overall, these results suggest that the heterolactic pathway may supply either one, or possibly two separate C-2 intermediates for secondary acetylation reactions. Further studies using ^{13}C- labelled

carbohydrates are needed to confirm whether C-2 units from the carbohydrate source are directly incorporated into the acetyl group of mousy N-heterocycles.

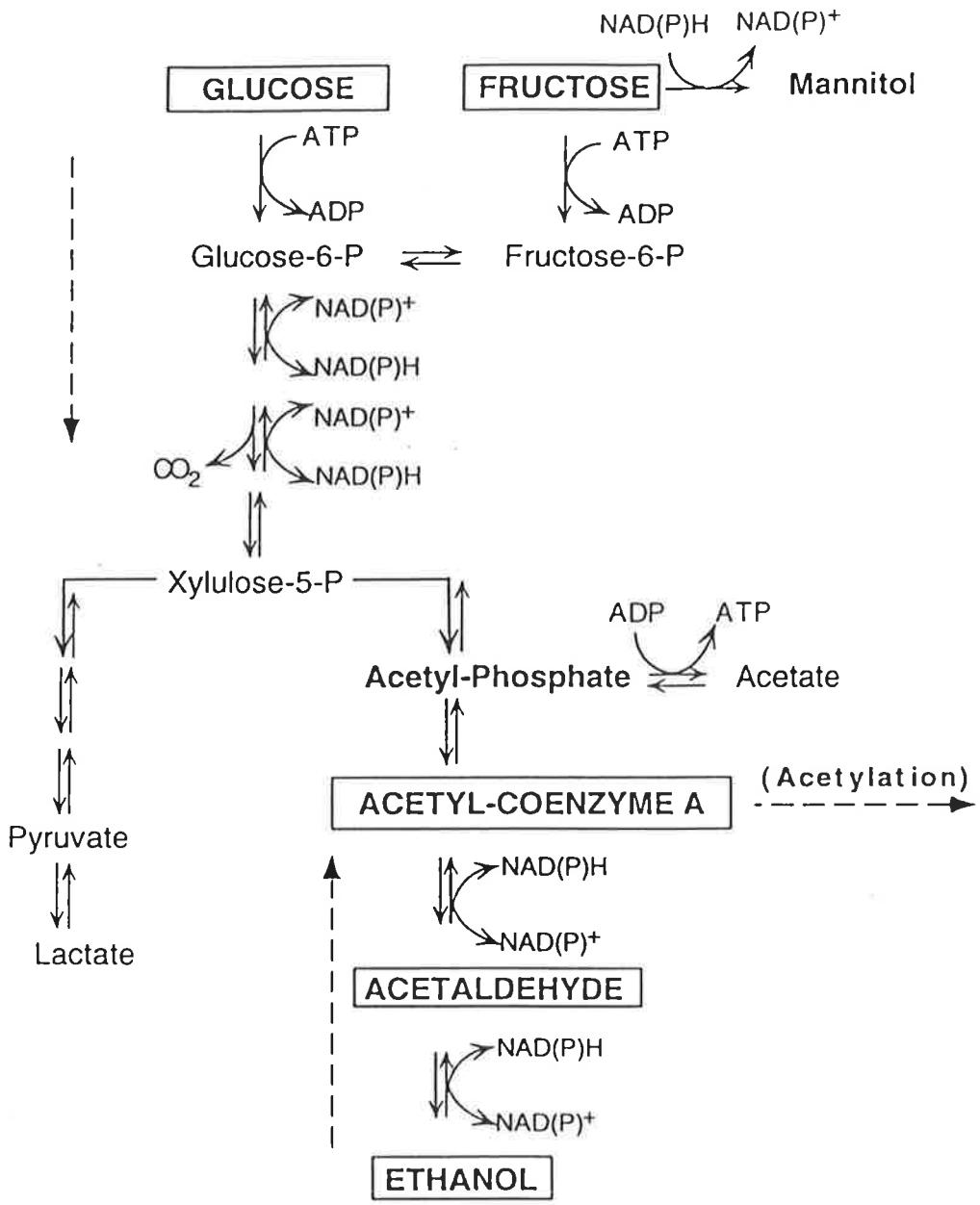


Figure 6.19 Scheme depicting the accumulation of C-2 intermediates in the heterolactic pathway under conditions of substrate and (C-2) end-product excess.

While the identity of the acylating C-2 intermediate(s) is not known, a likely candidate in this role is the acylating coenzyme, acetyl-CoA. This is a key molecule in many enzyme catalysed acetylations (Stryer 1975), and acyl-CoA derivatives are also common acylating reactants in the formation of pyrroline and piperideine based alkaloids (see below). An alternative source of a C-2 compound which may accumulate from the heterolactic pathway is acetyl phosphate. The identity of the acetylating compound(s) could be elucidated in further metabolic studies using labelled C-2 substrates. The use of inhibitors that are specific to certain reaction steps in heterolactic pathway, eg. towards alcohol and acetaldehyde dehydrogenase enzymes, may also be useful in this regard.

(iii) Nitrogen source: L-ornithine and L-lysine

Further experiments of this chapter have provided evidence for particular nitrogen substrates being involved in the formation of acetylated mousy N-heterocycles. Importantly, exogenous L-ornithine stimulated the formation of ACPY and repressed ACTPY and, conversely, L-lysine stimulated the formation of ACTPY and repressed ACPY. Although knowledge in this area of LAB metabolism is lacking, Tucknott (1977) and Heresztyn (1986) reported that L-lysine was a necessary substrate for the production of ACTPY by *Brettanomyces*. Furthermore, a colleague in this laboratory (Grbin 1998) has recently shown that L-lysine and L-ornithine were required substrates, respectively, for the formation of ACTPY and ACPY by *Brettanomyces* spp. These reports suggest similarities in the metabolism of amino acid substrates by both LAB and *Brettanomyces* spp. in the formation of acetylated N-heterocycles. In contrast, Romanczyk *et al.* (1995) established that glutamic acid and proline were required for the formation of ACPY by *B. cereus*. However, in the current study, these amino acids caused a significant decrease in the production of ACTPY and complete inhibition in the formation of ACPY by *L. hilgardii* DSM20176, suggesting that separate pathways of N-heterocycle metabolism occur in these different bacterial groups.

In this chapter, the stimulation of ACTPY and of ACPY formation by L-lysine and L-ornithine, respectively, in *L. hilgardii* DSM 20176 suggests that these two amino acids may play an integral role in the formation of these N-heterocycles. However, further studies using labelled L-lysine and L-ornithine would be required to establish whether these amino acids are directly incorporated into the respective N-heterocycles. Nevertheless, from the known pathways for the catabolism of the latter amino acids and, together with obvious structural similarities, the evidence here implicates a plausible mechanism for the biosynthesis of the mousy off-flavour compounds. The following is a discussion of this proposed scheme.

Concerning the metabolism of L-lysine, a diversity of catabolic routes for this amino acid have been proposed in plants, animals, fungi and bacteria (Reitz and Rodwell 1970, Mann 1987, Hammer *et al.* 1991). Of particular relevance here are those involving the formation of the cyclic intermediate, 1-piperideine, which has been demonstrated in certain *Pseudomonas* (Fothergill and Guest 1977) and *Streptomyces* spp. (Madduri *et al.* 1989). In this route, L-lysine is decarboxylated to cadaverine by lysine decarboxylase. Cadaverine is then deaminated by cadaverine aminotransferase to 5-aminoglutyraldehyde (5-aminopentanal), which exists in equilibrium with the cyclic structure, 1-piperideine. Further metabolism of 1-piperideine through to 5-aminovalerate, glutarate semialdehyde, glutarate and then other final catabolic end-products occurs. Moreover, Fothergill and Guest (1977) point out that the latter cadaverine route is analogous to that occurring with the lower homologue, putrescine, leading to the formation of intermediates including 1-pyrrolideine, and from which L-ornithine was suggested to be the initial substrate. The significance of 1-piperideine and 1-pyrrolideine to the current study is that the ring structures are susceptible to nucleophilic attack (acylation) at the C-2 position, hence establishing a key point for the generation of mousy N-heterocycles (see below).

It is interesting to note that the latter routes for the bacterial metabolism of L-lysine and L-ornithine closely resemble the initial steps of the metabolism of piperidine and pyrrolideine based alkaloids as described by Leistner and Spenser (1973) and Mann (1987). In this system, decarboxylation and deamination of the parent amino acids proceeds via the intermediacy of a lysine- or ornithine-pyridoxal phosphate complex. The alkaloid precursors are 4-amino-butanal, and 5-amino-pentanal, which exist in equilibrium with the cyclic imines, 1-pyrrolideine and 1-piperideine. Importantly, further elaboration of alkaloids concerns the enzyme mediated entry (such as Schiff-base formation, Mannich condensation and aldol type process) of a side chain at C-2 of the 1-piperideine and 1-pyrrolideine rings. Since these side chain reactions can involve acyl-CoA derivatives (Dalton 1979, Mann 1987), they are of major relevance to the current study, and provide suitable evidence from which a model for the production of acetylated derivatives can be proposed.

(iv) Proposed pathway for the formation of acetylated mousy N-heterocycles by lactic acid bacteria

The proposed scheme for mousy N-heterocycle formation by LAB is depicted in Figure 6.20, and involves the interaction of intermediates from two disparate pathways: (i) N-heterocyclic intermediates, 1-piperideine and 1-pyrrolideine, derived from the catabolism of amino acids, and (ii) acylating compounds such as acetyl-CoA accumulated from the

heterolactic fermentation. In the amino acid branch of this pathway, the intermediates 1-piperideine and 1-pyrrolidine are accumulated from L-lysine and L-ornithine via the cadaverine and putrescine pathways, respectively. Concomitantly, an acyl-carrier such as acetyl-CoA and acetyl phosphate could accumulate from the heterolactic fermentation of sugars when the cell is in the presence of ethanol. The 1-piperideine and 1-pyrrolidine intermediates are then subject to nucleophilic attack at the C-2 position from accumulated acyl-CoA (or similar) derivatives, possibly via an acetyl transferase, thus yielding the acylated mousy compounds, ACPY and ACTPY. Overall, in this proposed 1-pyrrolideine / 1-piperideine pathway, the resultant acetylated N-heterocycles are secondary metabolites of the basic amino acids, L-ornithine and L-lysine.

Although the above 1-pyrrolideine / 1-piperdeine pathway proposed for the formation of acetylated N-heterocycles fit the available evidence, consideration should also be given to other catabolic routes of the amino acid substrates, particularly those of lysine. Of relevance here is the pathway for the conversion of lysine to the 6-carbon cyclic imino acid pipecolate in aerobic bacteria (*Pseudomonas*) (Miller and Rodwell 1971, Chang and Adams 1971, Fothergill and Guest 1977). This pathway is initiated by conversion of L-lysine to D-lysine by lysine racemase and involves the following steps: D-lysine → 1-piperideine-2-carboxylate → L-pipecolate→ 1-piperideine-6-carboxylate. In a further series of reactions, 1-piperideine-6-carboxylate is then converted to L-glutamate. Also, L-lysine can be directly transaminated to 1-piperideine-6-carboxylate by an L-lyine-6-aminotransferase in *Achromobacter liquidum* and *Flavobacterium* sp., (Soda and Misono 1968, Soda *et al.* 1968; cited by Fothergill and Guest (1977)). Given the structural similarities of the latter cyclic intermediates to the 1-piperideine nucleus of ACTPY and, coupled with an additional decarboxylation step, it is conceivable that the pipecolate pathway could potentially supply the necessary C_5N sub-unit for generation of piperideine-based mousy compounds. However, this outcome is unlikely since the literature does not indicate the occurrence of a pipecolate decarboxylase and, furthermore, evidence from this chapter demonstrated that DL-pipecolate repressed the formation of ACTPY (and ACPY) by *L. hilgardii* DSM 20176. The potential role of 1-piperideinecarboxylate in the formation of piperidine alkaloids was also discounted by Mann (1987) on the basis of labelling studies. Another pathway for the catabolism of lysine conducted in most yeasts (other than *Saccharomyces cerevisiae*) involves initial acetylation (via acetyl-CoA) at the ε-amino nitrogen by a lysine N^6-acetyltransferase to yield N^6-acetyl-lysine (Large 1986). This suggests the possibility that acetylation of mousy N-heterocycles could also occur prior to cyclization. However, this is unlikely since, as Large (1986) points out, the acetyl group prevents cyclization of lysine catabolites. Furthermore, for the biosynthesis of ACTPY, acetylation of L-lysine would need to occur at a position other than the ε-amino group.

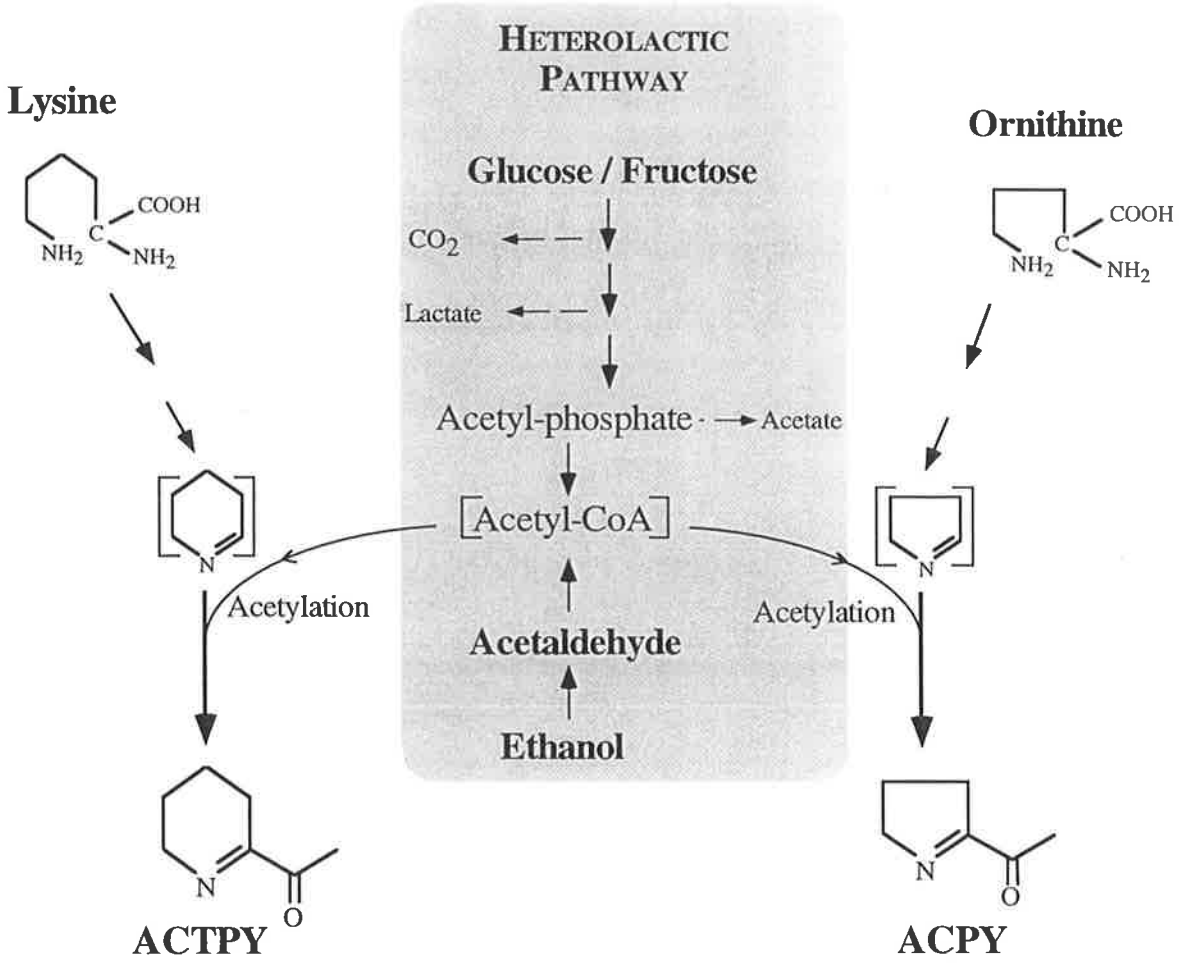


Figure 6.20 Proposed pathway for the formation of the acetylated mousy N-heterocycles, 2-acetylpyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY), by the heterofermentative lactic acid bacterium, *Lactobacillus hilgardii* DSM 20176

From the HCDI studies presented in this chapter, the ability of *L. hilgardii* DSM 20176 to produce mousy N-heterocycles in the absence of a nitrogen source indicates that a pool of the respective nitrogenous substrates and / or precursors had accumulated within the cell during preculture. Moreover, the repression of ACPY formation by L-lysine, and of ACTPY formation by L-ornithine indicates the existence of separate enzyme systems involved in the synthesis of the respective N-heterocycles, with each having distinct substrate specificity. The small (microgram per litre) quantities of acetylated N-heterocycles produced by *L. hilgardii* DSM 20176 in response to much larger (gram per litre) additions of carbohydrate and amino acid substrates also suggests the formation of these compounds is a minor metabolic route, with one or more of the pathways exhibiting weak activity. In this regard, high pH optima are often observed for enzymes involved in the metabolism of basic substrates (Fothergill and Guest 1977). Further studies are obviously required to confirm the metabolic route by which acetylated N-heterocycles are produced by LAB before rate limiting steps in their biosynthesis can be identified.

(v) Other factors of mousy compound formation

Another major factor affecting the production of mousy N-heterocycles in the HCDI assay was the presence of metal ions, particularly Fe^{2+}. That the generation of mousy compounds was highly dependent on the presence of Fe^{2+} and not on Mn^{2+}, Mg^{2+} or Ca^{2+} ions is perhaps not surprising as it is generally accumulated by cells at greater concentrations than other ions and also catalyses more essential reactions in living systems (Archibald 1986). While the exact role of Fe^{2+} in mousy compound formation is not known, the ease with which Fe^{2+} can donate and Fe^{3+} accept an electron (Archibald 1986) suggests that it could be of redox nature. Moreover, it is also not known why ACTPY formation was greater in the absence of either Mn^{2+}, Mg^{2+} or Ca^{2+} ions. Similarly, the observed increase in ACPY and ACTPY formation after removal of L-malic acid also cannot be explained here, suggesting further research to investigate the roles of metal ions and organic acids on mousy N-heterocycle formation is warranted.

In contrast to the formation of ACPY and ACTPY, it is noteworthy that ETPY production by *L. hilgardii* DSM 20176 in the HCDI assay was somewhat greater in the absence of amino acids. Furthermore, the lack of response in the production of ETPY from various treatments including different carbon source additions, extended reaction time and lack of incorporation of deuterium-labelled ethanol and acetaldehyde, suggests that the biosynthetic route for this compound by LAB is different to that for the acetylated N-heterocycles, in particular ACTPY. This contrasts with recent work by Grbin (1998) who found that U-$^{13}C_6$ + $^{15}N_2$ labelled L-lysine was incorporated into the piperideine ring structure of

ACTPY and ETPY by the spoilage yeast *Dekkera*, and considered that ETPY formation by this yeast probably resulted from the enzymatic reduction of ACTPY.

While the nature of metabolic route for ETPY biosynthesis for LAB is not known, it is relevant to note that other structurally related piperidine alkaloids including nigrifactin, produced by a strain of *Streptomyces*, and the hemlock alkaloids coniine (2-propyl-piperidine) and coniceine (2-propyl-tetrahydopyridine) are predominantly derived from acetate, and not from lysine (Mann 1987). Significantly, as previously discussed, *L. hilgardii* DSM 20176 was also observed to produce coniceine (2-propyl-tetrahydopyridine) in the presence of *iso*-propanol. Although the role of this alcohol in the latter metabolism is not understood, it would be advantageous for future metabolic studies of alkyl-substituted N-heterocycles to utilise other LAB as test strains, such as the *O. oeni* strains Lc5b, Lo-42 and Viniflora which were observed, in this chapter, to produce high concentrations of ETPY.

The high cell density incubation procedure has provided a convenient quantitative method to study the production of mousy N-heterocycles by LAB in a synthetic medium, and reflect the overall capacity of the bacteria to support this metabolism. Further studies are required, however, to ascertain the effects of lower pH (pH 3.3-3.5) and higher ethanol content (12%-14% v/v), which generally prevail in wine, on mousy N-heterocycle formation by LAB. Nevertheless, the evidence from this study has demonstrated that heterofermentative LAB, particularly *L. hilgardii*, can produce large amounts of mousy off-flavour compounds under anaerobiosis and in the presence of several components including ethanol and high concentrations of sugar (D-fructose). Hence, the opportunistic growth of certain *Lactobacillus* spp., especially in wines containing residual sugar or in stuck fermentations, having high pH and also minimal sulfite and moderate temperature (20° - 30° C), may provide ideal conditions for the rapid development of mousy off-flavour during the vinification process. It also remains to determine the mechanism by which other wine bacteria, in particular the homofermentative *Pediococci* and *Lactobacilli*, and also acetic acid bacteria homofermentative LAB and also acetic acid bacteria, can generate mousy off-flavour compounds.

6.5 Summary

A high cell density incubation technique was developed to enable systematic the study of the major factors that affect the generation of mousy off-flavour by LAB. By this procedure, mousy off-flavour was generated by LAB in a chemically defined basal assay medium, which essentially comprised D-fructose, ethanol, acetaldehyde (optional), L-ornithine, L-lysine, metal salts and citric and L-malic acids. Screening of LAB for the production of mousy compounds by the high cell density procedure corroborated with previous surveys that the most potent producers of ACTPY were the heterofermentative *Lactobacillus* spp., *L. hilgardii* and *L. brevis*, whereas the homofermentative *L. plantarum* and *Pediococci* were generally lacking in this ability. Most LAB also produced ACPY, and 3 of 5 strains of *O. oeni.* produced particularly high concentrations of ETPY (86.8 - 162.0 µg/L).

The essential components required for the production of the acetylated N-heterocycles ACPY and ACTPY were determined with the test strain *L. hilgardii* DSM 20176. These were identified to be: (i) a fermentable carbohydrate (D-fructose), (ii) ethanol and (iii) Fe^{2+} ions. L-Ornithine was found to stimulate the formation of ACPY and repress ACTPY and, conversely, L-lysine stimulated the formation of ACTPY and completely repressed ACPY. The omission of Mn^{2+}, Mg^{2+} and Ca^{2+} ions increased the production of ACTPY but had little effect on ACPY production. The production of ACPY and of ACTPY was stimulated by the omission of L-malic acid and also by a higher content of acetaldehyde (500 mg/L). Different carbon (D-glucose and D-xylose) and nitrogen (L-proline, L-glutamate and DL-pipecolate) source additions caused considerable reduction in the formation of ACPY and ACTPY compared to that obtained with control BA medium. In contrast to the acetylated N-heterocycles, the latter components generally had little effect on the formation of ETPY. Slight increases in the production of ETPY by *L. hilgardii* DSM 20176 occurred in the absence of amino acids.

Studies were also undertaken to determine the origin of the acetyl side chain of the ACPY and ACTPY. Replacement of ethanol in the assay medium with *n*-propanol led to the formation of the C-3 substituted analogue of ACTPY, 2-propionyltetrahydropyridine, but not of 2-propionyl-1-pyrroline nor 2-propyltetrahydropyridine. In most cases, further substitution of ethanol with *iso*-propanol or *n*-butanol, and of acetaldehyde with propionaldehyde or butyraldehyde did not produce C-3 or C-4 substituted analogues of mousy N-heterocycles. The reason for failure is not clear; the concentrations of products may have been too low to detect with the assay system used. An exception to this was the detection of 2-propyltetrahydropyridine from the *iso*-propanol substituted fermentation. Incorporation of d_6-ethanol into the acetyl side chain of ACTPY and of ACPY, and of d_4-acetaldehyde into the side chain of ACTPY confirmed that the C-2 compounds were

precursors of these acetylated mousy compounds. Some evidence of the incorporation of 2-*d*-D-glucose into ACTPY was also provided.

A pathway for the formation of ACPY and ACTPY by heterofermentative LAB is proposed. In this scheme, the metabolism of exogenous carbohydrate in the presence of ethanol (and acetaldehyde) force the accumulation of C-2 intermediates of the heterolactic pathway (eg. acetyl-coenzyme A). These C-2 compounds may then concurrently acetylate N-heterocyclic intermediates (1-pyrrolideine and 1-piperideine) derived from the metabolism of L-ornithine and L-lysine, thus leading to the production of ACPY and ACTPY. To confirm this hypothesis, further high cell density studies are required using other mass labelled substrates and including those of fructose, acetate, L-ornithine, and L-lysine. In addition, other metabolic studies are required to identify the acyl- and also N-heterocycle ring intermediates.

7. CONCLUSIONS

The main conclusions and recommendations of this thesis may be summarised as follows:

1. A diversity of LAB species, particularly heterofermentative *Lactobacillus* spp., *O. oeni* and *Lc. mesenteroides*, can induce mousy off-flavour in chemically undefined ethanolic and wine-based media. The homofermentative *Pediococcus* spp., however, are lacking in this ability. These results are of major commercial relevance and extends the range of wine LAB known with the potential to cause this type of spoilage. A possible link between the ability of LAB to produce mousy off-flavour and the mode of sugar catabolism is suggested.

2. The alkaline test strip procedure is a simple and effective method for the qualitative assessment of mousy off-flavour in bacterial fermentations. The sensitivity and reliability of this method is affected by a number of factors including variation between individuals and interference from other volatiles. The method has proven especially useful as a preliminary guide for assessing mousy off-flavour occurrence in large screening programs.

3. The tautomeric ACTPY, a known and potent causal compound of mousy off-flavour, is extremely difficult to quantify due to its intrinsic chemical and chromatographic instability. Optimal GC separation and resolution of ACTPY tautomers requires an amine specific fused silica HRGC column such as the Carbowax 20 CAM. Satisfactory chromatography of two other structurally related mousy compounds, ETPY and ACPY, with the Carbowax 20 CAM column indicates its overall suitability for the analysis of these N-heterocycles.

4. Efficient and artefact-free extraction and quantification of low concentrations (µg/L level) of mousy compounds from wine can be achieved with an optimised continuous liquid-liquid extraction procedure used in association with gas chromatography-mass spectrometry (CLLE / GC-MS). For future studies, the use of stable (deuterated) isotopes as internal standards would improve accuracy and precision of quantitation as well as the scope of extraction methods.

5. Three mousy N-heterocycles, ACTPY, ETPY and the newly discovered and highly potent ACPY, are unique compounds which only occur in wines spoiled by mousy off-flavour. Of these N-heterocycles, ACTPY occurs more frequently and at the highest concentration. More extensive analytical surveys of mousy wines are required to confirm the relative abundance of each compound. Such studies should also incorporate GC-sniff assessment of wine extracts to determine whether other compounds having mousy sensory properties are present.

6. Wine bacteria comprising strains of *Lactobacillus* spp., *O. oeni*, *Pediococcus* spp. and *Gluconobacter oxydans* produced one or more of ACTPY, ACPY and ETPY in undefined (Carr-MEt) medium. Particularly high concentrations of ACTPY are produced by the *Lactobacillus* spp., *L. hilgardii* DSM 20176 (259 µg/L) and *L. brevis* IV-1 (42 µg/L). The lowered production of mousy compounds by LAB in a chemically defined (S1) medium, however, reflects the considerable influence of medium composition on this metabolism.

7. Mousy off-flavour and mousy N-heterocycles are generated by LAB when inoculated at high cell density in a chemically defined basal assay medium which comprised D-fructose, ethanol, acetaldehyde (optional), L-ornithine, L-lysine, metal salts and citric and L-malic acids. Potent producers of ACTPY are the heterofermentative *Lactobacillus* spp., *L. hilgardii* and *L. brevis*, whereas homofermentative *L. plantarum* and *Pediococci* generally lack this ability, hence supporting the data from conventional culture methods. Most LAB tested produce ACPY and certain strains of *O. oeni*. produce particularly high concentrations of ETPY. The simplicity and effectiveness of the high cell density method renders it appropriate for screening programs aimed at selecting LAB strains for commercial use which lack the potential to produce mousy off-flavour.

8. Three essential substrates for ACPY and ACTPY production by high cell density incubation of *L. hilgardii* DSM 20176 are a fermentable carbohydrate (particularly D-fructose), ethanol, and Fe^{2+} ions. Ethanol and acetaldehyde provide a precursor which is incorporated into the side chain of acyl-substituted N-heterocycles; the acetyl group may also be derived from D-glucose. These results indicate that the side chain of acylated N-heterocycles is derived from C-2 intermediates of the heterolactic fermentation, such as acetyl-CoA, which are accumulated in the presence of excess ethanol. Furthermore, L-ornithine stimulated the production of ACPY, and L-lysine stimulated the production of ACTPY. The production of ACPY and ACTPY may therefore ensue by acetylation of 1-piperideine and 1-pyrrollideine intermediates derived from concurrent catabolism of L-ornithine and L-lysine. Further metabolic studies using labelled substrates are required to confirm the metabolic pathway for the metabolism of ACPY, ACTPY and also ETPY.

9. Wines which contain a fermentable carbohydrate, such as slow or stuck alcoholic fermentations, provide a unique environment which is conducive to the formation of ACPY and ACTPY by opportunistic, heterofermentative LAB. Fundamental winemaking practices including correct acid and pH adjustment and appropriate use of sulfur dioxide are prerequisites for preventing this spoilage during vinification.

8. APPENDIXES

Appendix 3.1 de Man, Rogosa and Sharpe - apple juice (MRSA) medium

Stock cultures of LAB strains were maintained and pre-cultured in a de Man, Rogosa and Sharpe medium (Amyl) which was modified by the addition of apple juice (MRSA medium). The MRSA medium consisted of (per litre):

Component	Amount
Yeast extract	4g
Bacteriological peptone	10g
Beef extract	8g
Dextrose	20g
Sodium acetate	5g
Tri-ammonium citrate	2g
D-potassium phosphate	2g
Magnesium sulphate	0.2g
Manganese sulphate	0.05g
Sorbitan mono-oleate complex	1mL
Apple juice*	200 mL
pH	5.5

* Berrivale Orchards, clarified, pasteurised, preservative-free

Appendix 4.1 Variability of relative response factors (RRf) determined for 2-acetylpyrroline (ACPY), 2-acetyltetrahydropyridine (ACTPY) and 4-acetylpyridine†

Data source	RRf			
	ACPY (I)††	ACTPY (I)††	ACTPY (11)††	4-acetylpyridine†††
Peak area				
Average	3.99	1.60	1.08	0.81
C.V.(%)	4.0	13.0	6.4	1.0
Peak height				
Average	3.84	1.66	1.04	0.82
C.V.(%)	4.0	4.1	2.8	2.9

† RRf values determined from replicate (n=5) GC-MS analyses of standard reference solutions over a period of five months:
General formula: RRf = [(RIC target compound / RIC of IS) x (Ion response of IS / Ion Response of target compound)]
GC-MS analysis performed using Carbowax 20 CAM column

†† Determined with respect to first internal standard (4-acetylpyridine)

††† Determined with respect to second internal standard (3-acetylpyridine)

Appendix 4.2 Variabiliy of GC-MS† quantifications of 2-acetylpyrroline (ACPY) and 2-acetyltetrahydopyridine (ACTPY) after replicate injections of a standard reference solution††

Data source	ACPY		ACTPY	
	µg/mL	Range (%)	µg/mL	Range (%)
Peak area:				
Total ion response (RIC)	73.7	2.7	125.1	1.1
Molecular ion	75.2	0.5	126.9	3.4
Peak height:				
Total ion response (RIC)	82.7	2.4	130.4	2.1
Molecular ion	82.1	2.5	130.4	1.2

† GC-MS analysis undertaken using Carbowax 20 (CAM) column

†† Concentrations were calculated from the ratio of total or molecular ion responses of target compounds, to respective responses of internal standard (4-acetylpyridine; 79.4 µg/mL). Calculations involving molecular ion data utilized appropriate relative response factors (RRf's). ACPY and ACTPY concentrations expressed as the sum of tautomers. Final data average of duplicate analyses.

††† Recovery of 4-acetylpyridine determined with respect to second internal standard (3-acetylpyridine; 74.3 µg/mL).

Appendix 5.1 Methods for the isolation and identification of lactic acid bacteria from wine

Procedures for the isolation and identification of LAB from wine were similar to those described by Fleet (1993). Wine samples (100µL) were spread plated onto the surface of MRSA+C agar [MRSA medium containing 15 g/L Bacteriological agar (Oxoid) and 100 mg/L cycloheximide to inhibit yeast growth]. Inoculated plates were incubated aerobically at 27 °C for 7 days, and colonies which developed were further purified and maintained on MRSA agar (without cycloheximide). Wine isolates which were Gram positive and catalase negative were classified as LAB. Further classification of LAB to genus level was carried out by microscopic examination of cellular morphology, and testing for gas production from glucose fermentation:

Pediococcus : cocci in pairs and tetrads; homofermentative
Oenococcus : coccobacilli in pairs and chains; heterofermentative
Lactobacillus : rods; homofermentative or heterofermentative

Appendix 5.2 Methods for the isolation of *Oenococcus oeni* strains from commercial malolactic fermentation preparations

Small quantities (ca. 0.1 g) of commercial MLF preparations were rehydrated in sterile distilled water (10 mL, 5 min) and subsequently spread plated (100µL) onto MRSA agar. Inoculated plates were incubated aerobically at 27 °C for 7 days and colonies which developed were taken as isolates of *O. oeni* strains following microscopic examination for purity.

Appendix 5.3 Culture of *Gluconobacter* Gb-86

An AAB strain, *Gluconobacter* Gb-86, was initially cultured in Glucose Yeast Extract (GYE) medium (Oxoid) for 7 days at 27 °C. Prior to experimentation, this strain was inoculated into MRSA medium and incubated for 7 days at 27 °C.

Appendix 6.1 The concentration of 2-ethyltetrahydropyridine (ETPY), 2-acetyl-1-pyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY)* produced by high cell density incubation (HCDI) of lactic acid bacteria (LAB) in basal assay medium**

LAB		N-heterocycle (μg/L)		
Genus / species	Strain	ETPY	ACPY	ACTPY
Oenococcus oeni	MCW	3.6 (3.0, 4.2)†	8.5 (2.7, 14.3)	11.9 (11.1, 12.6)
" "	DSM 20252	8.1 (7.3, 8.8)	11.3 (11.2, 11.4)	4.5 (4.1, 4.8)
" "	Lc5b	162.0††	57.1	47.9
" "	Lo-42	86.8 (85.4, 88.2)	23.2 (22.6, 23.8)	12.7 (8.6, 16.8)
" "	Viniflora	128.9 (94.8, 163.1)	54.7 (50.9, 58.5)	103.8 (87.4, 120.2)
Lactobacillus hilgardii	DSM 20176	4.6	31.6	579.9
" "	L21c	5.15 (nd††, 10.3)	35.5 (26.1, 44.9)	403.9 (156.9, 650.9)
Lactobacillus brevis	L17a	7.6 (4.8, 10.3)	24.4 (17.1, 31.7)	328 .1 (184.4 , 471.8)
Lactobacillus plantarum	L11a	0.2	15.6	2.9
Pediococcus damnosus	P1a	2.3 (1.1, 3.4)	nd (nd, nd)	0.7 (nd, 1.4)
Pediococcus parvulus	P6b	0.4 (0.1, 0.6)	37.0 (24.5, 49.5)	10.5 (7.9, 13.1)

* N-heterocycles quantified by GC-MS using the procedures described in section 5.2

** Basal assay medium used in this screening experiment omitted acetaldehyde

† Data obtained from analysis of duplicate HCDI assays: first value denotes the mean concentration, and values in braces are the concentration of each replicate

†† Concentration determined from a single HCDI assay

††† nd = not detected

Appendix 6.2 Variation in the concentrations of 2-ethyltetrahydropyridine (ETPY), 2-acetyl-1-pyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) produced by *Lactobacillus hilgardii* DSM 20176 from repeated high cell density incubation experiments performed in basal assay (BA) medium

Experiment	N-heterocycle (μg/L)		
	ETPY	**ACPY**	**ACTPY**
1	4.7 (3.0, 6.4)†	89.3 (84.8, 93.9)	1040.5 (1039.9, 1041.1)
2	5.8 (nd†††, 11.5)	39.1 (41.4, 36.9)	453.1 (422.7, 483.5)
3	6.3 (4.3, 8.3)	34.3 (32.9, 35.7)	502.6 (492.4, 512.8)
4	5.5††	40.9	721.9
5	nd††† (nd, nd)	62.1 (60.3, 64.0)	510.6 (442.5, 578.6)
6	2.0	34.5 (32.8, 36.3)	270.2 (242.8, 297.7)
7	1.5	45.3	469.3
Uninoculated BA medium	nd (nd, nd)	nd (nd, nd)	nd (nd, nd)

† Data obtained from analysis of duplicate HCDI assays: first value denotes the mean concentration, and values in braces are the concentration of each replicate

†† Concentration determined from a single HCDI assay

††† nd = not detected

Appendix 6.3 The effect of fructose concentration in basal assay (BA) medium on the production of 2-ethyltetrahydropyridine (ETPY), 2-acetyl-1-pyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by high cell density incubation of *Lactobacillus hilgardii* DSM 20176

Fructose content (g/L)	N-heterocycle (μg/L)		
	ETPY	ACPY	ACTPY
0	3.6 (0.4, 6.7)†	19.5 (15.8, 23.1)	7.5 (5.1, 9.8)
10	1.8 (0.5, 1.3)	19.5 (15.9, 23.1)	211.9 (173.5, 250.3)
50	4.7 (3.0, 6.4)	89.3 (84.8, 93.9)	1040.5 (1039.9, 1041.1)
100	2.5 (2.2, 2.8)	78.1 (71.7, 84.6)	1100.6 (948.5, 1252.7)

† Data obtained from analysis of duplicate HCDI assays: first figure denotes the mean concentration, and values in braces are the concentration of each replicate

Appendix 6.4 The effect of L-ornithine and L-lysine concentration in basal assay (BA) medium on the production of 2-ethyltetrahydropyridine (ETPY), 2-acetyl-1-pyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by high cell density incubation of *Lactobacillus hilgardii* DSM 20176

Amino acid content of BA medium (g/L)	**N-heterocycle (μg/L)**		
	ETPY	**ACPY**	**ACTPY**
BA medium without amino acids (control)	15.9 (12.7, 19.0)	24.5 (11.0, 38.0)	894.9 (724.2, 1065.7)
L-Ornithine, 1	3.8 (1.5, 6.1)	258.4 (222.7, 294.1)	536.8 (457.1, 616.5)
L-Ornithine, 5	3.0 (1.5, 4.5)	319.6 (264.0, 375.1)	707.5 (621.8, 793.2)
L-Lysine,1	5.1 (3.9, 6.2)	1.0 (nd, 1.9)	1450.6 (1253.8, 1647.5)
L-Lysine, 5	6.4 (5.6, 7.2)	nd* (nd, nd)	1497.2 (1478.1, 1516.3)
L-Ornithine, 5, plus L-Lysine, 5	4.7 (3.0, 6.4)	89.3 (84.8, 93.9)	1,040.5 (1,039.9, 1,041.1)

† Data obtained from analysis of duplicate HCDI assays: first value denotes the mean concentration, and values in braces are the concentration of each replicate

* nd = not detected

Appendix 6.5 The effects of ethanol and acetaldehyde content on the production of 2-ethyltetrahydropyridine (ETPY), 2-acetyl-1-pyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 in basal assay (BA) medium

Treatment	N-heterocycle (μg/L)		
	ETPY	ACPY	ACTPY
Experiment (I):			
BA medium (control)	nd* (nd, nd)	62.1† (60.3, 64.0)	510.6 (442.5, 578.6)
" " , without ethanol	Tr**	7.8 (7.3, 8.3)	56.7 (46.7, 66.7)
" " , without acetaldehyde	nd (nd, nd)	65.3 (62.6, 67.9)	464.5 (462.3, 466.7)
Experiment (II):			
BA medium (control)	5.8 (11.5, nd)	39.1 (36.9, 41.4)	453.1 (422.7, 483.5)
" " , without acetaldehyde	3.3 (1.8, 4.8)	38.8 (37.9, 39.8)	365.7 (295.6, 435.8)
Experiment (III):			
BA medium (control)	2.0	34.5 (32.8, 36.3)	270.2 (242.8, 297.7)
" " , 500 mg/L acetaldehyde	1.8 (2.8, 0.7)	45.8 (41.8, 49.8)	392.2 (358.9, 425.5)

† Data obtained from analysis of duplicate HCDI assays: first value denotes the mean concentration, and values in braces are the concentration of each replicate

* nd = not detected

** Tr = Trace amount

Appendix 6.6 The effect of metal ion content on the production of 2-ethyltetrahydropyridine (ETPY), 2-acetyl-1-pyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 in basal assay (BA) medium

Treatment	N-heterocycle (μg/L)		
	ETPY	ACPY	ACTPY
Experiment (I):			
BA medium (control)	5.8 (nd, 11.5)†	39.1 (36.9, 41.4)	453.1 (422.7, 483.5)
" " , without metal ions	2.7 (1.6, 3.8)	nd* (nd, nd)	16.7 (10.2, 23.2)
Experiment (II):			
BA medium (control)	2.0	34.5 (32.8, 36.3)	270.2 (242.8, 297.7)
" " , without $FeSO_4$	0.2 (0.1, 0.2)	2.0 (2.0, 2.1)	16.5 (15.6, 17.4)
" " , without $MnSO_4$	1.4 (1.1, 1.7)	34.2 (33.4, 34.9)	352.4 (327.3, 377.6)
" " , without $MgSO_4$	2.5 (0.5, 4.5)	32.0 (26.0, 38.0)	347.9 (285.8, 410.0)
" " , without $CaCl_2$	6.5 (3.4, 9.6)	32.8 (30.7, 34.8)	354.5 (332.4, 376.7)

† Data obtained from analysis of duplicate HCDI assays: first value denotes the mean concentration, and values in braces are the concentration of each replicate

* nd = not detected

Appendix 6.7 The effect of organic acid content on the production of 2-ethyltetrahydropyridine (ETPY), 2-acetyl-1-pyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 in basal assay (BA) medium

Treatment	N-heterocycle (μg/L)		
	ETPY	ACPY	ACTPY
Experiment (I)			
BA medium (control)	nd* (nd, nd)	62.1 (60.3, 64.0)†	510.6 (442.5, 578.6)
" " , without citric acid	2.5 (1.6, 3.3)	60.0 (59.9, 60.1)	553.5 (532.2, 574.8)
Experiment (II)			
BA medium (control)	2.0	34.5 (32.8, 36.3)	270.2 (242.8, 297.7)
" " , without L-malic acid	2.6 (2.3, 2.8)	46.1 (41.3, 51.0)	483.4 (467.9, 498.8)

† Data obtained from analysis of duplicate HCDI assays: first value denotes the mean concentration, and values in braces are the concentration of each replicate

* nd = not detected

Appendix 6.8 The effect of nitrogen source on the production of 2-ethyltetrahydropyridine (ETPY), 2-acetyl-1-pyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 in basal assay (BA) medium

Nitrogen source (g/L)	**N-heterocycle (μg/L)**		
	ETPY	**ACPY**	**ACTPY**
BA medium without amino acids (control)	3.6	33.8	853.5
L-Proline, 5	3.9	nd*	534.6
L-Glutamate, 5	4.2	nd	493.6
DL-Pipecolate, 1	1.8	12.8	396.9

* nd = not detected

Appendix 6.9 The effect of carbohydrate source on the production of 2-ethyltetrahydropyridine (ETPY), 2-acetyl-1-pyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 in basal assay medium

Carbohydrate source	**N-heterocycle (μg/L)**		
	ETPY	**ACPY**	**ACTPY**
D-Fructose (control)	5.5	40.9	721.9
D-Glucose	3.1	2.3	18.9
D-Xylose	2.2	2.6	30.2

Appendix 6.10 The effect of incubation time and anaerobisis on the production of 2-ethyltetrahydropyridine (ETPY), 2-acetyl-1-pyrroline (ACPY) and 2-acetyltetrahydropyridine (ACTPY) by high cell density incubation of *Lactobacillus hilgardii* DSM 20176 in basal assay medium

Incubation conditions	N-heterocycle (μg/L)		
	ETPY	ACPY	ACTPY
Aerobic:			
16 h (control)	6.3 (4.3, 8.3)†	4.3 (32.9, 35.7)	502.6 (492.4, 512.8)
72 h	2.3 (nd*, 4.6)	29.5 (25.0, 33.9)	555.8 (507.3, 604.4)
104 h	6.3 (3.5, 9.2)	33.8 (28.9, 38.7)	707.6 (607.1, 808.1)
Anaerobic:			
16 h	8.6 (8.4, 8.7)	35.2 (33.2, 37.1)	465.9 (442.7, 489.1)

† Data obtained from analysis of duplicate HCDI assays: first value denotes the mean concentration, and values in braces are the concentration of each replicate
* nd = not detected

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